A STUDY OF HEPATITIS C VIRUS AND ITS MOLECULAR EPIDEMIOLOGY IN THE LOCAL POPULATION OF THE FAISALABAD REGION.

BY

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To

The Controller of Examinations,
University of Agriculture,
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"We, the Supervisory Committee, certify that the contents and form of thesis submitted by Mr. Nasir Ahmad Regd. No. 88-ag-221 have been found satisfactory and recommend that it be processed for evaluation, by the external Examiner(s) for the award of degree."

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Member:
(Dr. Javed I. Sultan)
To
My Late Parents

To
My Wife and Little
Kids
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List of abbreviation:

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>ALT</td>
<td>Alanine-aminotransferase</td>
</tr>
<tr>
<td>AS</td>
<td>Antisense</td>
</tr>
<tr>
<td>ASO</td>
<td>Allele Specific Oligonucleotide</td>
</tr>
<tr>
<td>bDNA</td>
<td>Branched Chain DNA Assay</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxy Ribonucleotides</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immuno Sorbet Assay</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>LFT</td>
<td>Liver Function Test</td>
</tr>
<tr>
<td>(M-MLV)</td>
<td>Malony Murine Leukemia virus</td>
</tr>
<tr>
<td>N-PCR</td>
<td>Nested PCR</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>R-PCR</td>
<td>Regular PCR</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SGPT</td>
<td>Serum Glutamate-Pyruvate Test</td>
</tr>
<tr>
<td>SSPE</td>
<td>Sodium chloride sodium thiophosphate citrate</td>
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<tr>
<td>SDS</td>
<td>Sodium dodesyl sulphate</td>
</tr>
<tr>
<td>TMB</td>
<td>3, 3', 5, 5' tetramethylbenzidine</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
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INTRODUCTION
1. **INTRODUCTION**

Viral hepatitis is a major liver disease and health problem all over the world. It is caused by at least six distinct viruses named as hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis delta virus (HDV), hepatitis E virus (HEV) and a recently found hepatitis G virus (HGV) each belonging to entirely different family of viruses except HCV and HGV. They have little in common except the target organ they affect, the liver and a certain degree of shared epidemiology (Purcell, 1994).

The hepatitis C virus (HCV) was found to be major cause of non-A, non-B (NANB) hepatitis in the world (Choo et al. 1989). HCV have infected an estimated 170 million persons worldwide and represents a viral pandemic and it has five times more widespread infection as with the human immunodeficiency virus type 1 (HIV-1) (Lauer et al. 2001). HCV is a major cause of parenterally transmitted, acute and chronic hepatitis. This virus has been reported to be associated with the pathogenesis of autoimmune chronic hepatitis, liver cirrhosis, primary hepatocellular carcinoma and variety of other extra-hepatic disorders.

HCV infection is worldwide health problem. HCV has a high propensity for inducing life-long persistent infections that can progress to significant liver diseases including liver cirrhosis and hepatocellular carcinoma (Houghton et al. 1991, Lanford et al. 2001). Approximately 3% of the world’s population is chronically infected with HCV (Anon., 1997). It is responsible for parenterally transmitted, acute and chronic hepatitis. HCV infection is often indolent and asymptomatic and the general characteristics of this disease can be summarized as persistent infection in the majority of exposed individuals, associated with wide spectrum of histological damage and fluctuating biochemical
expression, low tendency towards spontaneous recovery, high prevalence of autoimmune phenomenon and a very weak or no immunity against re-infection (Camps et al. 1995).

The vast majority of persons who contract HCV infection become persistently infected, and most also develop chronic liver disease (Alter et al. 1998). The persistent infection (more than 50%) due to HCV usually results in chronic active hepatitis (Saito et al. 1990). The mechanism, by which such a high rate of persistent infection is established, appears to be related to lack of development of an effective neutralizing immune response. Like other viruses, the substantial heterogeneity of HCV genome is the result of mutations that occur during viral replication (Bukh et al. 1995).

Within an infected individual, the genetic heterogeneity of HCV consists of population of closely related, yet heterogeneous, sequences called quasispecies, which result from the rapid development of mutations in a hyper variable region (HVR1) within one of envelope proteins. Patients infected with HCV, mount a humoral immune response to epitopes of HVR1, but sequential changes in the consensus sequence of HVR1 during infection results in the generation of variants that are not recognized by pre-existing antibodies (Bukh et al. 1995, Farci et al. 1994)

HCV infection is common worldwide but there are different prevalence rates in different countries. Data on the incidence of HCV in the general population are scarce. Spontaneous viral clearance occurs in 10-25% of infected individuals after acute infection but the controversy yet exists regarding the frequency of spontaneous clearance during the natural course of HCV infection in the general population (Kondili et al. 2002)
In some areas of the world, the prevalence of HCV remains very high suggesting that some, yet unknown vector might be involved in its transmission in those areas. The incubation period for acute HCV infection following transfusion or accidental needle stick has been reported to average 6 to seven weeks, but may range 2 to 26 weeks (Sceff, 1991). The course of acute hepatitis is variable, although its characteristic feature is fluctuating Alanine aminotransferase (ALT) patterns. Normalization of ALT may occur and suggest full recovery, but is frequently followed by symptomless ALT elevations later, indicating chronic disease. In most cases patients with fulminate non-A non-B hepatitis, evidence of HCV infection has been found (Wright, 1993) and constitutes massive medical threat, underscoring the urgent need for anti-HCV vaccines and antiviral agents (Koshy et al., 1996).

HCV has been classified on the basis of genomic organization as a new genus (Hepacivirus) in the Flaviviridae family (Mayo et al., 1998). HCV is a single positive-stranded RNA virus having a distinct genomic organization (Overby, 1993). The genome of HCV consists, about 9400 nucleotides that code for a poly-protein of 3011 amino acids. The structural regions encode the core and envelope of the HCV while the nonstructural regions encode enzymes and membrane binding sites. Non-coding as well as core regions are highly conserved while other regions of the HCV genome are variable (Hess, 1994).

Although a certain degree of immunity appears to be induced following infection, it fails to control the infection. A number of possible reasons for the failure to mount a protective immune response are being studied and in particular, there is growing evidence that typically, HCV infections are associated with low viral titer which may account for low antigenic stimulus and rapid evolution of mutant viruses with altered B and T cell
epitopes and also virus particles are closely associated with immunoglobulin, thus could result in masking of antigenic determinants (Koshy et al. 1996).

Presently interferon is the only approved therapy for chronic hepatitis occurring as a consequence of HCV infection. Because interferon is expensive, has a large number of unwanted side effects and its efficacy is not guaranteed, many physicians have limited the use of this therapy to those with histologically advanced only (Van Thiel et al. 1995).

A number of factors may influence the progression of liver disease in patients with chronic HCV infection. In several studies, an association between disease severity and HCV genotype has been found. Patients with histological severe chronic hepatitis and cirrhosis are more likely to be infected with genotype 1b than with patients with mild or moderate disease (Nousabum et al. 1993). However another study did not support this association (Poynard et al. 1997). One factor strongly correlated with severe disease is alcohol-induced liver injury (Koff et al. 1995) and other factors including older age at infection, male gender and immunodeficiency (Poynard et al. 1997). Many advances have been made during last 10 years including characterization of HCV with its genetic diversity, development of third generation antibody diagnostic tests, better understanding of epidemiology and its treatment with alpha-interferon (Poel et al. 1994).

HCV as well as other members of *Faviviridae* family might enter the cells by binding to low density lipoproteins (LDL) receptors (Agnello et al., 1999). HCV circulates in the sera of infected individuals with significantly lower concentration than HBV (Farci et al., 1991), therefore HCV can not be detected without amplification technology. Presently the most efficient and sensitive technique in HCV detection is reverse transcriptase-polymerase chain reaction (RT-PCR). This procedure has
dramatically improved the sensitivity of many diagnostic techniques, since ELISA testing got many limitations including 2 to 12 months window of seronegativity after acute infection, and occasionally false antibody reaction (Caldwell et al. 1993). RT-PCR can be used for the conformation of HCV infection in individuals with positive anti-HCV antibody test, early diagnosis of acute HCV attack and follow up of antiviral drug treatment and screening of blood donors for safe transfusion.

