PREVALENCE OF HEPATITIS C VIRUS GENOTYPES IN LOCAL CENTRES OF ISLAMABAD

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Islamabad

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Prevalence of Hepatitis C Virus Genotypes in Local Centres of Islamabad

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CERTIFICATE

This thesis, **Prevalence of Hepatitis C Virus Genotypes in Local Centers of Islamabad**, submitted by **Mubammad Asim Ansari** is accepted in its present form by the Department of Bioinformatics and Biotechnology, **International Islamic University, Islamabad** as satisfying the thesis requirement for the degree of Master of Science in Biotechnology.

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To

Prof. Dr. Zafar M. Khalid

Chairman, Department of Biotechnology
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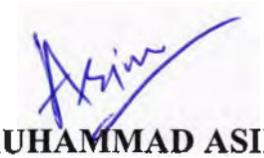
For his inspiration in the completion of this thesis

DECLARATION

I, **Muhammad Asim Ansari** declare that the material contained in this thesis, **Prevalence of Hepatitis C Virus Genotypes in Local Centers of Islamabad,** is my own and original work. It is being submitted for the degree of Master of Science in Biotechnology. It has not been submitted before for any degree or examination elsewhere.



MUHAMMAD ASIM ANSARI
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Abbreviations

ALT	Alanine Aminotransferase
cDNA	Complementary Deoxyribonucleic Acid
ddH_2O	Deionized Distilled Water
DEIA	DNA Enzyme Immunoassay
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
E1	Envelope Protein 1
E2	Envelope Protein 2
EIA	Enzyme Immunoassay
EtBr	Ethidium Bromide
FDA	Food and Drug Administration
HCV	Hepatitis C Virus
HCC	Hepatocellular Carcinoma
HVR	Hypervariable Region
IFN-α	Interferon- α
ISDR	Interferon Sensitivity Determining Region
LIPA	Line Probe Assay
NS	Non-structural Protein
PCR	Polymerase Chain Reaction
RdRp	RNA-dependent RNA Polymerase
RFLP	Restriction Fragment Length Polymorphism
RIBA	Recombinant Immunoblot Assay
RNA	Ribonucleic Acid
TAE	Tris Acetate EDTA
WHO	World Health Organization
UTR	Untranslated Region

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I bow my head in deep gratitude to **ALLAH, The Almighty**, Who is the intact source of knowledge and wisdom to me and all mankind. I owe my deep respect to **Prophet MUHAMMAD (Peace Be Upon Him)**, the city of knowledge and mercy for the entire universe.

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MUHAMMAD ASIM ANSARI

Abstract

Hepatitis C virus (HCV) is top of the list in the major causative agents of early, severe and prolonged hepatitis that subsequently leads to cirrhosis of liver and liver cell (hepatocytes) carcinoma. Roughly 170 million people, at the moment, are infected with HCV worldwide and majority of them is in chronic state of disease. The subject virus is a positive stranded RNA virus with an envelope covering it as a whole. The size of HCV genome is 9.5 kb. The unique type of error-prone replication of HCV leads to its extraordinary heterogeneous state of nature. 6 major genotypes and around 70 subtypes of HCV are discovered so far. The genotypes and subtypes of hepatitis C virus have variety of patterns as far as its prevalence rate is concerned according to geographical distributions in the world as well as within one region. These genotypes and subtypes also have difference in clinical features, signs, symptoms, and manifestations such as severity of infection, response to therapy (interferon) and disease progress. The detection of HCV genotype and its ethnic distribution is of great scientific importance just because to apprehend the epidemiology of HCV and the medical allegations of its genotypes and subtypes. In this study, 6551 individuals were screened for anti-HCV (antibody) presence by Electro-Chemiluminescence Immuno-Assay (ECLIA) method followed by HCV RNA detection, by using real time polymerase chain reaction (PCR) technique, from anti-HCV positive patients that were 350 in number. HCV genotypes were subsequently determined from 334 HCV RNA detected patients while 16 individual were found non-detected for HCV RNA. The predominant genotype of HCV in this group was determined as genotype 3 with subtype 3a and subtype 3b with (n=288) 86.23% and (n=26) 7.78% of respective prevalence rate. Genotype 1 with subtype 1a & 1b and genotype 4 were also present with lower prevalence rates as (n=13) 3.89%, (n=06) 1.80%, and (n=01) 0.30% respectively. The findings of our study were the endorsement of the former studies conducted in various cities and regions of Pakistan, indicating the predominant genotype 3 and subtype 3a in the capital city of Islamabad.

Keywords: Hepatitis C Virus (HCV), Genotyping, Subtype, Islamabad, Pakistan

1. INTRODUCTION

1.1 Epidemiology

Hepatitis C virus is one of the foremost bases of acute hepatitis and chronic liver disease, with cirrhosis and liver carcinoma. The infection caused by this deadly micro-organism varies in severity ranging from a minor ailment lasting a couple of weeks to a serious, enduring disease. The virus was discovered as an agent of NANB (non-A, non-B hepatitis) in the year 1989. It belongs to the Flaviviridae family with a plus-stranded RNA virus (Lindenbach and Rice, 2001).

About 170 million individuals are infected with HCV globally with majority being in the chronic state. A huge number of almost 4 million people are newly infected every year. (WHO, 2000) Since the innovation of HCV and its ability of universal features of epidemiology, HCV incidence rates are known (Table 1.1). HCV incidence rates in high HDI countries are as little as less than 3%. In the USA, only 1.8% of the inhabitants are infected with hepatitis C virus (Alter, 1997). The HCV occurrence rate varies from 0.20 to 3.00% in Europe.

About 500,000 people expire every year from hepatitis C-linked liver diseases (WHO, 2004). In Pakistan, around 10 million people are supposed to be infected with hepatitis C (Hamid *et al.,* 1999). Because of extraordinary genetic variability, the HCV isolates are clustered into six genotypes based on their genetic variety (Ridzon *et al.,* 1997) and all six isolates have <72% homology with regards to main division in phylogenetic tree. Amongst these genotypes, a couple of strains are existing in specific regions while certain are present globally. Genetic heterogeneity is a unique characteristic of HCV and this may have significant inferences in the diagnosis, pathogenesis, treatment, and vaccine development. The detection of HCV genotypes, subtypes and isolates have been supportive in

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understanding the progress and the epidemiology of the HCV, and is an significant factor in the pre-treatment assessment of patients (Akhund *et al.,* 2008). In Pakistan, scarce studies have been piloted in order to figure out the presence of various HCV genotypes in various cities from 4 provinces.

1.2 Transmission

The course of the HCV infection is variable from country to country and region to region. The main path of transmission in the countries with high HDI is the intravenous drug users (IDU), whereas in the countries with low HDI, the major source of infection includes transfusions with HCV infected blood and blood products and un-hygienic medical practice procedures. (Maheshwari and Thuluvath, 2010). In a recent study published in Lancet, data from seventy seven countries were reviewed. Astonishingly, twenty five of them which included the USA had very high incidence rates of HCV in the IV drug users, i.e. from 60% to 80% (Nelson *et al.,* 2011)

It is interesting to note that the main route of transmission of hepatitis C virus evolve over the period of time, e.g. the transfusion of blood and blood products has emerged as the main source of transmission after the World War II. However people noticed that in the early ages of WW II, generalised jaundice was observed in the individuals who used re-cycled unsterile needles and apparatus. This was actually the start of HCV spreading in that period of time.

In two separate studies of very high HCV infection, i.e. 80% was reported in the patients receiving blood and blood products from infected donors of blood (Estehan *et al.,* 1991; Vrielink *et al.,* 1995). This fact specifies the paramount significance of an appropriate blood screening procedures and improvement of the sensitive and specific detection system of HCV.

The transmission of HCV via sexual route is still debatable (Tohme,

Holmberg, 2010). Although a linkage has been developed among the high risk behaviour, numerous sexual partners and HCV, there has been no definite proof that HCV is transmissible via sexual intercourse. This is also proven through a study where individuals who had reported transmission via sex hide their IV drug use (Wilkins *et al.,* 2010).

The spread of hepatitis C around the globe made us aware of the use of IV infusion of infected blood components and products such as clotting factors and immunoglobulins etc as a root of HCV infectivity (Power *et al.,* 1994 and Yap *et al.,* 1994). Stringent screening of blood and blood products and the use of recombinant clotting factors is an ideal combination to decrease the transmission rate of HCV through blood transfusions certainly lessens the occurrence of HCV due to transfusion of blood products.

The items used by individuals in routine including the tooth-brushes, shavers, and equipment used for manicure and pedicure can become infected with blood and transmit the virus if the customer had HCV (Lock *et al.,* 2006). Apposite attention should be given to any clinical condition which may result in bleeding, e.g. sores & cuts. However, it is vital to note that HCV cannot be transmitted through the routine contact, e.g. via kissing, touching, eating together, or through cooking apparatuses. Neither is it spread via food or water.(Wong and Lee, 2006)

The hospital acquired HCV infection from patient-to-patient, health care worker-to-patient and vice versa occurs intermittently. In a single reported case, the use of the same unsterilized colonoscope earlier used for a HCV infected patient caused the transmission of hepatitis C virus (Bronowicki *et al.,* 1997). There are several other cases of HCV spread among patient-to-patient after compromised dialysis technique (Jadoul 2000; Taskapan *et al.,* 2001). In a study conducted at Islamabad, Pakistan, out of 97 patients on haemodialysis, 23 (23.7%) were found anti-HCV positive, the mean duration of dialysis was 2.9±2.7 years. The period and frequency of haemodialysis technique may also affect the spread of HCV in dialysis

recipients.

Organ transplant to recipient from HCV infected organ donor permanently transmits HCV (Pereira *et al.,* 1991; Terrault *et al.,* 1995). Newly transplanted liver is infected by circulating viruses or by viruses that are created at extra-hepatic places in liver transplant patients. However, there is still room for unceasing discussion about the risk factor of organ transplantation other than liver from HCV-infected persons.

Mother-to-foetus (maternal) transmission of HCV is proven at a rate of 0-8% (Halfon *et al.,* 1999; Zanetti *et al.,* 1995). As the time of transmission is still unidentified, hence, there is no methodology to stop it then and there. The circulating antibodies from the mother enters and continue activity in the child till 1 year of age, hence the occurrence of HCV infection in the child must be confirmed with PCR during this span rather than antibodies detection.

1.3 Infection Manifestations and Natural History

The HCV infection results in acute signs and symptoms in 15% of the individuals (Maheshwari, 2008). The symptoms are usually insignificant and imprecise, such as low appetite, tiredness, nausea, pain in joint and muscle, and losing weight and very occasionally acute liver failure (Wilkins *et al.,* 2010). It is critical to note that majority of the individuals does not develop the jaundice. The HCV infection disappears naturally in 10–50% of the individuals who are mostly young females (Shiffman, 2012)

It is very unfortunate that after the stage of primary and severe infection, 80-85% of the infected patients develop chronic and long term HCV infection. HCV is the foremost reason of long-term viral hepatitis in several countries due to its capability to cause insistent and recurrent infection by idiopathic aetiology. The

development of chronic HCV infection is very slow in terms of signs and symptoms that initiates with the beginning subclinical or nonspecific to mild manifestations of fatigue, generalised weakness and generalised illness. The patients with acute state of infection also suffer from weight loss, occasional nausea, vomiting, breathlessness and abdominal pain. Serum SGPT/ALT values may vary in 70% of the patients while rest of 30% patients may have normal values of serum SGPT/ALT.

If we noticed the few chronic cases of HCV infected patients, 20% of total HCV infected patients have developed cirrhosis of liver and it will lead to mortality in 25% of those chronically infected patients with cirrhosis of liver. It is not so clear whether rest of the patients will converted into cirrhosis or persist in chronic infection phase. Marked fatigue, generalised weakness, intestinal haemorrhage and jaundice specify end-stage liver disease in the cirrhotic patients. Once the patient develops last-stage liver disease, the therapy will depend on the liver transplantation or other possible interventions. The last-stage chronic hepatitis C is one of the causative agents of liver transplant cases (Detre *et al.,* 1996).

The key source of hepatic carcinoma in many countries is chronic HCV infection. About 1-5% of the individuals having chronic HCV infection progress to hepatocellular carcinoma (HCC). HCC mostly occurs in patients with cirrhosis of liver and with long period of HCV infection (Hoofnagle 1997). Hepatitis C virus also causes immuno-complex facilitated extra-hepatic syndromes such as glomerulonephritis, keratoconjuctivitis sicca, cryoglobulinemia, porphyria torda, (Johnson *et al.,* 1993; Tsukazaki *et al.,* 1998).

The scientists have developed a number of estimations for risk progression in HCV infected (chronic) individuals. Variances in estimates might be because of viral, host and environmental factors and also due to challenges faced during such type of research (shown in Table 1.1). Upcoming studies start from the beginning of the syndrome that will go parallel with the disease progression. Retrospective studies are done with patients who have confirmed liver disease. There are 3 types of studies

related to natural history to outline clinical results of HCV.

Table 1.1: Problems in Conducting an Ideal Natural History (Adapted from Seeff, 1999)

Requirement for Study	**Struggle in Designing HCV Study**
*Precise determination of disease onset and extent	* Onset of acute infection unrecognized in >80% of patients and must be concluded * Commonly asymptomatic; most acute infections are lost
*Inclusion of full spectrum of acute illness	* Frequently asymptomatic with normal ALT
*Inclusion of full spectrum of chronic illness	* Matched control group not likely without capability to identify acute onset
*Construction of matched control group without HCV	* Treatment of chronic HCV infection now common
*Evaluation of clinical outcome unchanged by treatment	* Period from acute infection to overt chronic liver disease can extent to 20-40 years
* Constant assessment to resolution or disease endpoints	

The first twenty years of the HCV infection displays a very benign clinical characteristic resulting in relatively medium level of mortality and morbidity. But after 30 to 40 years of infection, severe manifestations appear, e.g. cirrhosis, end-stage liver infection or hepatocellular carcinoma. Currently the characteristics of every patient are not forecasted which can affect the natural history such as male gender, old age infection, use of alcohol, co-infection with other viruses like HIV, HBV and added features also to be revealed.

The countries where HCV incidence rates are high belong to low HDI countries (Arthur *et al.,* 1997). In some developed countries, certain geographical regions exhibit high HCV incidence rates (shown in Table 1.2), e.g. In Ararhiro region of Japan where around 45% of subjects with greater than 41 years of age had HCV infection (Kiyosawa *et el.,* 1994).

Table 1.2: Hepatitis C Prevalence Globally (Adapted from World Health Organization, 2000)

WHO Region	Total Population (Millions)	Hepatitis C Prevalence (%)	Infected Population (Millions)	Number of countries by WHO Region where data not available
Africa	602	5.3	31.9	12
America	785	1.7	13.1	7
Eastern Mediterranean	466	4.6	21.3	7
Europe	858	1.03	8.9	19
South-East Asia	1500	2.15	32.3	3
Western Pacific	1600	3.9	62.2	11
Total	**5 811**	**3.1**	**169.7**	**57**

A literature review on the prevalence of HCV genotypes in Pakistan revealed 34 published articles. They had been conducted in all provinces except the federal capital, Islamabad. In these 34 studies, 28,400 hepatitis C infected patients were studies for genotyping. The distribution of these subjects was 3, from Punjab, 2,868 cases from Sindh, 1,200 cases from Khyber Pakhtunkhwa (Afridi *et al.,* 2013) and 152 individuals from the province of Balochistan, while 20,417 individuals were investigated from other parts of Pakistan (Attaullah *et al.,* 2011). The current study was done to find out the molecular epidemiology of various HCV genotypes and subtypes existing mainly in the federal capital, Islamabad.

The presence of the hepatitis C can be tested through the serological or molecular parameters available. The serological tests identify/detect the antibodies against the HCV by using known antigens (shown in Figure 1.1). Antibody detection assays include the Enzyme linked immunosorbent assay (ELISA/EIA) which is easy to perform and cost effective also. Similarly rapid ICT assays are also available which are lesser sensitive hut can be performed with our electricity or trained manpower. They are called rapid point of care tests.

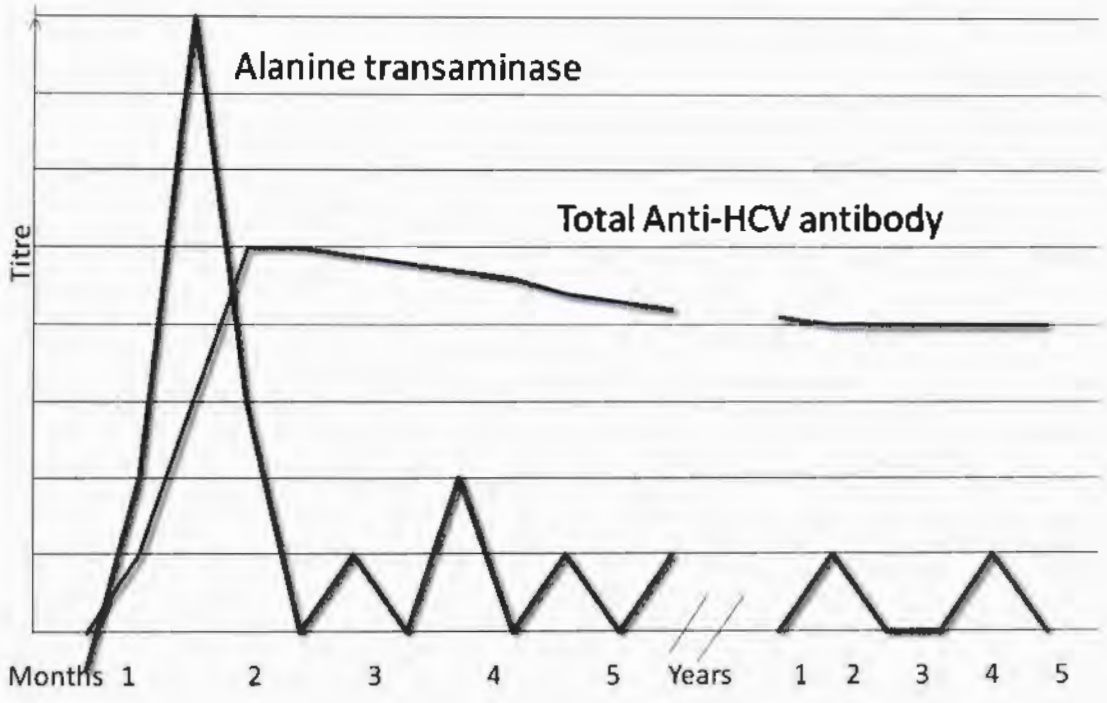

Figure 1.1 Serological profile of HCV infection

ELISA/EIA can be performed in three different ways and most common of them include the recombinant HCV antigens, c22-3 (core region encoded), c200 (NS4 and NS3 encoded) and NS5 (NS5 encoded). A positive ELISA or immunology based test does not imitate the current HCV infection however it may interpret as the previous infection after auto recovery. Likewise a negative ELISA test does not eliminate the likelihood of HCV infection as antibodies to HCV might not be made at the same point time when test is conducted. As a result of this scenario, complimentary or confirmatory tests have also been established such as Recombinant Immunoblot Assay (RIBA) which is a blood test that detects specific antibodies against the hepatitis C virus. Because it is more specific and sensitive than immunology testing of hepatitis C screening tests.

Though RIBA is considered as a confirmatory test for anti-HCV positive tests however PCR technique is much more common and better approach for the confirmation of HCV infection as far as immunology testing is concerned. New CDC guidelines for HCV testing and diagnosis also state that you must go for PCR testing if anti-HCV found positive in any patent. If HCV RNA also detected by PCR then we can call it a confirmed HCV infected case otherwise clinical manifestation can rule out the repetition period of immunology testing or PCR itself.

Most famous molecular diagnostic assay for HCV infection is Real Time PCR, quantitative DNA and branched DNA. The main objective is to sense HCV nucleic acid by applying a scientific method called amplification. The hepatitis C virus RNA is detectable in the blood by the PCR normally 1-2 weeks subsequent to infection, whereas the antibodies may take considerably extended time to develop and thus be identified in serum (Ozaras and Tahan, 2010). The qualitative testing of HCV RNA by using PCR is a very specific and sensitive method that measures 100 to 1000 copies of HCV RNA per ml of plasma. But this methodology also has few limitation including, it only confirms the presence or absence of HCV-RNA. Whereas the quantitative PCR and branched DNA methods attempts to determine the number of copies of HCV-RNA per ml of the plasma of the patient.

Till the end of 2015, scientist have not been able to developed an approved vaccine against HCV, although there have been many attempts to develop such a vaccine. Many of them are under development and some have also shown significantly positive results (Halliday *et al.,* 2011).

Injectable Alpha-Interferon (IFN-α) single-therapy and/or its combination with oral Ribavirin is the ultimate treatment of choice that is accredited by FDA, USA against HCV infection. IFN-α enhances the immune system of the patient to response effectively by increasing its efficacy in addition to its ability to promote the lytic activity T cells. The approved scheme of IFN-α therapy is 3,000,000 units of IFN administrated sub-cautiously thrice a week for at least up to 12 months. Normalized serum SGPT/ALT values and absence or low viraemia level of HCV RNA from blood stream is the indicators that endorse the response of IFN therapy that can be observed in the 50% infected population generally.

The rest of 50% people may undergo a recurrent episode after the termination or compromised regime of therapy. Dosage optimizations and/or certain chemical intervention in IFN may improve the response influx but it does not affect the reoccurrence rate. Various earlier studies suggest that elevated concentration of alpha-IFN will reduce the resistance to alpha-IFN based on the manifestation that include generation of heavy amounts of RNA as 300 billion copies daily (Neumann *et al.,* 1998). If we follow the regime of combined therapy with oral ribavirin there is one guanosine analogue that has also very effective in compromised-treated patients and relapsed infection individuals (Davis *et al.,* 1998; McHutchison *et al.,* 1998).

Both alpha-IFN mono-therapy and combined therapy with oral ribavirin also has some challenges. Injectable alpha-IFN and oral ribavirin have certain significant adverse effects such as IFN should be administrated sub-cutaneously and the price of therapy is higher than routine price (Stättermayer *et al.,* 2014). It has also been noticed that HCV genotype 1b and genotype 4 are found very least

responsive against the conventional combined therapy of INF and Ribavirin (Abdel-Ghaffar *et al.,* 2015). Such challenges subsequently lead to further research to establish more effective method that produce high quality therapeutic agent for HCV infection. Development of anti-HCV therapy is based upon the through study of HCV life cycle since there are many developmental steps in the life of HCV that can be targeted. The main targeted points are viral attachment to the cell wall and its entry into the hepatocyte, RNA translation, protein generation, RNA replication, entire viral assembly and transformation into the hepatocyte. (Figure 1.2). The conventional anti-HCV therapy targets the main non-structural proteins of the virus like NS5B, NS3 or NS4.