Although our understanding of the molecular biology of HCV has progressed rapidly, little is known about its general characteristics because in vivo studies have been limited to HCV infected chimpanzee only. Development of efficient in vitro culture systems for HCV propagation or readily available in vivo models are priorities for its comprehensive study. HCV replication has been described in liver tissue as well as in peripheral blood mononuclear cells (PBMC) from infected patients (Willems et al. 1994).

Chronic HCV infection and type-II diabetes mellitus may also cause devastating long-term complications in a significant minority of affected patients. From the hepatologist’s viewpoint, type-II diabetes mellitus seems to be more common in HCV infected persons. It has been demonstrated earlier that the patients with HCV infection are five times more likely to have type-II diabetes mellitus than those who are not. regardless of sex, body mass index, or severity of liver disease (Allison et al. 1994).

Enzyme Linked Immunosorbent Assay (ELISA) licensed test for the detection of HCV antibodies (anti-HCV) has been evolved since 1990 and third or even forth-version assay is now available that detects anti-HCV antibodies in 95% or more of patients with HCV infection (De-Medina et al. 1995) and are the only screening tests in most clinical laboratories of the world, which are not so specific and sensitive enough especially, for
early diagnosis of HCV infection. Their efficacy is still limited by several factors; a. these assay do not detect anti-HCV in all infected persons, b. these assays do not distinguish between onset of acute illness and seroconversion due to 2-12 months of window period of seronegativity after acute infection and occasionally gives false antibody reaction (Caldwell et al. 1993). These assays also give false results in persons who have cleared/recovered from HCV infection and in patients on antiviral therapy.

Although vast studies on HCV like HIV are being carried out all over the world due to its challenging infection and subsequent chronic disease, no or very little data is available about HCV infection and its molecular epidemiology in the local population of Pakistan especially, in this area. Present study has been designed to know more about HCV infection and its molecular epidemiology in liver disease patients, HCV patients on anti-viral therapy, diabetics as well as in volunteers coming to HCV screening camp from the local population of this region. It was also taken to see the response rate of combined (Interferon plus ribazole) therapy against the prevalent genotypes of HCV in the area.

Objectives:

1. To find the prevalence/incidence of HCV infection and its genotypes in the local population.
2. Evaluation of RT-PCR results of HCV infection with the biochemical and serological findings.
3. Epidemiological study of the role of HCV genotypes in the management and treatment of HCV infection by antiviral therapy.
4. To investigate any relationship between HCV infection and clinically diagnosed diabetes mellitus type-II.
REVIEW OF LITERATURE
2. REVIEW OF LITERATURE

Hepatitis C virus (HCV) is a blood-born pathogen that poses a significant threat to public health worldwide. A significant number of parenterally transmitted viral hepatitis cases in the 1980's could not be ascribed to any of the then known hepatitis viruses (Hepatitis A virus, hepatitis B virus and delta virus). HCV was shown to be cause of most cases of non-A non-B (NANB) (Choo et al., 1989) who also determined genetic organization diversity and the nucleotide sequence of the RNA genome for the first time. In the present section, research about HCV and its molecular epidemiology have been reviewed under different topics.

2.1. Clinical Features and Natural History.

HCV has not very specific clinical features and it varies from individual to individual depending upon the age, gender, viral load and genotype etc. In substantial number of cases, the infection is asymptomatic and clinical symptoms appear only on the last stages of the disease. The incubation period for acute hepatitis following HCV infection has been reported to 6 to seven weeks on the average but may range from 2 to 26 weeks (Scheff et al., 1991). Children and adults with acute hepatitis are typically asymptomatic or have a very mild clinical illness. Persistent HCV infection develops after the onset of acute hepatitis in most persons (≥85%), even in those with no biochemical evidence of active liver disease (Alter et al., 1992).
Locasciulli et al. (1995) reviewed HCV serum markers and liver disease in children with leukemia and summarized serologic profiles and clinical features of HCV infection. According to them the pattern of liver disease was rather homogenous and resulted in persisting ALT elevation during chemotherapy often with drastic reduction during high-dose chemotherapy, followed by sharp exacerbations of liver cell necrosis. ALT normalized after chemotherapy withdrawal in most cases, despite persisting viremia. They observed that among a series of 119 patients followed for at least 10 years after therapy, none had developed clinical decompensated liver disease and only 6% still had abnormal ALT levels.

Alberti et al. (1999) reported that HCV infection might be resolved without any clinical signs of liver disease in individuals exposed to low dose inoculums and that those cases might have developed T cell immunity even in the absence of anti-HCV seroconversion. Rates of complete biochemical and virological resolution of acute hepatitis C range between 10 and 50%, were probably affected by the route of infection, size and type of inoculums and acute phase clinical features. Chronic HCV infection might develop with or without ALT abnormalities and with or without chronic inflammation and increasing fibrosis in the liver. Studies conducted in patients who acquired hepatitis C by blood transfusion about 15-25 years ago indicated that 20-30% of them had now progressed to cirrhosis, including 5-10% with end stage liver disease and 4-8% who died of liver-related causes.

The study on natural course of HCV infection, 18 years after an epidemic outbreak in a plasmapheresis center was carried out by Datz et al. (1999). The aim of the study was to determine the natural course of HCV infection in a well-characterized group of patients. HCV infection was confirmed using second and third generation ELISA test
kits. HCV RNA was detected by a polymerase chain reaction (PCR). Eighteen patients had abnormal liver enzymes and 17 were HCV RNA positive, all of whom were infected with genotype 1a. Ninety per cent of the cohort showed evidence of chronic HCV infection with 50% having progressive liver disease and 20% cirrhosis after 18 years of acute infection. It was concluded that host factors were important in determining disease outcome.

Clinical significance of HCV genotypes and quasispecies was studied by Farci et al. (2000). It was found that the quasispecies nature of HCV provides a large reservoir of biologically different viral variants that might have important clinical implications for viral persistence by immune escape mechanisms, for the generation of antiviral drug resistance, and for the development of an effective vaccine. The article reviewed the state of the art on the biologic and clinical implications of the genetic variability of HCV.

Jonas, (2001) studied that hepatitis C infection in children is associated with a unique set of challenges for clinicians and investigators. The natural history of this infection in most children was either more benign or significantly prolonged than that of infection acquired in adulthood. Reasons for this difference in natural history must be explored and possibly even exploited in the care of adult patients with HCV infection. Identification of appropriate pediatric candidates for treatment and definition of optimal therapy for these children are required. There were important differences in the clinical features, natural history, and response to therapy between pediatric and adult patients with HCV infection.

Kyrlagkitsis et al. (2003) studied liver histology and progression of fibrosis in individuals with chronic hepatitis C and persistently normal ALT. They performed a
retrospective review of patients with chronic HCV infection and persistently normal ALT to compare clinical and histological features with those in patients with abnormal liver biochemistry. Ninety-one HCV RNA-positive patients with persistently normal ALT who had a liver biopsy between 1993 and 1999 were identified. Clinical, histological, and epidemiological features in the group were compared with those found in 94 patients with abnormal ALT. Although overall necroinflammatory score and fibrosis were significantly lower in those with normal ALT, none had normal liver histology, and 15 (16%) patients with normal ALT were found to have significant necro-inflammation with a score of 5 or greater.

2.2 Epidemiology of HCV

Lin et al. (1994) studied the possible role of high-titer maternal viremia in perinatal transmission of HCV. At delivery, maternal blood was taken and anti-HCV titer was determined and HCV RNA measured in each serum sample by reverse transcriptase polymerase chain reaction (RT-PCR). The results implied that high-titer maternal viremia and normal spontaneous delivery might allow more HCV to infect the neonate intrapartum, therefore establishing perinatal transmission.

Feucht et al. (1995) demonstrated that tear fluid of the HCV carriers could be infectious. Up to 20 to 40% chronically infected patients with HCV; the mode of transmission was still unknown. It was concluded that smear infection with tear fluid might play role in virus transmission.

Majid et al. (1995) investigated prevalence of HCV infection among a group of intravenous drug users (IVDUs) in West Suffolk (England) and compared with the
prevalence of infection with hepatitis B virus (HBV) and human immunodeficiency virus (HIV). It was found that HCV antibodies were present in 59% of those tested; by comparison 22% had antibodies to HBV and 1% antibodies to HIV. HCV RNA was found in 44% of those with HCV antibody. HCV genotype 1 was the most prevalent although both genotypes 2 and 3 were also represented.

Paccagnini et al. (1995) investigated perinatal transmission and manifestation of HCV infection in a high-risk population in 70 mother/infant pairs. Seventy-six percent of the mothers were co-infected with human immunodeficiency virus (HIV) and 79% had a history of drug addiction. HCV RNA was detected in (20%) infants: 12% in infants born to HIV-negative mothers; and 23% (12 of 53) in infants to HIV-positive mothers. The rate of vertical transmission was significantly higher in vaginally delivered infants than in those delivered by cesarean section (32% vs. 6%; P < 0.05).

Tanzi et al. (1995) studied hepatitis C, its modes of transmission and preventive measures in Italy, where it accounted for approximately 20% of all cases of acute viral hepatitis. It was more frequent among young persons (15-24 years old) and in males than in females (3:1). Since the implementation of anti-HCV screening and restrictions on donor eligibility, the incidence rate had been dropped and changes in the HCV transmission patterns were also observed.

Simmonds, (1996) reviewed virus assembly and release, the variability of HCV and its classification into genotypes, the geographic distribution of HCV genotypes, and the biologic differences between HCV genotypes. Molecular epidemiology of HCV infection and HCV genotyping assays in terms of reliability and consistency were also
discussed. The study would be valuable in documenting the spread of HCV in different risk groups and evaluating alternative routes of transmission.

Feucht et al. (1997) determined the prevalence of HGV and HCV infection by RT-PCR in 777 individuals with and without risk factors for viral transmission via blood. From the results they concluded that transmission of HGV and that of HCV were favored by similar risk factors.

Kocabas et al. (1997) studied HBV and HCV infections in 137 Turkish children with cancer, undergoing chemotherapy using ELISA and PCR techniques. 47.45% patients with cancer were positive for hepatitis B surface antigen (HBsAg). HCV specific antibody (anti-HCV) was detected in 5.8% patients. Ten (13.9%) of the 72 patients, negative for HBsAg, had circulating HBV-DNA and 7 (5.4%) of the 129 cancer patients, negative for anti-HCV, had circulating HCV-RNA. It was concluded that HBV and HCV infections were common among Turkish children with cancer.

Noguchi et al. (1997) conducted an epidemiological study in 857 adult inhabitants of K area, in Japan, an area endemic for HCV. The prevalence of antibody to HCV was 26.3%. The HCV genotype 1b was the most prevalent (89.3%). Nucleotide sequences of the HCV core region from 23 participants with type 1b showed that the isolates were distributed into more than 1 group. No significant differences in the presence of anti-HCV prevalence were observed between the wives of men positive for anti-HCV (33.3%) and age-matched women (36.4%), or vice versa. The findings suggested that the various strains of HCV type 1b were transmitted via medical procedures.
Smith et al. (1997) used molecular techniques to see the epidemiology of HCV at several different levels. At a global level, the time of divergence of the diverse HCV genotypes isolated from different geographical regions was estimated. Analysis of virus sequences provided evidence for a common source of infection in several outbreaks of HCV infection. Finally, sequence analysis was used to investigate the vertical or horizontal transmission of HCV.

Pybus et al. (2001) used a new model of HCV spread to investigate the epidemic behavior of the virus and to estimate its basic reproductive number from gene sequence data. Significant differences in epidemic behavior among HCV subtypes suggested that these differences were largely the result of subtype-specific transmission patterns. Their model built a bridge between the disciplines of population genetics and mathematical epidemiology by using pathogen gene sequences to infer the population dynamic history of an infectious disease.

Ding et al. (2003) carried out a molecular-based epidemiological survey of hepatitis viruses, including HBV, HCV, and HEV, in Harbin, China, in 358 subjects consisted of 132 healthy blood donors and 226 liver disease patients. The infection rate among healthy subjects was 14.4% for HBV and 2.3% for HCV while among liver disease patients; the infection rates were 72.6% for HBV and 7.5% for HCV, respectively.

Molecular epidemiology of HCV in Uzbekistan was investigated by Kurbanov et al. (2003). Preliminary serological screening of 1,269 subjects revealed 6.5% anti-HCV-positive in a general population, 27.1% in patient groups, and 51.7% among intravenous drug users. HCV genotypes results of 104 anti-HCV positive subjects showed that
Genotype 1b (64.2%), was the most prevalent while genotype 3a was identified in 25.0% cases.

Marx et al. (2003) determined the association between sexual exposure and HCV infection in urban Chennai, India. It was found that HCV infection was not associated with number of sex partners for men or women. Women were more likely to be HCV infected if they reported previous genital ulcer disease. Men were more likely to be HCV infected if they were HSV-2 infected (AOR, 3.85; 95% CI, 1.18-12.6) or reported having had sex with men (AOR, 3.61; 95% CI, 1.00-13.1).

Polatti et al. (2000) analyzed vertical transmission of HCV and follow-up of newborns from infected mothers in 36 babies born to HCV-positive and HIV-negative pregnant women at the University of Pavia. All babies proved HCV-Ab positive at birth, but only one case (2.7%) proved infected by PCR analysis. Different patterns of HCV-Ab clearance were noted in the 35 non-infected babies. Of 24 babies from HCV-RNA-positive mothers, HCV-Ab reached zero in 24 months while in 11 babies from HCV-RNA-negative mothers, the antibodies disappeared at 12 months. It was concluded that the risk of vertical transmission in babies born to HCV-RNA negative mothers was very low and the clearance of maternal antibodies was set within 12 months of follow-up. Mothers positive to HCV-RNA had a higher risk of transmitting the virus to their offspring.

Abacioglu et al. (2000) investigated the molecular epidemiology of HCV type-2a infections in patients undergoing haemodialysis in a Turkish hospital. Of nine HCV-infected patients, four were infected with type 2a, four with type 1b and one with type 1a viruses. Since type 2 HCV infections in the Turkish population were rare, the possibility
of nosocomial infection was investigated by the PCR in the NS5b region. The results showed that the patients were infected with the same HCV type 2a strain. Seroconversion and clinical data suggested that those patients might have been infected on different occasions by possibly more than one mode of transmission.

Pybus et al. (2001) investigated the epidemic behavior of HCV and estimated its basic reproductive number from gene sequence data. They found significant differences in epidemic behavior among HCV subtypes and suggested that the differences were largely the result of subtype-specific transmission patterns.

Dore et al. 2003 evaluated epidemiology of HCV infection in Australia and reviewed evidences, including HCV notification data obtained through public health surveillance systems and HCV seroprevalence surveys among high-risk populations. Over the period 1990-2000 approximately 160,000 notifications of HCV infection were received by State and Territory health jurisdictions. Approximately 210,000 people were estimated to be living with HCV infection in Australia, with an estimated 80% having acquired their infection through injecting drug use. An estimated 16,000 new infections occurred annually. Despite the widespread introduction of needle and syringe programs in the late 1980s, HCV transmission continued at high levels among current injecting drug users (IDUs).