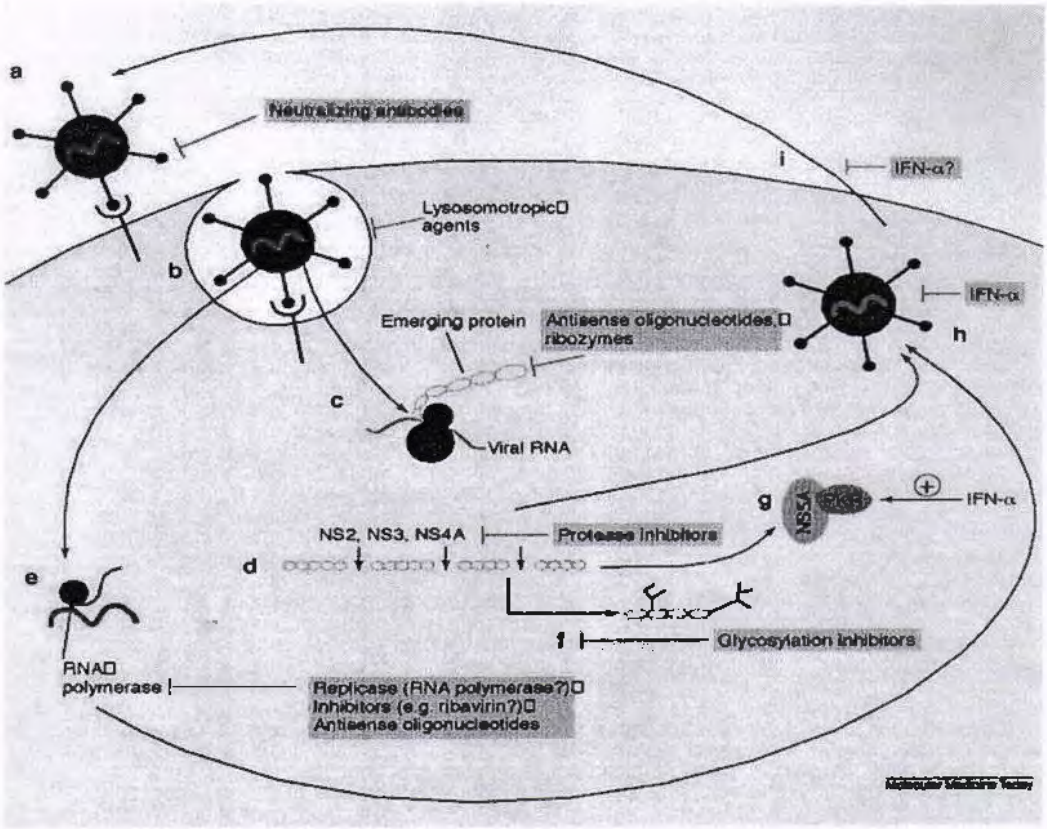

Fig. 1.2: To block HCV replication, novel therapeutic strategies. (Adapted from Rosen and Grecth, 1999)

1.5 Variability of HCV Genome

HCV is composed of a core portion of genetic material which is if RNA type and covered by a compact icosahedral protective cover of protein and further encapsulated in a lipid envelope of cellular origin. Two viral envelope glyco-proteins, E1 and E2, are embedded into the outer lipid envelope. HCV is a single-stranded RNA genome. The genome is consists of a single open reading frame of RNA that is 9.5 kb long. This single RNA frame is translated to produce a single but unique protein product, which is afterward further processed to generate smaller active proteins. At the 5' and 3' terminals of the RNA there are the UTR, which have not been translated into proteins but are vital for the translation and replication of the viral RNA. The 5' UTR has a special biding site for ribosome (IRES — Internal ribosome entry site) that actually starts the translation of nearly 3000 amino acids (shown in Figure 1.3)

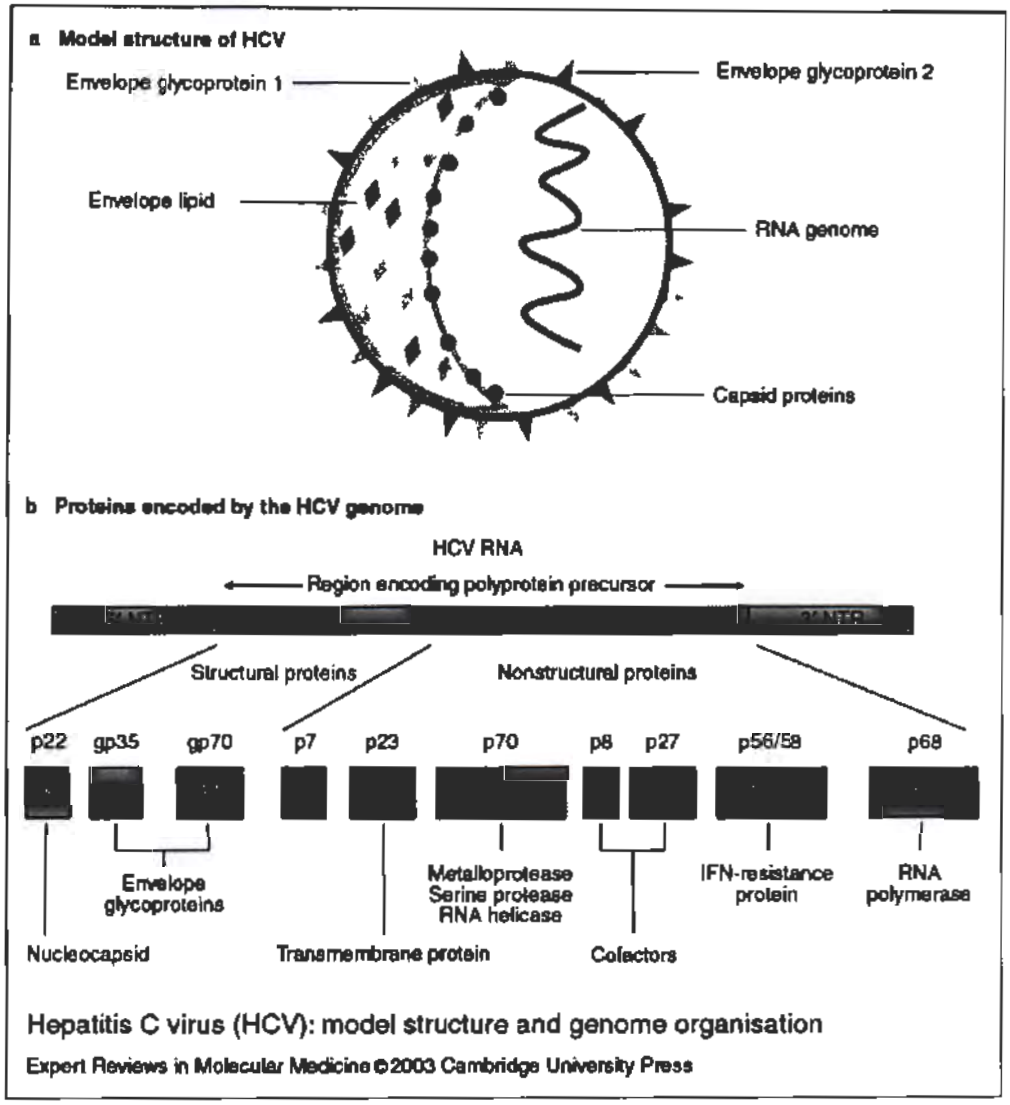

Fig 1.3: HCV model structure and organization of the genome (adapted from Expert Reviews in Molecular Medicine © 2003 Cambridge University Press

The core portion of HCV contains a four-way helical junction that is combined within a predicted pseudoknot. The assembly of this core portion constrains the open reading frame for 40S ribosomal subunit. The large pre-protein is divided by the help of viral proteases into the 10 smaller proteins that actually allow viral replication within the host hepatocyte or develop a mature viral genome. HCV also produces few structural proteins those include Core structural proteins such as E1 and E2 and nonstructural proteins include NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

HCV belongs to the genus Hepacivirus which is a member of the family Flaviviridae. Till now, it was known as the only member of this genus. But recently a member of this genus has also been discovered in dogs (canine) named hepacivirus. There is also another virus in this genus that actually infects horses. Many other viruses in this genus have also been reported but in bats and rodents.

Alike other viruses, HCV is also gradually become inactivated in outside of the host cell. The heat can significantly reduce the viral lifespan which found outside from the body. The virus can remain viable and infectious outside the body for around two weeks at 25°C while only two days at 37°C. Whereas the virus may found active for more than one month at the temperatures that is less than 4 °C. Upon heating upto 60 °C the hepatitis C virus can be inactivated within 5 minutes.

Depending upon this trifold hierarchical structure, there are six main genotypes of HCV that are numbered from 01 – 06 and around 70 subtypes that are lettered as a, b, c in the order of its discovery (Fig 1.4).

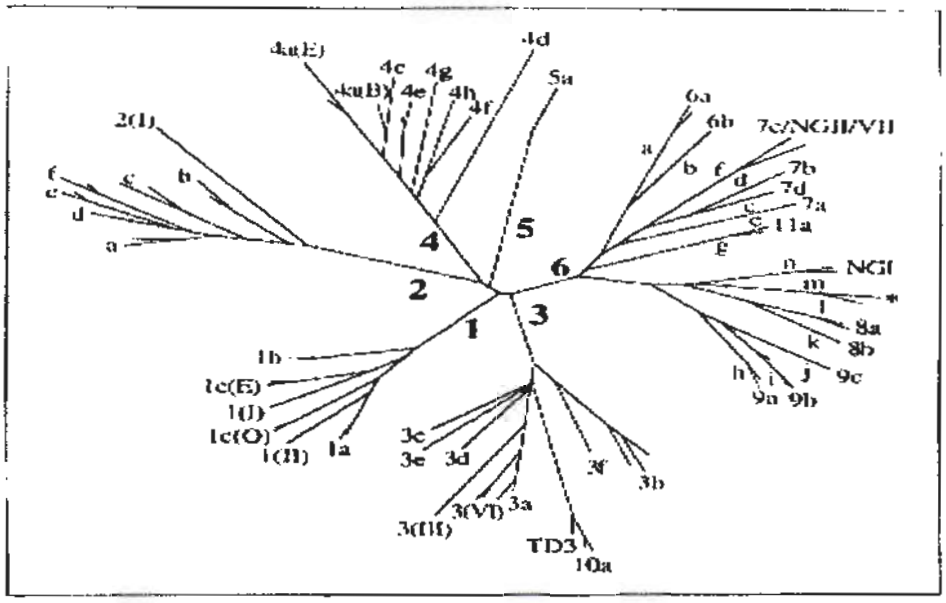

Fig 1.4: The phylogenetic analysis of nucleotide sequences from part of the HCV NS5B region amplified from samples of HCV-infected blood donors and hepatitis patients from numerous countries. Six main groups of sequence variants are found corresponding to types 1-6; each group comprises a number of more closely related subtypes (a, b, c...). Nomenclature of types 1-5 and 6a follows the consensus proposal for classification of HCV. Lately the revealed variants from South East Asia cluster with type 6a but are labelled according to the original publications. TD3/type 10a from Indonesia groups likewise with type 3 variants. (Adapted from Reesink, 1998)

Objectives of the Study

The HCV virus has around 70 subtypes owing to its six focal genotypes and their heterogeneous combinations. These subtypes vary between regions to region even within the country. In order to comprehend the source and propagation within the population, the investigation of genotype circulation is significant for epidemiological studies at the molecular levels. Main Objectives of current research include:

1. To determine the active HCV infection among HCV antibodies positive samples collected from various centres of Islamabad.
2. To determination of genotypes and subtypes of active HCV positive samples.
3. To outline the recommendations to clinicians in selecting right antiviral therapy on the basis of genotyping data.

2. Literature Review

The HCV has no historical imprint identifying the evolution of the virus like the other viruses. the current information construct an incomplete picture for HCV history when weighed against the distribution and genetic relatedness of the specie. The genetic relatedness analysis is the calculation of viral divergence time by sequence changing rate per time.

The rate of change of sequences in short time frames are calculated by various studies conducted on the animal model or individuals infected with HCV years after transmission (Abe *et al.*, 1992; Okamoto *et al.*, 1992a). The 1977 Ireland usage of antirhesus D immunoglobulin and infected individuals from same batch provided opportunity to determine the sequence changing rate. The E1 and NS5 areas were assessed for sequence change and the answer came out as 4.1 and $7.1x10^{-1}$ per site per year in 17 and 20 years respectively (Smith *et al.*, 1997). There was no change in this rate among the individuals.

If we presume that the sequence changing rate remains persistent over a period of time, the time of divergence can be calculated in the same subtype. It is calculated as 60-70 years in the case of subtype 1b variant by Smith *et al.*, while studying NS5 sequence from around the world including US, Europe and Asia on a sample of 40 sequences, no morphological grouping was identified. The results yielded faster initial spread of type 1b whereas lower diversity in type 3a. The divergence time for 3a was 40 years as calculated by NS5 region distance where as it was 90-150 years in case of type 2a-c. The factors that affect the subtype diversification are still elusive however long travel distance two to three centuries ago is considered to be the contributor for HCV diversification (Reesink, 1998).

Silent substitution is used normally to calculate the sequence changing rates due to several reasons such as: The consistency of result obtained even by using

phenotypical variants specie as expected from the amino acid change. Furthermore, the HCV proteins variability and morphological constraints have no effect on ultimate rate if calculated by the silent substitution. The only constraint in employing this tool is its inability to calculate the genotypic time of divergence (Nouroz *et al.*, 2015). The multiple substitutions pose a tricky subject when viewed in the large evolutionary gaps between the genotypes. The algorithms applied to correct this issue work but are prone to underestimation of the evolutionary years. The issue is still encountered if the results for a smaller rate are extrapolated and used for a longer period of time.

HCV genotype cannot be traced back to a focal point in time and demographics with the current level of information at our hands. However, several studies indicate no significant difference among the types in different geographical area. The long term area specific infections are heavily dependent upon the combined factors of distribution and variability.

2.1 The Concept of Quasispecies

The HCV expresses another phenomenon that is the quasispecies which are linked genetic makeup of heterogeneous origin (Bukh *et al.*, 1995; Eigen 1996; Holland *et al.*, 1992; Martell *et al.*, 1992). The quasispecies are composed of a master sequence or the common sequence prevalent in the population and various other less prevalent sequences. Such sequence is also called consensus sequence that sprouts from directly sequencing of PCR products and overlapping the most prevalent nucleotide in the genomic sequence. This sequence detected via the above method does not reflect a quasispecies genome but it identifies the major diversity in the proportion of quasispecies that have been the study population.

The HCV NS5B has a unique ability for heterogeneity owing to the RNA-dependent RNA polymerase (RdRp) that can result in versatile subtypes. Since there is no proofreading in the genetic code of NS5B, appearance of mutations is fairly common. The HCV has the RNA that can replicate itself to produce 300

billion copies of it within 24 hours. The RNA takes 4-7 hours to replicate itself constituting the heterogeneity of HCV (Lam *et al.,* 1997; Neumann *et al.,* 1998). One hypothesis proposed the presence of HCV quasispecies as the basis for viral sustainability. Adding into this is the high turnover rate of the RNA and absence of proof reading provides window for viral replication and progression of minor mutant forms.

The humeral response of the body causes the neutralization of HCV. However this neutralization is partial and many minor viruses escape the antibodies which are designed against the major components of the virus sequence (Kato *et al.,* 1993; Shimizu *et al.,* 1994; Weiner *et al.,* 1992). These minor variants replicate and their prevalence is responsible for the persistent infection with the HCV. The cell mediated response is more agile in its functionality and can target numerous epitopes in a given time (Koziel *et al.,* 1992; Koziel, 1997). The wide ranging targeting of the cell mediated immune response makes the understanding of this arm of immunity intangible requiring full genome analysis for detection of mutations.

The viral persistence of HCV can also be explained by the occurrence of decoy antigens. As the name suggests, the decoy antigens engages the immune system eliciting a response against the virus that does not eliminate the virus from the infected individual. This difference in eliciting various degrees of response is dependent on difference in genomic sequence. The sub optimal response to such antigens not only allows the virus to persist, but it also distributes the response of immune system to various regions of HCV and bypasses the response to the lesser evident epitopes (Forns *et al.,* 1999).

Apart from the viral persistence, the quasispecies are also alleged to play a pivotal role in the determining the response to drug therapy, thus greater the documented complexity of quasispecies, greater its resistance to the IFN reported (Farci *et al.,* 1997). The selection might turn in favour of IFN resistant variant with

the increasing complexity of the quasispecies. Similarly, the quasispecies and its effect on the natural history of virus and infections has been neglected but evidence suggest the increasing severity of infection with higher complexity quasispecies. (Farci *et al.,* 1997; Honda *et al.,* 1994).

The disease progression is effected by the quasisepicies hence when HCV positive patients of liver transplant were followed for a period of time, the patients were seen to either retain the initial HCV main variant that infected the individual in the first place, or new quasispecies were present. If the individual has retained the main variant HCV strain, then the disease progression is swift, however the disease progress is relatively slower in quasi-specie. Thus the different strains of HCV have different grades of pathogenicity and the identification and proper research into the role of quasispecies and their effect on the characteristics of the virus and disease progression needs to be done.

2.2 Clinical Significance of HCV Genotypes

Even though most evolutionary drift in the nucleotides sum up to a considerable difference among the specie; these differences are considered to be neutral. However similar scenario cannot be applied to HCV where the subtypes are significantly different and illicit biological differences (Simmonds, 1997).

The major genotypes of the HCV virus are similar to most of the positive RNA viruses including mode of spread. A major glitch in this principle is the Pesti-viruses which are closely related to HCV but have different variants amounting three in number and showing stark differences in host, mode of transmission and disease progression. Thus it is safe to conclude that different genotypes could confer biological variability, same is the case with HCV viral genotype variabilities.

The major techniques of nucleotide comparisons and genotyping have been established as an alternative to cell culture system due to unavailability, failure of

propagation and neutralization of HCV. These techniques have now replaced the previous to characterise different variants of HCV.

The clinical significance of HCV genotypes have been done in various studies the points of interest include the HCV genotype and its suggested effect on response to IFN treatment, the disease progression and effect on transplant. These points have been elaborated and summarised.

The evolvement of HCV and its natural history through time and spread in epidemiological setup is only possible through un-coding and understanding its genetic information. The HCV genotyping was able to solve the conundrum of vertical transmission by identifying same genetic makeup of virus in 6 out of 7 mother and new born infant thus confirming transmission. Similarly inter spouse transmission was also shown in the same fashion (Akahane *et al.*, 1994; Ohto *et al.*, 1994). In all these studies, the HCV was isolated from the infected individual and the source and sequencing of the HCV virus clarified the source.

Emergence of new cases of hepatitis C infection remains the major concern hence the HCV diagnosis remains integral part of HCV management and prevention. The method employed for diagnosis of HCV is via ELISA through the detection of anti HCV antibodies in the infected person's serum by using the recombinant proteins in genotype especially 1a, these proteins are however mainly present in subtype 1a, this raises concern among many about the accuracy of this test in detecting hepatitis C where the patient has been infected with other variants of HCV.

Type 1 infections are detected with better sensitivity by HCV antibody detection with First and second generation as compared to type 2 and 3 (Harrison and Zuckerman, 1997). Type 4 and type 6 have a decrease in reactivity of 40% and 35 % (McOmish *et al.*, 1993). Similarly the type 1 samples were more reactive by 5 folds as compared to type 2 and type 3 samples on ELISA third generation as

concluded by Dhaliwal and colleagues (Dhaliwal *et al.,* 1996). The detection of patients affected with genotypes other than 1 can be severely compromised in 3 generation genotype specific methods since the false negatives have not been taken into account and hence inefficiency remains uncalculated.

Universally approved treatment for hepatitis C infection is Interferon-α (IFN-α). The response rate is a mere ~50% and is heavily influenced by human as well as viral factors such as age, genotype, histological type and viremia. These factors are predictors of response time and sustenance and also the results of treatment such as viremia and histology (Chemello *et al.,* 1994; Martinot-Peignoux *et al.,* 1995). The innate ability of the viral components provides HCV with intrinsic property that confers IFN-α sensitivity. Low response has been seen in genotype 1a, 1b, 3a, 2 and 4, 1b and 4, while a higher response has been seen in 1a, 3a, 2 (Chemello *et al.,* 1994; Tsubota *et al.,* 1994; Yoshioka *et al.,* 1992).

The subtype 1b has been the focus of rigorous scrutiny due to its high prevalence in countless countries. A 39 amino acid (2209-2248) long region was identified by Enomoto *et al* by the comparison of complete length genome among the N55A carboxy terminus. (Enomoto *et al.,* 1996) This chain is associated with interferon sensitivity and is called interferon sensitivity determining region (ISDR). IFN-α resistance is also attributed to same sequence of ISDR area in HCV- 1b strains (HC- J4, HCV- JTa and HCV- J). Variation of just 14 amino acids in HCV-1b and type 2 ISDR region along with 4 amino acids deletion (Kurosaki *et al.,* 1997) leads to sensitivity to IFN. Enomoto`s study and its result were replicated in various other studies such as (Chayama *et al.,* 1997; Halfon *et al.,* 2000) but there are inconsistent data in several studies (Hofgartner *et al.,* 1997; Khorsi *et al.,* 1997; Squadrito *et al.,* 1997). Interferon sensitivity due to wild ISDR occurrence needs to be studied farther.

The IFN and its effect on genotype has been studied in genotypes 1a, 1b, 2, 3a and 4, however any such study is lacking in subtype 5 or 6 with no clarification

on resistance to IFN.

Through investigation has been carried out to identify the HCV genotype and liver disease progression. Cross-sectional studies have been conducted to identify the relationship between the various genotypes and disease progression to HCC and end stage liver disease. The various parameters are compared and contrasted for co-relation.

The histopathological manifestations are also linked with certain genotype such as quicker progression of subtype 1b to severe form of cirrhosis and chronic hepatitis (Booth *et al.,* 1995; Kasahara *et al.,* 1995; Watson *et al.,* 1996; Islam *et al.,* 2015) similar results were examined in subtype 1b progression to hepatocellular carcinoma (Bruno *et al.,* 1997; Silini *et al.,* 1996; Zein *et al.,* 1996). These studies have been deemed controversial on accounts of other studies that have displayed no association between subtype 1b infection and earlier progression to cirrhosis (Dusheiko *et al.,* 1994; Mita *et al.,* 1994) or HCC (Lee *et al.,* 1996; Yotsuyanagi *et al.,* 1995).

The subtype 1b is by far the most aggressive form of HCV causing severe disease and cirrhosis as compared with its other subtype 1a and type 2 infections; hence the difference in genotype or subtype can also effect the disease progression. The observation of difference in genotype, demographics and disease progression has yielded positive results such as the early advancement of type 4 infections into HCC and liver cirrhosis in the Middle East and Europe and the relatively slow progression of disease in Central Africa. There seem to be to be no other correlation documented in this context.