2.3. Molecular Biology.

Cloning of the cDNA of the HCV genome from a healthy carrier in an aboriginal community of Taiwan, with high prevalence of HCV infection, was done by Chou et al. (1991). They compared the nucleotide and deduced amino acid sequences from the 583
nucleotides long cDNA with those of previously reported clones. The sequences have 93.7%, 93.1% and 80.4% homology at the nucleotide level and 96.9%, 96.7% and 91.85% homology at the amino acid level, respectively.

Houghton et al. (1991) discussed the molecular biology of the HCV, its diagnosis, and control of the viral disease. A specific diagnostic assay for the diagnosis of anti-HCV antibodies was developed, using purified peptides derived from the recombinant yeast expressing a small part of the HCV genome. The data obtained by using this immunoassay indicated that HCV was the predominant cause of post-transfusion NANB hepatitis around the world and a major agent of chronic liver disease and hepatocellular carcinoma (HCC).

Tokita et al. (1994) characterized by sequencing five isolates of HCV -RNA from patients with chronic liver disease in Nepal, that were not classifiable into the known genotypes using PCR with type-specific primers deduced from the HCV core gene and compared with each other and with reported sequences of HCV isolates of various genotypes. They were more similar to a reported HCV isolate (NZL1) of genotype V/3a (in 81.6 to 84.1% of their nucleotides and 85.7 to 88.7% of the deduced amino acid sequence) compared with the genotypes I/1a to IV/2b (in 69.3 to 74.7% and 72.3 to 77.4%, respectively). Hence they were considered to be variants of the third major group (group 3). The five HCV isolates shared 81.3 to 85.2% of nucleotide sequence and 85.4 to 89.3% of deduced amino acid sequence. Thus they were substantially different from each other.

Tagawa et al. (1995) studied the infection of human hepatocyte cell lines with HCV in vitro. Hepatocyte related cell lines, namely human embryonic hepatocyte cell
line (WRL68) and hepatoblastoma cell line (Hep G2), were tested for the ability to support HCV replication in vitro. The replicative intermediate of the minus strand HCV RNA was newly transcribed from the inoculated virus, and was stable in WRL68 cells over a period of 62 days. In Hep G2, transcription of the minus strand HCV RNA was detected until 39 days after infection, but transcription and secretion of viral progeny could not be demonstrated.

Koshy et al. (1996) evaluated HCV protein epitopes for the development of vaccine. The specificity of the immunoresponses of the infected patients suggested that responses directed at certain viral epitopes might be associated with less aggressive disease and possibly good interferon response and virus clearance. The identification of such epitopes might hold the key for future development both of prophylactic and therapeutic vaccines.

Ito et al. (1996) established a system for cultivation of HCV in primary hepatocytes culture from patients with chronic hepatitis C that resulted in release of high titer infectious virus. A significant amount of HCV RNA was observed in the cells and supernatant during cultivation. Negative-strand RNA, regarded as a maker of viral replication, could be detected by a small strand-specific RT-PCR method and the HCV core protein could be detected by immunofluorescence microscopy. The results indicated that HCV could replicate in cultured hepatocytes, and infectious virion were released into the supernatant. This cultivation system should facilitate the study of HCV genomic heterogeneity, infection and replication.

Theamboonlers et al. (2002) performed phylogenetic analysis based on the virus core region and identified distinguished HCV genotypes 1-6 as well as subtypes in
Thailand. Among 100 plasma samples randomly selected from blood donors positive for antibodies to HCV (anti-HCV) 90 (90%) were found positive for HCV RNA and 77 of them were subjected to nucleotide sequencing. The following types and subtypes were identified in that group: 1a in 16 samples (20.8%), 1b in 14 samples (18.2%), 3a in 29 samples (37.7%), 3b in 5 samples (6.5%), and 6a in 13 samples (16.9%). Although that study allowed identification and characterization of HCV among blood donors, more extensive studies were needed to explore the HCV distribution in other population groups and in geographical regions.

Cantaloube et al. (2003) examined HCV evolution in the largest cohort of HCV-infected blood donors (BDs)/blood recipients (BRs) reported to date (25 pairs). A molecular analysis of partial sequences in the E1 (envelope) and NS5-B (polymerase) genes showed that the evolution of dominant strains was qualitatively and quantitatively different in BDs and BRs. The evolutionary rate was significantly higher in BRs, in which most substitutions observed were synonymous. These findings should be taken into account for the modeling of the long-term evolution of HCV and their possible contribution to improve our understanding of HCV natural history.

Van-Compernolle et al. (2003) found that CD81 was a receptor for HCV envelope glycoprotein E2. Although the binding of HCV-E2 with CD81 was well documented the role of that interaction in the viral life cycle remained unclear. Host specificity and mutagenesis studies suggested that the helix D region of CD81 mediated binding to HCV-E2. The results provided an important proof of concept that CD81-based mimics could disrupt binding of HCV-E2 to CD81.
2.4 Genetic Diversity.

Genetic organization, diversity and the nucleotide sequence of the RNA genome of the human HCV from overlapping cDNA clones were determined by Choo et al. (1991). The sequence had single large open reading frame that could encode a viral polyprotein precursor of 3011 amino acids. The nucleotide sequence upstream of that large open reading frame had a substantial similarity to the 5' termini of pestivirus genomes. The polyprotein also had significant sequence similarity to helicases encoded by animal pestivirus, plant potyvirus and human flavivirus. Significant genome diversity was apparent within the putative structural gene of different HCV isolates, suggesting the presence of closely related but distinct genomes.

Chou et al. (1991) cloned the cDNA of HCV genome from a healthy carrier in an aboriginal community in Taiwan with high prevalence of HCV infection. The nucleotide and deduced amino acid sequences from the 583 nucleotides long cDNA were compared with those of previously reported clones. They had 93.7%, 93.1% and 80.4% homology at the nucleotide level and 96.9%, 96.9% and 91.8% homology at the amino acid level, respectively.

Han et al. (1991) characterized the terminal regions of HCV-RNA and identified conserved sequences in the 5' untranslated region and poly (A) tails at the 3' end. Their analysis of those sequences showed that nucleotide sequence in the 5' untranslated region was highly conserved among HCV isolates of widely varying geographical origin. The polyprotein synthesis was initiated through internal ribosome binding site at nucleotide
342. The relative position of short open reading frames in some regions of HCV genome was similar to that of pestivirus genome.

Dusheiko et al. (1994) studied sequence variability of HCV and its clinical relevance. Cloning of RNA amplified from patients infected with HCV confirmed the heterogeneity of hepatitis C. The variability of HCV suggested a two-tiered classification: the nomenclature comprised 'types' corresponding to the major branches in a phylogenetic tree and 'subtypes', corresponding to the more closely related sequences within some of the major groups. In many European countries genotype distributions varied with the age of patients, reflecting rapid changes in genotype distribution with time within a single geographical area.

Davidson et al. (1995) surveyed major genotypes and subtypes of HCV using RFLP of sequences amplified from the 5' non-coding region. Using the enzymes HaeIII-Rsal and HinfI-Mval, followed by cleavage with BstU1 or ScaI, it was possible to identify and distinguish HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5 and 6 in 723 blood donors in 15 countries; the largest survey to date, covering a wide range of geographical regions (Europe, America, Africa and Asia). The results, combined with a review of the existing literature, indicated the existence of several distinct regional patterns of HCV genotype distribution.

Kao et al. (1995) found quasispecies of HCV and genetic drift of the hypervariable region in chronic type C hepatitis. The results showed that quasispecies of HCV existed and patients with different courses of chronic hepatitis might have different sequential changes in virus titer and genetic drift of HVR-1.
Silini et al. (2003) studied sequence variation in the hypervariable region 1 of HCV and post-transplantation recurrent hepatitis. They investigated the sequence diversity of hypervariable region 1 of HCV in liver transplant recipients and correlated it with the recurrence of hepatitis. Twenty-six patients were considered during a 2-year period; all had graft reinfection and 14 patients developed hepatitis recurrence. Sequence diversity within single sera and over consecutive samples was analyzed quantitatively by matrix comparison and phylogenetic analysis. Sequence diversity over consecutive samples was reduced in patients who experienced hepatitis recurrence and in those infected with genotype 1b.