There are many factors responsible for the unavailability of HCV vaccine in the market. The reasons being firstly, the virus's capacity to replicate, start and sustain infection even with intact immune response of the human body, secondly the diverse and heterogeneous nature of the virus encompassing 6 main genotypes

arranging it in 70 subtypes. This heterogeneity has been proposed to possess the difference in the antigen eliciting factor of HCV epitope. Hence the type 1 derived antibodies to NS4 and NS3 proteins are not identified by non-type 1derived proteins.

Identification of various genotype subtypes identification and their respective antibodies pressed the need for manufacturing of multivalent vaccine for HCV. Isolation of focal genotypes immunogens or reactive epitopes needs to be addressed in order to manufacture effective vaccines in a diverse epidemiology with numerous variations of genotype prevalent. The identification and knowledge of the prevalent subtypes of HCV in risk groups, communities, regions and countries also becomes imperative in preventing the spread of hepatitis and minimising the new cases of hepatitis infection. Group 1b is the major subtype as evident by prevalence rate and poor response to clinical treatment.

2.3 HCV Genotyping Methods

Methods for rapid and specific genotyping are being employed establishing the HCV genotype. The target is to identify the diversity of genomic variation. The targeted genome significantly affects the development of the technique. Not only should the types and subtypes be identifiable by the region, it should also be able to develop sensitive probes or specific primers for a wider range as well. Core, 5' UTR, E1 and NS5Bregions are utilised for the analysis of genotype. The methods employed are InnoLipa probe assay, specific PCR, RFLP and sequence and evaluation of phylogeny of the region pertaining to subgenome. The techniques are chosen on the basis of nature of study, monetary retrains, the pros and cons and population of the sample. The concordance in employing the different techniques for the HCV assessment has been studied and evaluated. The 6 different genotypes have been evaluated and a concordance rate of 93% was found among the techniques.

A component of core region is amplified initially with nonspecific primers followed by subset-specific primers in type1a, 1b, 2a and 2b (Okamoto *et al.*, 1992b). Agarose gel electrophoresis is employed to examine the various lengths of augmented PCR yield. The length of cut fragments determines the genotype. This basic method is employed with variations to identify more subtypes such as use of NS5B region. (Chayama *et al.*, 1993; Ohno *et al.*, 1997; Okamoto *et al.*, 1993).

Nokao and his associates (Nakao *et al.*, 1991) analysed the HCV genome via genotyping with RFLP. Extensively used site by the RFLP analysis is 5'UTR and NS5B region. The PCR products are converted into fragments by different restriction endonucleases; these are type or subtype specific restriction enzymes targeting the amplified PCR products. The cut fragments are segregated and then genotype identified by employing electrophoresis profile of them.

The variations in the 5'UTR region of the HCV viral genotypes is utilised by line probe assay (INNOLIPA HCV II, Innogenetics, Zwijndrecht, Belgium), an assay by reverse hybridization. The various genotypes such as 1a, 1b, 2a-2c, 3a-3c, 4a-4h, 5a and 6a are isolated and identified by attachment of nucleotides probes numbering 16 via the poly (T) to a nitro cellulose membrane base. This tail is an addition to the structure by the deoxynucleotidyl transferase. 5'UTR is then used to attain PCR products that are biotin labelled, these PCR components are finally reverse hybridized and identification is completed.

DEIA targets core specific genotype probes and works on the same principle as the InnoLipa Assay. The agreement percentage in LIPA-I and DEIA comes around 87.5%. Detection of subtypes 1a, 1b, 2a, 2b, 2c, 3a, 4, and 5 is possible from the kit of DEIA (GEN-ETI-K DEIA Kit; Sorin, Soluggia, Italy).

2.4 Direct Sequencing

Theoretically speaking, accurate detection of HCV genotype is done by

genome sequencing of entire genome and phylogeny construction. In reality, the genome length is too extensive and the procedure too costly. Thus a more feasible method is used where only a few subgenomic sequences of the whole genome is used as a representative the analysis for E1, NS5B, and 5'UTR and the sequences are correlated with whole genome based phylogenetic analysis in different regions of the genome. Simmonds *et al.* on the basis of this finding argued that phylogenetic assessment of at least 2 genomic region is necessary in order to give the HCV genotype. (Simmonds *et al.,* 1994) since the 5'UTR region is the basic genome sequence, it might be similar in the six genomic subtypes.

Currently the gold standard for assessment of HCV genotype is through NS5B, core and E1 region amplification which is followed by genetic sequence evaluation and construction of phylogenetic structure as a confirmation. This procedure, though widely accepted, has its short comings such as the need for various primers sets in order to minimise inefficient amplifications of some regions in genotype resulting in detection of genotypes or quasispecies. Variegated infections will not be recognized in a large scale genotype study. The costly procedure and heavy reliance on large man power limits the usage of this method.

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4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Phlebotomy Consumables

All phlebotomy consumables were procured from below mentioned sources;

Material	**Manufacturer/Supplier**
Disposable syringes (5CC)	Terumo Medical Limited, Philippine
Alcohol swabs (Propanol)	Future Scientific, Pakistan
Blood collection tubes	Vacuette by Greiner, Germany
Saniplast bandages	Uniferoz Pvt Ltd. Pakistan

4.1.2 Equipments & Analysers

All equipments & analysers were used from below mentioned sources;

Material	**Manufacturer/Supplier**
Centrifuge machine (Swing)	Hettich, Germany
Automated Pipette 05µL	Eppendorf, Germany
Automated Pipette 10-100µL	Eppendorf, Germany
Automated Pipette 100-1000µL	Eppendorf, Germany
Cobas E411 (Analyser)	Roche Diagnostics, Switzerland
Cobas AmpliPrep (Analyser)	Roche Diagnostics, Switzerland
Cobas TaqMan	Roche Diagnostics, Switzerland
Cobas 4800 System	Roche Diagnostics, Switzerland

4.1.3 Reagents and Chemicals

All reagents, enzymes and chemicals were obtained from the sources below:

Material	**Manufacturer/Supplier**
Taq Polymerase	Roche Molecular Diagnostics, Pakistan
dNTPs	Roche Molecular Diagnostics, Pakistan
100bp DNA Marker	Roche Molecular Diagnostics, Pakistan
Gene ruler DNA Ladder	Roche Molecular Diagnostics, Pakistan

Nucleotide Removal Sol.	Roche Molecular Diagnostics, Pakistan
cDNA Synthesis Kit	Roche Molecular Diagnostics, Pakistan
HCV RNA Isolation Kit	Roche Molecular Diagnostics, Pakistan
Big Dye Cycle Sequencing Kit	Roche Molecular Diagnostics, Pakistan

4.1.4 Plastic Disposables

These are the main disposables plastic items used in the experiment:

Microcentrifuge Tubes

Tube size 0.2 µL

Tube size 1.5 µL

Tube size 2.0 µL

Glass Test Tubes

Tube size 05 mL

Tube size 15 mL

Tube size 50 mL

Auto-Micropipette disposable Tips

Auto-Micropipette disposable Filtered Tips

4.2 METHODS

4.2.1 Patients

Venous blood of 6551 patients, from 3 different centres; Pakistan Institute of Medical Sciences (PIMS), Kulsum International Hospital (KIH) and Islamabad Diagnostic Centre (IDC), Islamabad; were collected by using disposable syringes into special sample collection tubes after following all aseptic techniques. These blood samples were centrifuged at 4000 RPM for 8-10 minutes to get clear and hemolysis free serum for subsequent testing. All serum samples were tested for HCV antibody on a special and sophisticated analyser named Cobas E411.

4.2.2 Electro-Chemiluminescence

Cobas E411 (Fig 4.1) by Roche Diagnostics is the fully automated with regular sample loading facility and random access system for immunoassay analysis used to detect HCV antibody. This analyser followed the working principal termed as Electro-Chemiluminescence (ECL). This technique is the most refined form of ELISA and its principal states that an enzyme that converts an analyte to a final reaction product that will emits photons of light rather to develop any colour. Luminescence is defined as the emission of light from a substance as it comes from an electronically excited (higher) state to the ground (lower) state. The multiple forms of luminescence (bioluminescence, chemiluminescence, photoluminescence) differ in the way the excited state is reached. For instance, photoluminescence is simple fluorescence; the excitement is initiated by light source at a specific wavelength. Bioluminescence is categorized by the use of a bioluminescent compound such as luciferin and firefly luciferase enzyme. Chemiluminescence is the light produced by a chemical reaction. The chemiluminescent agent is excited by the oxidation and catalysis forming substances. When the excited substances return back to their stable ground (lower) state, a photon of light is released, which

is spotted by the luminescent signal meter. Thus by this sophisticated way we could have the concentration of HCV antibody.

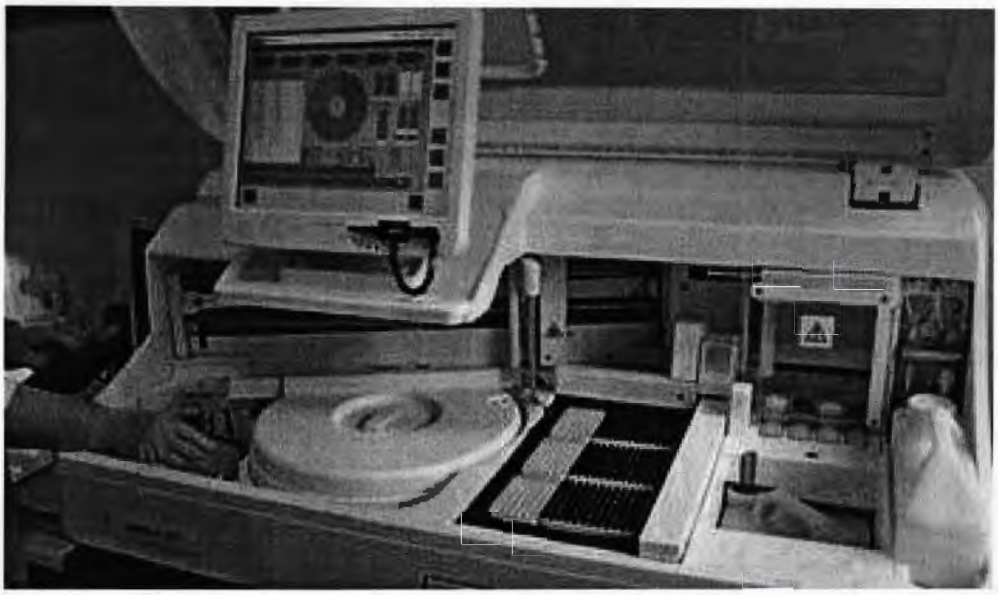

Fig 4.1: The method to detect HCV antibody is patent of Roche diagnostics

Sandwich ELISA principle: Total duration of the test is: 18 minutes.

- In primary incubation 40 µL of patient's sample, 60 µL of a testing reagent containing biotinylated HCV antigens and 60 µL of another testing reagent that contains HCV antigens labelled with a ruthenium complex. All these 3 substances react in a sophisticated way to form a sandwich complex.
- In secondary incubation: After addition of a specific substance named streptavidin – coated microparticles, the complex is bounded to the solid phase by the interface of biotin and streptavidin.
- The final reaction mixture is then extracted into the measuring chamber where the microparticles are magnetically seized onto the surface of the electrode or wall. The unbound material is then washed and removed with the help of Pro-Cell and Pro-Cell, special kind of solution. After applying a particular strength of voltage to the electrode then produced the chemiluminescent emission which is suhsequently measured by a photomultiplier or photo-amplifier.
- Results are obtained automatically by the software application after comparing the electro-chemiluminescence signal recorded from the reaction product of the sample mixture with the signal of the cutoff value formerly obtained by assay calibration (Fig 4.2)

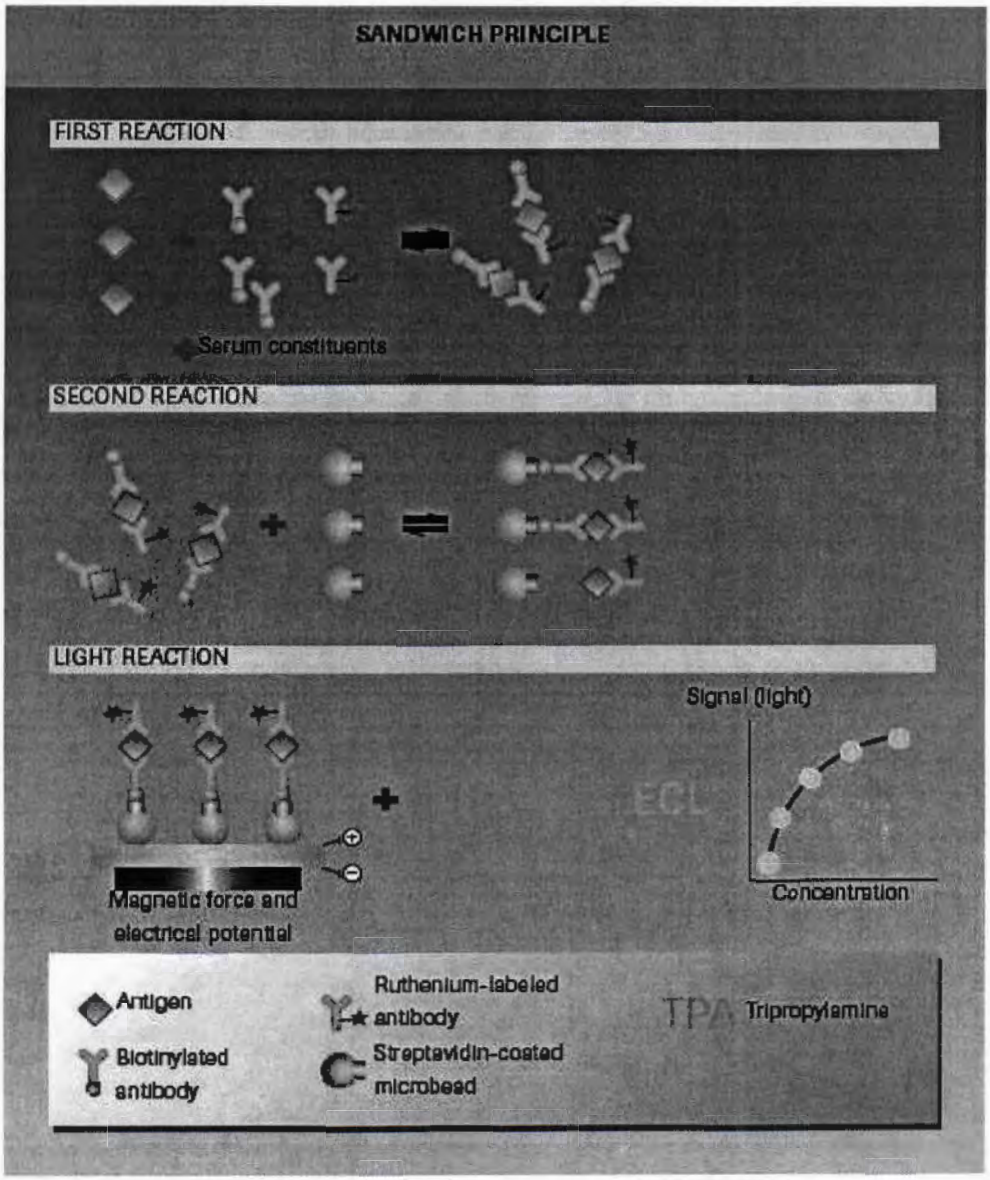

Fig 4.2: Reaction of Anti-HCV in Cobas E411 (adopted from www.roche.com)

Reagent kit of Anti-HCV was taken out from refrigerator and put into the analyser at specific place which is also refrigerated. Work lists were prepared according to the sample IDs. All 6551 serum samples were run on the analyser according to the manufacturer guidelines. These samples were run on different time intervals, in different numbers and at different centres. Serum samples were immediately freezed at -40°C after testing. Analyser gave the results after 18 minutes automatically. 350 out of 6551 serum samples found Positive or Reactive for Anti-HCV. These 350 samples were subsequently tested for HCV RNA by Real Time PCR technique. All appropriate ethical protocols were considerations and followed while handling the samples.

4.2.3 HCV RNA Extraction and cDNA Synthesis

Isolation of HCV RNA of 350 samples was accomplished by guanidium thiocyanate method (Ausubel *et al.*, 1987) after few little changes. 500µl serum sample was taken for RNA extraction after recently prepared lysate buffer, composed of guanidine-HSCN solution, mercaptoethanol and carrier RNA (1µg/ml). Retrieval of HCV RNA was achieved by using isopropanol (alcohol) precipitation method. Retrieved HCV RNA was re-mixed in 10µl DEPC-doubled distilled water and incubated at 95°C for 3 minutes to denature the secondary structure of HCV RNA. Extracted RNAs were further used in the synthesis of cDNA.

Primary strand of complimentary-DNA was prepared by using a specific kit "First Strand cDNA Synthesis Kit" (MBI Fermentas, Catalogue # K1612). Synthesis of cDNA was accomplished by adding 15µl re-suspended HCV RNA in a total 20 µl reaction volume by using 0.2 µg of specific primer, 40 unit of M-MuLV Reverse Transcriptase enzyme, 20 unit of Ribonuclease Inhibitor and 1mM (each) deoxyribonucleotide for 1 hour at 37 degree celsius.

4.2.4 Synthesis of Oligonucleotide

A sole and unique pair of primer (Enomoto *et al.*, 1990) was actually used to intensify HCV RNA isolates from 350 serum samples. The principal sequences of primers that were used are also discussed in Table 4.1. In the NS and 5B reverse primer there is one T›A transversion at the 9th nucleotide position. Oligonucleotides that used in PCR procedure and in the sequencing step were taken from molecular division of Roche Diagnostic Limited, Pakistan. The quantities of the oligonucleotides were obtained by using oligodeoxy-nucleotide quantitative estimation by using the spectrophotometer at the end.

Table 4.1: PCR and its Sequencing Primers

Position of 5' base relative to HCV genomic sequence in Choo *et al.*, 1991

Primer	**Position of 5' base***	**Sequence (5'›3')**
NS5B Forward	7904	TGGGGATCCCGTATGATACCCGCTGCTTTGA
NS5B Reverse	8304	GGCGGAATACCTGGTCATAGCCTCCGTGAA

4.2.4 Polymerase Chain Reaction (PCR)

NS-5B section of HCV RNA genome was used to detect genotype of HCV derivatives of 350 serum samples to amplify DNA segments from NS-5B region of single primer pair (Enomoto *et al.*, 1990). Because of lower viral load of HCV RNA, double-step PCR technique with the same principal primer was established to conduct the procedure. By double-step PCR technique, a 400 bp DNA segment covering region from 7904 to 8304 (Position of 5` base relative to HCV genomic sequence in Choo *et al.*, 1991) was amplified with *Thermus aquaticus* Polymerase enzyme. To evade the risk of cross contamination, different places of the core testing area were signed as pre and post PCR laboratories for respective step of the double-step PCR technique. Each area was consists of a set of dedicated reagents, chemicals, disposables and analysers. (Fig 4.3)

To optimize the primary studies an NS-5B DNA fragment, commercially cloned HCV 1b isolate was used in this testing procedure. Multiple concentrations of DNA template (NS-5B clone), dNTPs, primer and $MgCl_2$ were tested simultaneously at one point of time. Augmented PCR circumstances are present in Table 4.2.

Table 4.2: Augmented PCR circumstances

* Each PCR step was performed as 30 cycles.

Fragment	1st PCR Mix	2nd PCR Mix	PCR
NS5B	¼ of total cDNA	4/50 of the PCR	94 °C 4'
	50 pmol/µl primer	product	94 °C 40"
	1X Taq Polymerase Buffer	50 pmol/µl primer	58 °C 40"
	60µM dNTP	1X Taq Polymerase Buffer	72 °C 40"
	2.5 units of Taq Polymerase	60µM dNTP	72 °C 10'
		2.5 units of Taq Polymerase	

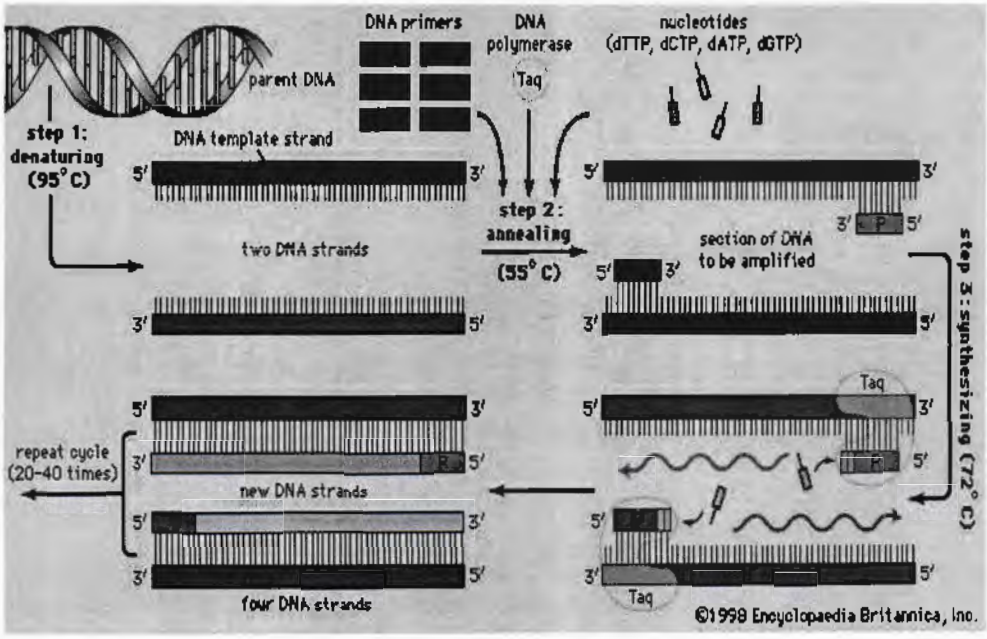

Fig 4.3: PCR Reaction

4.2.5 Gel (Agarose) Electrophoresis method for DNA Fragments

Obtained DNA segments were divided by Agarose gel electrophoresis method at specific concentration of 1.7% of w/v in 1x TAE (Maniatis *et al.*, 1982). 0.20-0.40 µg of EtBr per ml of the agarose gel was used to envision of the obtained DNA. Isolates of DNA were then loaded in the wells of chamber with the help of loading buffer. The electrophoresis method was conducted by giving the gel a 100 voltage for electricity for 01 hour of time. The products obtained by PCR were visualized by UV illuminometer at 302 nm wavelength and the photographs of the gel were taken by a colour printer. The length of DNA fragments was detected by comparing NS-5B fragments with the molecular weight of standards that were loaded and run on the same agarose gel. DNA weight indicators were used in this study that made the detection of DNA fragments very easy.