2.5 Serology and Diagnosis of HCV.

Castillo et al. (1992) optimized several procedures for the detection of serum HCV-RNA, using different regions of HCV genome. They found that the best method for the RNA extraction was guanidine isothiocyanate followed by de-naturation step prior to the polymerase chain reaction. The region of HCV genome amplified influenced the percentage of positivity of HCV in the serum.

Bukh et al. (1993) found high prevalence of HCV-RNA in patients and failure of commercially available antibody tests to identify a significant number of patients with HCV infection. The results indicated that current commercially available antibody tests did not accurately reflect the HCV status in dialysis patients.

Magrin et al. (1992) detected serum HCV-RNA and assessed the response to alpha-interferon (IFN) in anti-HCV positive chronic hepatitis patients. Out of 22 anti-HCV positive patients, 11 were responders and 11 were nonresponders to alpha-
interferon. They also found that PCR primers from the 5' noncoding (NC) region were more sensitive (95%) than primers from a nonstructural (NSS) region (32%) in detecting HCV-RNA. Viremia generally decreased during IFN treatment, but no HCV markers clearly distinguished responders from non-responders before IFN treatment.

Riezuboj et al. (1992) devised a new serological assay to detect antibodies against hepatitis C, based on a recombinant protein (BHC10) that incorporated structural and nonstructural viral antigens. The results were compared with another assay using recombinant non-structural viral antigen (c100). The assay with BHC10 had higher sensitivity (96.8%) compared with c100 antigen (83.3%). Furthermore less false positive results were found when BHC10 was used.

Fathalla et al. (1994) studied the prevalence of HCV infection in a province of Saudi Arabia by 2nd generation and supplemental EIA tests. It was concluded that the incidence of HCV antibodies was very high among the patients on haemodialysis programs and among Egyptian workers in that area.

Goergen et al. (1994) established a procedure of detection and quantification of HCV replication, using one step competitive RT-PCR and a solid phase colorimetric method. In 47 serum samples from 28 patients with chronic HCV infection including 5 repeatedly HCV positive patients under interferon therapy, viral titer was determined. The sensitivity and specificity of the procedure were identical to those of conventional nested-PCR. As both internal and external standards were used, that reliable and time saving test system might be routinely applied for monitoring antiviral treatment or for studying the relation of plus- and minus-stranded HCV-RNA in infected tissues.
Hao et al. 1994 used ELISA and second generation recombinant immunoblot assay (RIBA) to study the patterns of specific antibody response in 10 cases of post-transfusion hepatitis (PTH) during a period of 36-38 weeks after blood transfusion. Nine cases were positive with serum anti-HCV, including 8 cases positive with serum HCV-RNA. Antibodies to core protein of HCV showed a higher positive rate and were detected 1-3 weeks earlier than those to the putative nonstructural (NS) protein. Anti-HCV IgM to core protein were detected 1-4 weeks earlier than anti-HCV IgG and the detective absorbent values of anti-HCV IgM were positively correlated with the levels of serum ALT (P < 0.01). "Passive transfer" of anti-HCV was found in 3 cases. The facts suggested that HCV was the major causative agent of PTH cases and anti-HCV IgM to core protein was a putative serological marker not only for early diagnosis of HCV infection but also for demonstration of active HCV infection.

Lavanchy et al. (1994) evaluated third-generation assay for detection of anti-HCV antibodies and compared it with the presence of HCV-RNA in blood donors reactive to c100-3 antigen. It was found that circulating HCV-RNA was in high proportion of reactive samples.

Liang et al. (1994) demonstrated the presence of hepatitis B and hepatitis C genomes in blood donors detected by polymerase chain reaction amplification. Their results indicated that low level HBV viremia; otherwise undetectable by conventional method was prevalent in blood donor groups.

Yeh et al. (1994) detected anti-HCV antibodies at early stage in acute hepatitis C virus by Western blot (Immunoblot) using a recombinant HCV core protein fragment.
Tisminetzky et al. (1995) compared genotyping and serotyping methods for the identification of hepatitis C virus types. HCV genotyping using a dot blot assay with type specific probes derived from the 5'-UTR of the HCV genome and HCV serotyping using an ELISA system in which type specific antibodies against NS4 region were detected. Overall, a good correlation of the two methods was observed. Mixed infections were not detected by either method. It was concluded that molecular and serological methods were equivalent in determining the viral genotype.

Heermann et al. (1996) described some improvements in PCR amplification of HCV, such as simplification of specimen preparation, and elimination of false negative reactions influenced by point mutations. After rapid chemical denaturation of the clinical specimen with guanidine isothiocyanate and simultaneous hybridization of primers to template HCV RNA, the product was fixed to streptavidin-coated magnetic beads and potential inhibitors were removed in easy washing steps. The low cost of the procedure allowed the quantitation of templates by testing of dilute series as is common in microbiological laboratories.

Mayerat, et al. (1996) developed a sensitive and reproducible one-step competitive reverse transcriptase (RT) PCR assay, which allowed HCV-RNA quantitation in plasma over a broad range of values. RNA samples and an internal standard were reverse transcribed and co-amplified with the same primers in the same tube. A standard curve was obtained from both internal standard and known amounts of wild type HCV-RNA transcript. The assay, with a detection limit of 2500 copies per ml, was markedly more sensitive than the bDNA assay. This assay was convenient for following up patients with low viremia, a common situation with alpha interferon treatment.
Tsuzurahara et al. (1997) detected MAGE-4 protein in the sera of patients with HCV associated hepatocellular carcinoma and liver cirrhosis. Specific ELISA test was employed for the detection of MAGE-4 protein in the sera of disease patients, healthy men and women and those undergoing prostate cancer screening. MAGE-4 protein level was significantly higher in sera of patients with HCV-associated HCC and HCV-associated liver disease patients than those of healthy persons. Thus the serum MAGE-4 protein level might be useful as a marker for identification of patients suffering from HCC that was undetectable by presently available methods.

Garinis et al. (1999) compared enzyme-linked immunosorbant assay III, recombinant immunoblot third generation assay, and PCR method in the detection of HCV infection in haemodialysis (HD) patients One hundred sixty-one HD patients were tested for the presence of anti-HCV antibodies by the ELISA III. HCV RNA was determined by an HCV RT-PCR method. All reported results, designated as discrepant anti-HCV (+) and/or HCV RNA (+), were further investigated by means of a quantitative HCV RT-PCR assay. Reported results obtained from ELISA III and qualitative RT-PCR assays were HCV positive for 16/161 patients (9.93%) and were designated as anti-HCV (+)/HCV RNA (+). 16 anti-HCV positive/161 HD patients were confirmed by the RIBA 3rd.

Shioda et al. (2000) evaluated changes in serum immunoglobulins M (IgM) and G (IgG) hepatitis C virus (HCV) core antibodies after HCV infection in acute hepatitis. Serum IgG HCV core antibody titer was measured using enzyme-linked Immunosorbant assay (ELISA) kit (CSTRC, Kumamoto, Japan). IgM core antibody titer was measured using horseradish peroxidase-labeled monoclonal anti-human IgM as the secondary
antibody for the ELISA kit. Serum HCV RNA was detected using the 5' non-coding region as the primer according to the reverse transcriptase (RT) nested-PCR and competitive RT-PCR method. IgM antibody was detected when Alanine aminotransferase (ALT) peaked, showing the closest correlation with the changes in ALT. The results indicated the usefulness of detection of serum IgM antibody in diagnosis of acute hepatitis C and the follow-up observation of hepatitis C.