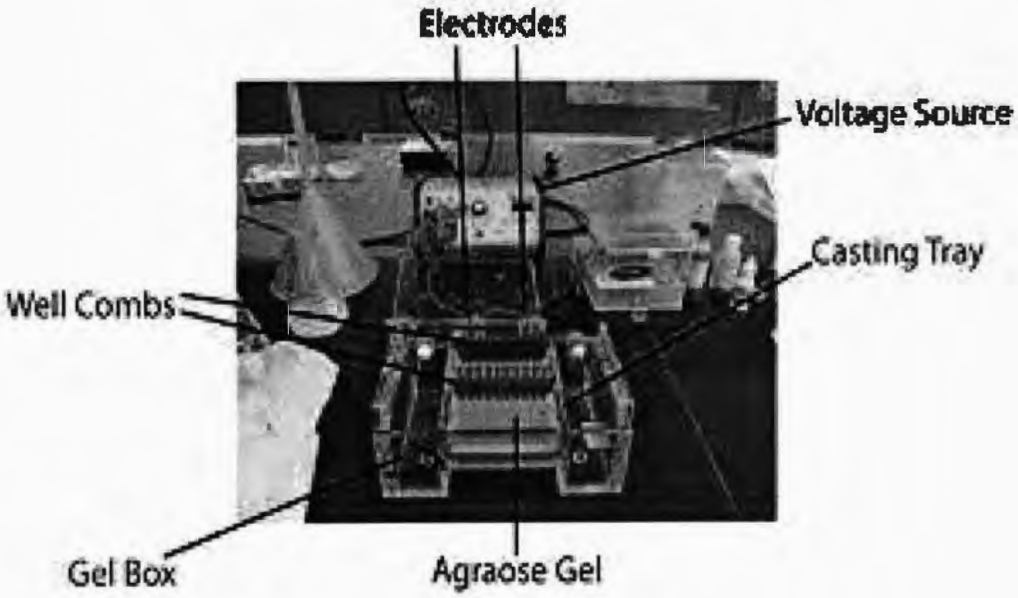

Fig 4.4: Agarose Gel Assembly

4.2.6 DNA Sequencing

The extracted DNA segments were treated with specific and unique "Nucleotide Removal Kit" to remove excess debris of dNTPs and $MgCl_2$ etc. After purifying the final substances, the PCR product was detected by comparing with known standards by using agarose gel electrophoresis procedure. The concluding PCR products having the concentration of 20-100 ng/µl were then sequenced with the same principal primer pair by a DNA sequencer.

To obtain the sequencing reactions "Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit" was used in the laboratory. Each cycle of sequencing method used a unique thermostable DNA polymerase enzyme and di-deoxynucleotides were used in the reaction of polymerization to establish a pure set of DNA fragments with di-deoxynucleotides at the 3' terminus of each fragment. Since each of the four di-deoxynucleotides was labelled with a specific coloured fluorescent dye, the reaction might be took place in the single tube and the sequence may be checked easily. Electropherograms for each sample was examined and potential doubts in the sequence data were addressed by comparing the results of either forward or reverse primers as well as by comparing the HCV sequences in the NCBI database. Genotype of every sample was analysed after sequence analysis.

4.2.7 Analysis of DNA Sequences and Genotype Determination

334 serum samples were run on Cobas 4800 System to detect genotypes of HCV RNA. Cobas 4800 System was consists of 2 fully automated components (Fig 4.2):

4.2.7.1 x 480;

This is a fully automated analyser in which nucleic acid either RNA or

DNA is extracted from the primary samples. User only needs to put samples into the analyser and it will perform all the respective tasks automatically. There is no manual intervention by user so the quality of the product is of very high without any cross-contamination.

Samples were given a unique identity number to track the ID which is very crucial while interpreting the results.

4.2.7.2 z 480;

This is also a unique type of automated analyser used for amplification and detection nucleic acid and genotype. It consists of a best quality thermal cycler that performs all the amplification steps automatically with very accurate time span and concise temperature requirements.

Uniquely identified samples were inserted into the sample bay of this analyser. Analyser examined the samples for HCV genotypes and subtypes by following the procedure mentioned below:

The extracted NS-5B sequences were then compared with genotype-specific reference sequences by Clustal W program (http://workbench.sdsc.edu). The arrangement of the sequences was followed to establish the generic phylogenetic grid to detect the genotypes of the HCV isolates. The grid can also be verified by using the account named and password of "hcv" on this website (http://workbench.sdsc.edu). Genotype of each isolate was determined as the genotype of reference sequence when they follow under same phylogenetic grid.

Fig 4.5: Cobas 4800 System, fully automated analyser for Genotyping

5. RESULTS

5.1 HCV Antibody Results

Total 6551 patients were tested for HCV antibody. This huge sized testing procedure was conducted in 3 different centres of Islamabad; Pakistan Institute of Medical Sciences (PIMS), Islamabad Diagnostic Centre (IDC) and Kuslum International Hospital (KIH). The selection of patients was purely on random basis. 3832 (58.49%) patients were male whereas 2719 (41.51%) were female. (Fig 5.1)

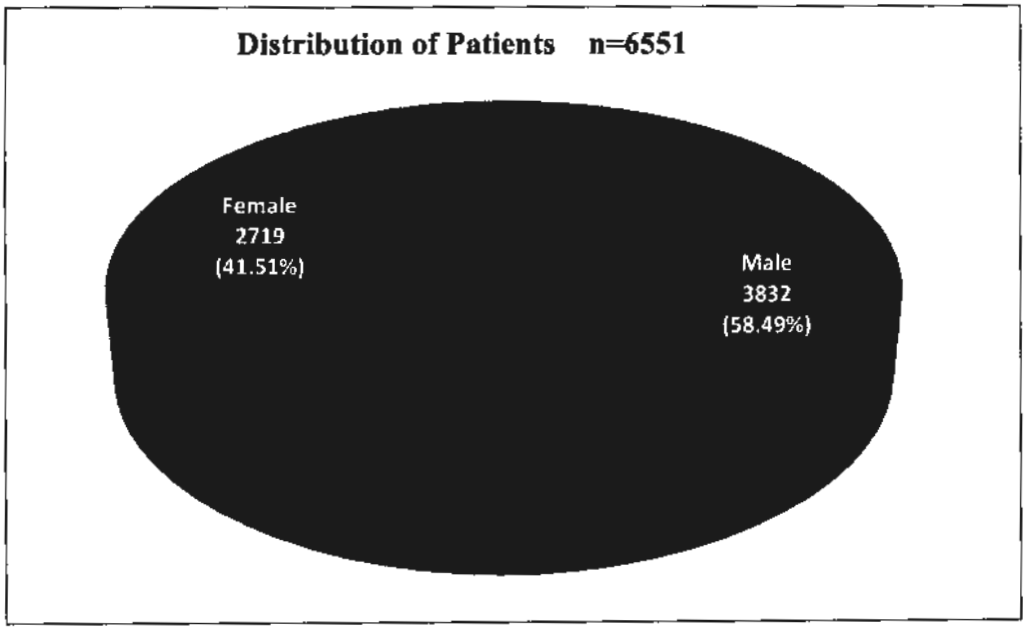

Fig 5.1: Distribution of Patients, Male and Female

Out of 6551 only 350 (5.34%) patients were found Positive/Reactive for HCV antibody (Anti-HCV) and 6201 (94.66%) were given clearance from further subsequent testing. Among 350 HCV antibody positive patients, 212 (60.57%) were male and 138 (39.43%) were female patients.

Table 5.1: Distribution of Anti-HCV Positive Patients, Male and Female

	Anti-HCV Positive Patients (Total n=6551)
Total	350 (5.34%)
Male	212 (60.57%)
Female	138 (39.43%)

5.2 HCV RNA Detection

350 Anti-HCV Positive patients then tested for HCV RNA. HCV RNA was detected in 334 (95.43%) patients while serum samples of 16 (4.57%) patients were negative for HCV RNA. (Fig 5.2)

Gender distribution among HCV RNA detected patients was 202 (60.48%) were male whereas 132 (39.52%) were female. (Fig 5.3)

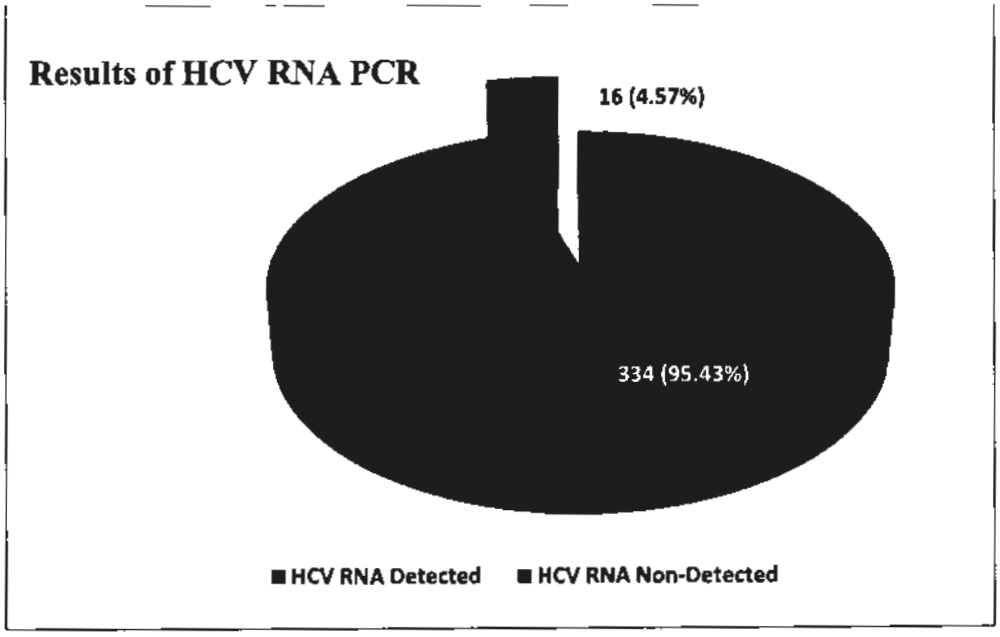

Fig 5.2 Results of HCV RNA (Detected & Non-Detected)

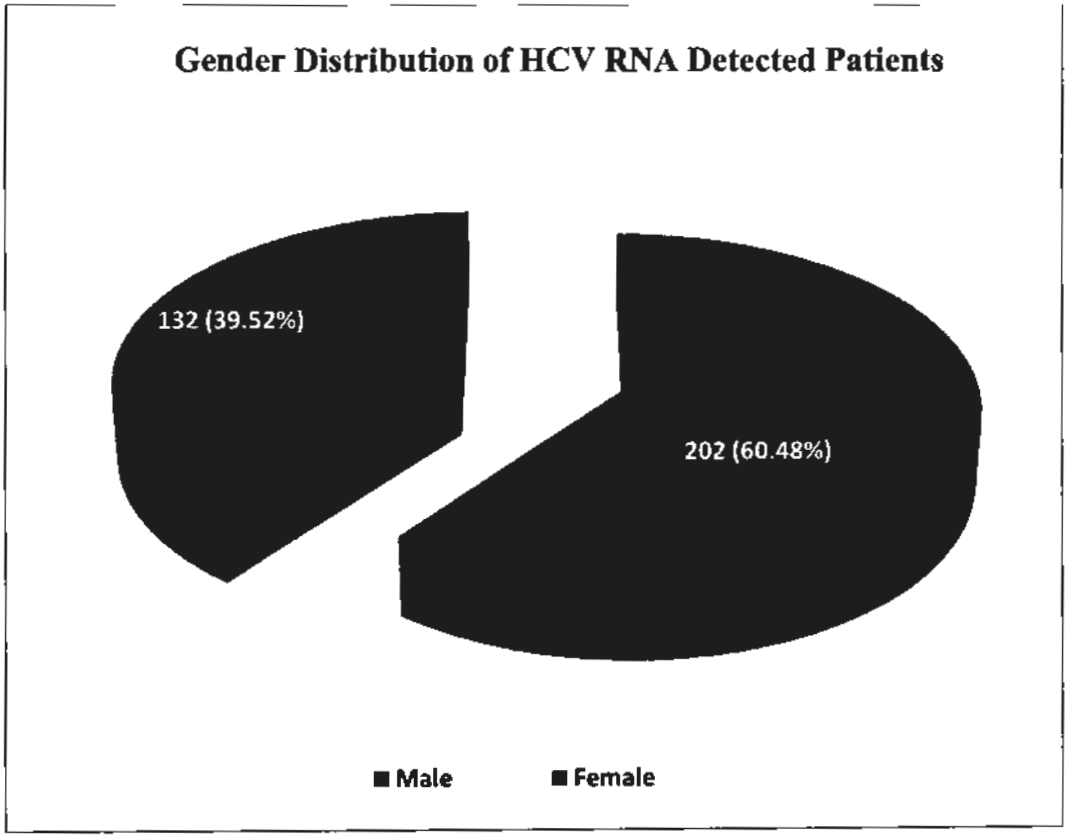

Fig 5.3 Gender Distribution of HCV RNA Detected (Male & Female)

Table 5.2: Distribution of HCV RNA Detected Patients, Male and Female

Distribution of HCV RNA Detected Patients (n=334)		
	Number	**Percentage**
Total	334	
Male	202	(60.48%)
Female	132	(39.52%)

5.3 HCV Genotype Detection

In our laboratory, HCV RNA isolates obtained from 334 HCV RNA Detected patients whom blood specimen were collected from 3 different centres of Islamabad were analysed to determine their genotypes. Among 334, 202 (60.48%) were males while 132 (39.52%) were females.