Chinchai et al. (2003) Compared different methods to genotype HCV type 6 variants HCV genotype 6a was found frequently in Southeast Asia. In Thailand, however, genotype 6B variants might exist which posed a genotype 1-like sequence in the 5' non-coding region. Four different methods; INNO-LiPA assay, two RFLP assays on the core region (using different restriction enzymes) and phylogenetic analysis of the core sequences were compared. Samples from 17 chronic HCV patients from the Netherlands and Thailand and 18 anti-HCV positive blood donors recruited from Thailand were tested. The INNO-LiPA could not distinguish genotype 6 variants. The RFLP methods used could not, or only in combination with 5'NCR genotyping methods, identified type 6 variants.

Khan et al. (2003) determined HCV seropositivity among chronic liver disease patients in Hazara, Pakistan. Hepatitis tests of 614 people (436 males and 178 females) were performed by DOT immune-chromatographic method for anti HCV antibodies. A total of 251 (40.8%) sera tested positive for anti HCV antibodies, including 184 males (73.3%) and 67 females (26.7%). Of 436 males, 184 (42.2%) tested positive, while among females, 67/178 (37.6%) tested positive. However the male/female ratio referred for testing was 2.4:1. It was concluded that there was a high frequency of HCV seropositive individuals of both sexes among patients referred for chronic liver disease.
Gault et al. (2003) evaluated, the performance of a new serotyping (HC03) (Abbott Murex Laboratories) assay for detection of anti-HCV type-specific antibodies in serum samples using a selected panel of 180 HCV-RNA-positive sera. HC03 was more sensitive than the current HC02 version, typing 53 (37.6%) of 141 samples that were not typable with HC02. Furthermore, the HC03 specificity was 94.1%. This new version of the test might improve the quality of the serological approach to HCV type determination.

2.6 HCV Infections and ALT Level.

Prieto et al. (1995) demonstrated, the existence of HCV carrier state in healthy persons using PCR technique. HCV-RNA was detected in the serum of 65% of the anti-HCV-positive donors while HCV RNA was detectable in 75% of the donors with elevated ALT values and in 59% of those with normal ALT levels. In summary, the prevalence of HCV RNA in serum appeared to be correlated with the severity of liver injury and was greatest in chronic active hepatitis. The detection of serum HCV-RNA in 36% of the donors with minimal changes on biopsy, all of them with normal ALT levels suggested that a healthy carrier state might exist.

Munoz et al. (1996) evaluated the prevalence of antibodies to anti-HCV and the relation of transaminase (ALT) levels and viraemia to liver damage in 55,5877 blood donors in Spain. The prevalence of anti-HCV detected by ELISA-2 was 0.93%. Serum ALT was abnormal in 61 of the 160 volunteers (38.1%). Of these, RIBA-2 was positive in 96.7% and HCV RNA was detectable in 96.1%. Serum ALT was normal in the remaining 99, 70.7% being RIBA-2 negative and 98.3% HCV RNA negative. The
majority of biopsies (85.6%) showed chronic hepatitis. This study demonstrated that in blood donors screening for anti-HCV, a positive ELISA-2 test, when associated with abnormal ALT levels, was effective in recognizing subjects with active infection detected by HCV RNA and liver disease.

Murashima et al. (1996) reported the clinical significance of the hypervariable region (HVR) in the N-terminus of the E2/NS1 region, which encodes the putative envelope glycoprotein (gp 70) of HCV. They studied the relation between HVR changes and elevation of the alanine aminotransaminase (ALT) level due to liver cell injury as well as the persistence of HCV infection. Three patients (carrier group), HCV RNA positive and had normal ALT levels since five years and three patients with high ALT levels were studied. The E2/NS1 region, including HVR-1 and HVR-2, was amplified using the RT-PCR method. PCR products were cloned and nucleotide sequences were determined using the didoxynucleotide chain termination method. No clear correlation was found between the ALT levels and the number of nucleotide substitutions in HVR-1. The number of nucleotide substitutions in HVR-1 during the five years was greater than in other regions. It was concluded that HVR-1 changes were probably important factor in persistent viral infection than liver cell injury.

Chang et al. (2000) studied serum ALT activity in relation to HBV and HCV infection among drug abusers in Taiwan. That survey included 769 male drug abusers aged 14-59 years. The prevalence of HBsAg seropositivity was 21.5%, and anti-HCV seropositivity was 27.2%, respectively. Drug abusers with HBsAg or anti-HCV had higher serum AST and ALT levels than those without HBsAg and anti-HCV. The prevalence of raised ALT and AST (> or =45 IU/liter) in the HCV-positive group was more significant than in the negative group, while that of the HBsAg-positive group did
not reach statistical significance. Their results indicated that HCV infection played an important role in the etiology of raised ALT activity among drug abusers, while HBV infection played a minor role. ALT screening still remains a simple and valuable method in the early recognition of HCV infection.

Kyrlagkitsis et al. (2003) studied liver histology and progression of fibrosis in individuals with chronic hepatitis C and persistently normal ALT. They performed a retrospective review of patients with chronic HCV infection and persistently normal ALT to compare clinical and histological features with those in patients with abnormal liver biochemistry. Ninety-one HCV RNA-positive patients with persistently normal ALT who had a liver biopsy between 1993 and 1999 were identified. Clinical, histological, and epidemiological features in the group were compared with those found in 94 patients with abnormal ALT. Although overall necroinflammatory score and fibrosis were significantly lower in those with normal ALT, none had normal liver histology, and 15 (16%) patients with normal ALT were found to have significant necroinflammation with a score of 5 or greater.

2.7 Genotyping Study.

Apichartpiyakul et al. (1994) analyzed HCV isolates among healthy blood donors and drug addicts in Chiang Mai, Thailand. HCV-RNA was detected in 92% anti-HCV positive blood donors and 83% in drug addicts by PCR for a portion of the NS5 region. Subtype analysis revealed that HCV type-3a was the prevailing genotype (30%), followed by HCV type-1a (21%).
Gerotto et al. (1994) studied the HCV genotypes in 22 patients with anti-LKM-1 autoantibodies. It was found that 77% of the patients were infected by hepatitis C virus genotype-1 (G1), 18% by genotype-2 (G2) and 5% by genotypet-3 (G3), thus excluding the association of the autoimmune reaction with a particular viral type. Prevalence of genotype-1 and 2 were significantly different from those patients with chronic hepatitis C who were negative for anti-LMK-1, as those were more rarely infected with G1 and more frequently with G2. Sequence analysis of the amplified 5' UTR provided identification of peculiar and identical nucleotide substitutions in two out of four patients with G2.

Tsiminetzky et al. (1994) studied HCV genotypes in Italian patients with chronic hepatitis C after cloning and sequencing part of the 5' un-translated region of the virus. The results indicated that 42% patients were infected by HCV type-1 (G1), 45% by genotype-2 (G2), and 4% by genotype-3 (G3).

Chan et al. (1995) determined quantitative branched DNA (bDNA) assay and genotyping for hepatitis C RNA in Chinese patients with acute and chronic hepatitis C. The samples that were positive by RT-PCR were also positive by bDNA assay. Combination of two methods clearly demonstrated changes in HCV RNA titers during and after interferon treatment. The most common genotype of HCV injection was Okamoto type II (Simmond type 1b, 60%), followed by type III (type 2a, 16.5%) and type IV (type 2b, 8.2%); mixed or untyped were noted in 15.3%.

Silini et al. (1995) found that HCV type 1a and 3a infections were more frequent among intravenous drug users than in 125 age-matched controls (48.8% and 21.1% vs. 17.6% and 11.2%). Analysis of hepatitis C virus genotypes showed that, in the general population, hepatitis C virus types 1a and 3a were more prevalent among patients
younger than 40 years of age than in older individuals (17.6% and 11.2% vs. 1.4% and 0.6%). The findings suggested that HCV types 1a and 3a were recently introduced in Italy, presumably via needle sharing among intravenous drug users.