The findings of HCV genotyping from 5' UTR described that the most predominant genotype in Islamabad was **3 (Three)** in total as found in 314 (94.01%) patients while its subtype distribution was 288 (86.23%) and 26 (7.78%) for **Subtype 3a** and **Subtype 3b** respectively. Genotype **1 (One)** was the second most prevalent type and was found in 19 (5.69%) patients while its subtype distribution was 13 (3.89%) and 06 (1.80%) for **Subtype 1a** and **Subtype 1b** respectively. Genotype **4 (Four)** was also detected with very less frequency that was only 01 (0.30%). Prevalence table is showing this entire information (Table 3.3a).

The studied patients were also divided into 4 age groups in 3 different point of study such as total number of patients (n=6551), anti-HCV Positive patients (n=350) and HCV RNA Detected patients (n=334) . The age groups were;

A. 1 – 20 years,
B. 21 – 40 years,
C. 41 – 60 years and
D. 61 – 90 years.

Table 5.3: HCV genotype prevalence in Islamabad

	Genotype 3a	Genotype 3b	Genotype 1a	Genotype 1b	Genotype 4
Number of cases	288/334	26/334	13/334	06/334	01/334
Percentage (%)	(86.23)	(7.78)	(3.89)	(1.80)	(0.30)
Gender (M/F)	207/81	19/07	08/05	04/02	01/00
Percentage (%)	(72/28)	(73/27)	(62/38)	(67/33)	(100/00)

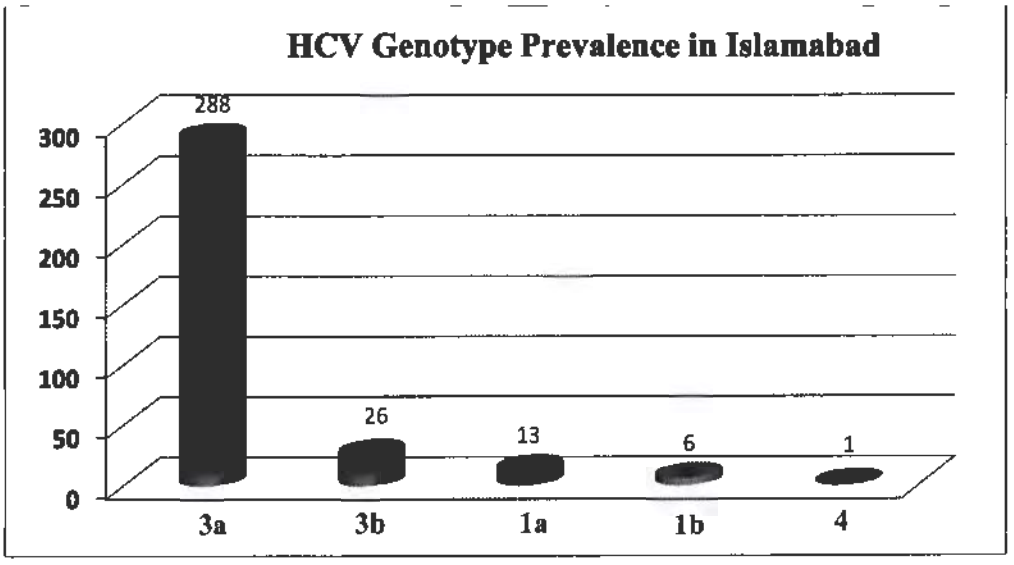

Fig 5.4: HCV genotype prevalence in Islamabad

Table 5.4: Distribution of studied patients in multiple Age Groups

	1 – 20 Years	21 – 40 Years	41 – 60 Years	61 – 90 Years
Total Number	11/6551	1987/6551	3567/6551	986/6551
Percentage (%)	(0.71)	(30.33)	(54.45)	(15.05)
Anti-HCV Positive	00/350	88/350	211/350	51/350
Percentage (%)	(0.00)	(25.14)	(60.29)	(14.57)
HCV RNA Detected	00/334	21/334	199/334	114/334
Percentage (%)	(0.00)	(6.29)	(59.58)	(34.13)

6. DISCUSSION

During the execution of current research study, genotyping of the hepatitis C virus was accomplished. The study subjects included HCV-Ab positive individuals from three different healthcare centres Pakistan Institute of Medical Sciences, Kulsum International Hospital and Islamabad Diagnostic Centre.

The results of this study showed that 89.71% (n=314) of anti-HCV positive patients are confirmed by HCV RNA detection while 10.28% (n=36) anti-HCV positive patients do not have active hepatitis C virus within their blood. Genotype 3 is predominant with its subtype distribution as subtype 3a found 58.86% (n=206) and subtype 3b found 23.71% (n=36). Due to high percentage of HCV-3 in total it is easy to conclude that this HCV genotype is found abundant in our population. Though over all prevalence of HCV infection is high but it is a sigh of relief that majority of cases are treatable and curable. Since genotype 3 responds to conventional therapy of combination Interferon and ribavirin hence all the patients with this genotype can be recovered from this HCV infection provided any secondary infection will not interfere.

Our outcomes affirmed that prevalent genotype in Islamabad is subtype 3a. The number of specimens which could have been used for amplification was restricted due to variability in the NS5B region. Already distinguished 3a subtype test was not amplified from the NS5B region; in any case, one subtype 2a and seventeen subtype 1b tests were amplified besides affirmation from NS5B locale. Similarly 10 extra examples which were not utilized as a part of past research. Genotypes were resolved just from locale of NS5B and every one of them was observed as subtype 1b. One hundred percent concordance was observed among the outcomes of these two strategies and they allowed a right portrayal of genotypes.

The genotypes of hepatitis C virus particular or transient dispersions. In this manner, the increased rate of 3a subtype may be prevalent in Islamabad and northern areas of Pakistan. To decide the dominating genotype in Islamabad, genotyping concentrates on with sera gathered from various districts of Islamabad

ought to be tested.

If we see around the world, especially in Europe, the frequency of 1b subtype raises from north to south. In another European country, Finland, predominance is 8%, whereas in Sicilian, it ranges to 91% of the total (Harrison & Zuckerman, 1997; Messina *et al.,* 2015). The supremacy of 3a subtype in Islamabad might relate with the overall conveyance of the 3a and 3b subtypes in Pakistan. Lower frequencies of various genotypes might be clarified as the intermittent instances of transmission of genotypes from other topographical locales, where endemicity of these genotypes is on a rise.

Taking the predominance rate of 3a subtype infections in Islamabad as high as 58.86%, noteworthy consideration should be particularly given to impacts of 3a subtype on finding, malady movement and medical management to add to an appropriate wellbeing administration strategy. A major critical medical part of 3a subtype infection is the open relationship among infection with 3a subtype and higher danger of advancement of liver illnesses. In the event that we consider the connection of illness term between sickness seriousness in HCV infections, the genuine result of the HCV diseases in Islamabad in near future is expected to be more extreme which might be reflected as the expansion in the mortality and grimness value as the screening of hostile to HCV-Abs have been done since the year 1990.

The interferon-α is the main treatment regime for the HCV infection and is very expensive. The expense every year of life spared in non-cirrhotic patients with interferon is PKR 300,000 to 500,000. The reaction to treatment relies on upon a few elements, for example, age, the genotype and the viraemia. In this way it is useful to incorporate the prescient variables, for example, genotype in the money saving advantage examinations of HCV treatment to get ready arrangements to expand the affectivity in administration of hepatitis C infection. Infection with 1b subtype is among the prescient variables connected with the small proportion of response to the treatment regime. Majority of the infections in Islamabad were attributed to genotype 3. It is valuable to incorporate different variables into notice

before the start of treatment to decide possible responders. Patients affected by the 3 genotype and low viraemia level, more youthful age, deprived of cirrhosis and infection not older than five years are liable to react to IFN. On the other hand, beforehand it has been reported that reaction of patients affected by genotype 3, to interferon might differentiate between nations. To outline an appropriate administration arrangement, the reaction rate in Islamabad ought to be known also. As an outline, the financially savvy strategy for the administration of HCV infection might incorporate the prescient components expressed above on the off chance that we reflect economy of Islamabad.

Among the key matters in HCV infection, is the aversion of new cases by the assistance of analytic tests. The genotype particular affectability of the indicative tests restrains the adequacy of tests in detection of infections with genotypes other than type 3. As the subtype 3a is the transcendent genotype in Islamabad took after by 3b, the screening against HCV antibodies in blood aids the indicative tests and is liable to diminish the quantity of new instances of hepatitis C. It is additionally identified with actuality that the significant transmission course decided in HCV affected patients with endless hepatic ailment in Islamabad is contaminated transfusion of blood and blood components.

The resistance of HCV-3a to IFN-α and expanded danger of improvement of cirrhosis and hepatocellular carcinoma (Kanda *et al.*, 2013) might become major issues in the administration of endless hepatitis infection in Islamabad. In the event that we include the expense and noteworthy symptoms of the IFN treatment the significance of screening hostile to HCV antibodies turns out to be clearer. The pervasiveness of the hepatitis C virus, rises along with the reduced financial and instructive level, it ought to be reflected in planning an appropriate administration strategy against this viral disease and expansion to the prevalent genotype along with the clinical properties of HCV-1b infection. Though in endeavours to advancement of local administration arrangements changes in genotype appropriation after sometime ought to be reflected too. Important game plans ought to be done after genotype determination at time interims.

7. CONCLUSION

This study was designed to check the prevalence of HCV Genotypes in Islamabad because genotype determination is a vital aspect prior to start anti-viral therapy against hepatitis C. Since HCV infection rate is increasing quite aggressively in Pakistan and recurrence infection after unsuccessful therapy is also one of the contributing factors in that rate. After knowing HCV genotype before start of treatment is very beneficial to cure the infection within appropriate time span as well as it will also help in lowering the HCV infection prevalence rate in Pakistan. This study concluded that the predominant HCV genotype in Islamabad is 3 (Three) with its subtype distribution of 3a and 3b followed by 1 (One) and its subtype classification 1a and 1b that is alike to other cities where such experiment was conducted so far.

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