Tisminetzky et al. (1995) compared genotyping and serotyping methods for the identification of HCV types. HCV genotype using a dot blot assay with type specific probes derived from the 5'-UTR of the HCV genome and HCV serotyping using an ELISA system in which type specific antibodies against NS4 region were detected. Overall a good correlation of the two methods was observed.

Bernier et al. (1996) determined HCV genotypes in Montreal, Canada for 358 viremic individuals, by restriction endonuclease analysis of PCR products and phylogenetic analysis of core gene sequences. Types 1, 2, and 3 occurred in 62.8, 14.2, and 13.5%, respectively. Moreover types 4, 5, occurred in 3.9 and 4.5%, respectively while 6a, 7a, and a novel genotype each occurred in 0.3%.

Soetjipto et al. (1996) found differential prevalence of HCV subtypes in healthy blood donors, patients on maintenance haemodialysis, and patients with hepatocellular carcinoma (HCC) in Surabaya, Indonesia. The prevalence of anti-HCV antibodies was 2.3, 76.3 and 64.7% in the healthy blood donors, patients on maintenance haemodialysis and patients with HCC, respectively. HCV-2a was most common (52%) in healthy blood donors while in the patients with HCC and haemodialysis; HCV-1b was the most common genotype (57% and 31% respectively). Subtype prevalence was not different between HCC patients with advanced liver cirrhosis and those without advanced cirrhosis.
Gayowski et al. (1997) determined HCV genotypes in liver transplant recipients by direct sequencing of the NS5 region with type-specific primers and response to interferon-alpha therapy. The results indicated that genotype 1a (66%, 32/47) was the predominant genotype. Type 1b was found in 25% of patients and type 2b was found in 9%. Histopathological recurrent HCV hepatitis developed in 53% (25/47) of the patients after transplantation. There was a trend toward a higher incidence of major infections in patients with type 1b (75%) versus type 1a (48%) and type 2b (50%). The response of interferon-alpha did not differ significantly among the genotypes. They concluded that the incidence, time to recurrence, and response to interferon-alpha therapy did not differ between the various genotypes in their liver transplant recipients. However, there was a trend toward higher infectious morbidity and overall mortality in patients with genotype 1b after transplantation.

Schreier et al. (1996) used various methods for HCV typing, including amplification of certain genomic regions using universal or type/subtype-specific primers, restriction fragment length polymorphism analysis, differential hybridization, nucleotide sequencing, and serologic genotyping. HCV genotypes and their subtypes coexisted in various geographic locations but showed different prevalence. The identification of genotypes/subtypes was useful for studies on the molecular epidemiology and pathogenesis of HCV infection.

Marshall et al. (1997) determined HCV genotypes in the United States by cleavage fragment length polymorphism analysis. They described the application of a new DNA-scanning method, which has been termed Cleavage Fragment Length Polymorphism (CFLP; Third Wave Technologies, Inc., Madison, Wis.), for genotyping HCV. CFLP analysis resulted in the generation of structural fingerprints that allowed
discrimination of different DNA sequences. They analyzed 251-bp cDNA products generated by reverse transcription-PCR of the well-conserved 5'-noncoding region of HCV and also determined the genotypes of 87 samples by DNA sequencing. Blinded CFLP analysis of those samples was 100% concordant with DNA sequencing results. The study demonstrated the suitability of that technology for HCV genotyping and suggested that it might provide a low-cost, high-throughput alternative to DNA sequencing or other more costly or cumbersome genotyping approaches.

Naoumov et al. (1997) examined the role of HCV replication and different genotypes, in the progression of cirrhosis to hepatocellular carcinoma in patients with histologically proven cirrhosis. HCV-RNA was detected in 31 of 72 (44%) patients who developed hepatocellular carcinoma, significantly more frequently than in 17 of 72 (23%) controls with cirrhosis. It was concluded that persistent hepatitis C virus was closely associated with hepatocellular carcinoma development in cirrhosis, and there was no preferential role of individual HCV genotypes.

Zali et al. (2000) determined HCV genotypes in the Islamic Republic of Iran. Serum samples from 21 HCV infected individuals were tested primarily by second generation of enzyme Immunosorbent assay and the two-stage PCR method. For determining the five most common variants, second-generation methods for genotype specification were used. The prevalence of specific genotypes in 15 samples was as follows: Type I/1a in seven cases, Type II/1b in three cases and Type V/3a in four patients. One sample disclosed Type 4.

White et al. (2000) demonstrated a simplified method of genotyping HCV by heteroduplex mobility analysis (HMG) with PCR fragments derived from the 5' un-
translated region or NS5 region of HCV genome. Genotypes determined by direct sequencing method correlated exactly with genotypes determined by hero-duplex mobility analysis within the groups 1, 2, 3, 3b/4, and 6. HMG was also used to simplify the identification of mixed infection with two HCV genotypes.

*Das et al. (2002) studied geographical distribution of HCV genotypes in India* with aims at determining and comparing the prevalence of different genotypes from different parts of India (North, South, East and West). A total of 153 samples representing different regions have been genotyped in our lab. A high prevalence of genotype 3 (> 76%) and very low prevalence of genotype 2 (< 2%), as a whole was documented. However, genotype 3a has been found to be the highest (50%) with a decreased frequency of approximately 25% in the case of 3b, approximately 14% in 1b and approximately 10% in 1a, whereas a minimal number (~4%) of genotype A total of 153 samples representing different regions have been genotyped in our lab. A high prevalence of genotype 3 (> 76%) and very low prevalence of genotype 2 (< 2%), as a whole was documented. However, genotype 3a has been found to be the highest (50%) with a decreased frequency of approximately 25% in the case of 3b, approximately 14% in 1b and approximately 10% in 1a, whereas a minimal number (~4%) of genotype 4 has been found only in Southern and Western India.

*Molin et al. (2002) examined HCV genotype distribution and its determinants in 318 consecutive HCV-RNA positive patients.* Subtype 1b infection was the most prevalent (35.5%), followed by subtype 1a (22%), 3a (21.4%) and genotype-2 (21.3%). Subtypes 1a, 1b and 3a had a comparable prevalence (30-35%) in the 0-15-, 16-30- and 31-45-year age groups. In subjects older than 45 years, genotype 2 prevalence increased, whereas subtype 1a and 3a infections decreased markedly. In this age group types 1b and
2 accounted for a prevalence of more than 90% in a comparable proportion. The community acquired infections and infections in patients with a history of transfusion were caused mainly by subtype 1b (38.5% and 66.6%, respectively).

Solmoné et al. (2002) devised a simple and reliable method for detection and genotyping of HCV-RNA in dried blood spots stored at room temperature. The method was useful in large field studies, particularly in settings where collection, preparation, storage, and shipment of samples at controlled temperature could be difficult.

Djebbi et al. (2003) genotyped HCV circulating in Tunisia. HCV isolates from 93 patients living in Tunisia, including 16 hemophiliacs, were genotyped by INNO LiPA and partial sequencing of the 5' untranslated region of the viral genome. In non-hemophiliacs, subtype 1b was largely predominant (79%), types 1a, 2a, 2b, 3a and 4a occurred much less frequently at 5, 7, 3, 3 and 1% of cases, respectively. In the group of hemophiliacs, a co-dominance between subtypes 1a and 1b was noticed (38%). Type distribution of HCV in Tunisia differed from that reported in other countries of the Mediterranean and Middle East regions.

Re et al. (2003) studied HCV genotypes in Cordoba, Argentina. Unexpectedly high prevalence of genotype 2 was observed. 96 anti-HCV positive subjects were taken and HCV-RNA was detected in 60 samples by RT-nested PCR of the 5' non-coding region (5'-NCR). Genotyping was performed by restriction fragment length polymorphism analysis of 5' NCR region combined with PCR using type-specific primers of the core region. Overall, genotype 2 was the most prevalent (55.0%), followed by genotypes 1 (38.3%), and 3 (5.0%). Within genotype 1, subtype 1b was the most prevalent. These figures differed from other cohorts from East-Argentina where genotype
1 has been found as the most prevalent. This indicated that regional differences of genotype distribution might exist between Central and East Argentina.

Shobokshi et al. (2003) determined the molecular epidemiology of HCV in the Kingdom of Saudi Arabia (KSA). Four hundred and ninety-two histological proven chronic HCV patients prospectively recruited from all regions of KSA, between November 1999 and March 2002, were genotyped and sub-typed using amplified products of specific primers from the 5-UTR region in a reverse transcription polymerase chain reaction (Roche Diagnostics, Switzerland) followed by a reverse hybridization technique (Innolipa HCV II [Innogenetics, Belgium]). Sixty-two percent of Saudis were found to be genotype 4. Other genotypes were 1 (24.1%); 2 (7.4%); 3 (5.9%); 5 (0.3%); and 10 (0.3%). There were no differences in distribution patterns between sexes and ages. It was concluded that 86% of Saudi chronic hepatitis C cases were due to genotypes 1 and 4.

2.8. Prevention and Treatment.

Beloqui et al. (1993) found that N-acetyl cysteine enhances the response to Alpha-interferon in chronic HCV patients. They studied that in all HCV patients, glutathione levels were depressed in plasma and mononuclear cells in comparison to controls. In IFN-unresponsive patients, the addition of N-acetyl cysteine, a glutathione precursor, resulted in a steady decrease of ALT values in all patients, with complete normalization in 41% of cases after 5-6 months of combined therapy.

Garson et al. (1995) evaluated a wide range of clinical, demographic, and virological parameters in relation to therapeutic outcome in a group of 30 Italian patients.
with chronic hepatitis C. All patients received 3 MU leukocyte-derived IFN-alpha three times a week for 6 months and were then followed prospectively for at least 12 months. 53% of patients responded initially, but a sustained response was observed in only 17%. Responders were found to be significantly younger than non-responders (45.6 +/- 3.1 vs. 55.4 +/- 2.7), and less frequently cirrhotic (2/16 vs. 7/14). Sustained responders had a mean pretreatment HCV-RNA titer approximately tenfold lower than that of those who did not have a sustained response, but the difference was not statistically significant. HCV genotype was found to be significantly associated with both initial and sustained response. Patients infected with HCV-2a were more likely to respond (89%) than those who were infected with HCV-1b (37%), and they were also more likely to sustain that response (33% vs. 6%).

Kohara et al. (1995) studied that HCV genotypes-1 and 2 responded to interferon-alpha with different virological kinetics. Levels of HCV viremia were determined by quantitative RT-PCR. Infection with HCV genotype-2 or low pretreatment HCV viremia levels in subjects infected with genotype-1 was associated with favorable (complete and sustained) responses to IFN-alpha treatment. HCV viremia levels in genotype-2 infection were significantly lower than in genotype-1 infection.

Mizokami et al. (1996) studied genotype, serum level of HCV-RNA and liver histology as predictors of response to interferon-alpha 2a therapy in Japanese patients with chronic hepatitis C in 43 patients with chronic active hepatitis C. Response to recombinant interferon-alpha 2a (504 million units in total) was defined as complete and sustained CR-->SR, n = 12), complete response followed by relapse (CR-->Rel, n = 17), and no response (NR, n = 10). Patients who showed CR-->SR had a lower HCV-RNA level (0.438 x 10(6) eq/ml) compared to CR-->Rel (2.452 x 10(6) eq/ml, p = 0.008) and
NR (4.88 x 10^6 eq/ml, p = 0.009). A higher proportion of patients with CR-->SR had type 2a HCV (67%) compared to the CR-->Rel (28%) and the NR (0%). There was a trend for type 1b HCV infection to have higher serum HCV-RNA levels. There was no correlation between pretreatment HCV-RNA level and alanine aminotransferase.

Yeh et al. (1996) studied nucleotide sequence variation in the hyper-variable region (HVR) of the HCV in the sera of chronic hepatitis C patients undergoing controlled interferon- therapy. Serum ALT activities in five out of ten patients treated for six months with IFN- fell to normal range, when HCV was not detected in the sera of three patients. The nucleotide sequence variation in HVR of HCV in the sera of 5 patients who responded to the -IFN therapy was relatively less than those who did not respond to the -IFN therapy. The results indicated that the effectiveness of the IFN- therapy was related to the sequence variation of HVR of HCV. It was concluded that the higher rate of sequence variation in HVR of HCV was compatible with a lower degree of effectiveness of IFN.

Reciprocal interactions between human immunodeficiency virus and HCV infections were studied by Zylberberg et al. (1996). They found that although hepatitis C virus (HCV) and human immunodeficiency virus (HIV) shared the same routes of transmission, have high levels of viral replication and a more severe histopathologic course but HCV infection did not seemed to be accelerate the progression of HIV infection. Interferon alpha (IFN-alpha) in co-infected patients led to a similar rate of primary responses, but sustained responses were less frequent.

Everhart et al. (1997) conducted a survey on the management of HCV infection by the American Digestive Health Foundation among United States physicians who were
most familiar with the disease. Those physicians frequently managed HCV patients. The Physicians generally agreed with the recommendations of the Consensus Development Conference Panel regarding prevention of transmission, minimizing alcohol consumption, and managing patients with typical presentations.

Kato et al. (1997) studied the levels of serum HCV-RNA, the HCV genotype before interferon therapy and the kinetics of serum HCV-RNA at the initial stages of therapy to determine their utility in predicting the therapeutic efficacy of interferon in 44 patients with chronic hepatitis C infection. They also looked at the efficacy of repeated interferon treatment in relation to the kinetics of serum HCV-RNA. Detection of serum HCV-RNA by the reverse transcription nested polymerase chain reaction assay after the completion of interferon therapy indicated relapse at its earliest stage. In patients who experience relapse, repeated treatment with an appropriate dose of interferon before an increase in viral levels might increase the proportion of complete responses.

Schalm et al. (1997) studied therapy of hepatitis C patients with cirrhosis. The prognosis of compensated cirrhosis caused by hepatitis C was relatively good. 18% of patients had developed hepatic de-compensation and 7% hepatocellular carcinoma. Overall 5-year survival rate was 91%. Treatment with alpha interferon appeared to decrease the incidence of hepatocellular carcinoma in patients who achieve a sustained remission. Virological measurements during therapy showed that only 22% of patients become HCV-RNA negative by 4 weeks and thereafter, there is a high rate of breakthrough. In small studies, the combination of interferon and ribavirin led to sustained biochemical and virological response rates of 21%, more than twice the rates achieved with interferon alone. The prognosis of decompensated cirrhosis caused by
hepatitis C was poor, with a 5-year survival rate of only 50%. The efficacy of interferon in patients with decompensated cirrhosis was not well documented.

Naoumov et al. (1997) examined the role of HCV replication and different genotypes, in the progression of cirrhosis to hepatocellular carcinoma in patients with histologically proven cirrhosis. HCV RNA was detected in 31 of 72 (44%) patients who developed hepatocellular carcinoma, significantly more frequently than in 17 of 72 (23%) controls with cirrhosis. It was concluded that persistent hepatitis C virus was closely associated with hepatocellular carcinoma development in cirrhosis, and there was no preferential role of individual hepatitis C virus genotypes.

Lucidarme et al. (1998) found that age limit was an important factor in progression of cirrhosis from the time of HCV. The results of this study suggested that progression to cirrhosis was slower in cases of viral contamination before 30 years of age than later on and thus the age at the time of contamination was an important predictive factor of progression to cirrhosis.

Riflet et al. (1998) reported psychiatric suicidal impulses as side effects in 5 HCV patients treated with alpha-interferon. Suicide was attempted in 4 cases after interferon withdrawal and was responsible for 2 deaths. The increased risk of psychiatric side effects due to interferon as well as their severity suggested that interferon should be administrated with caution.

Vanrossum et al. (1998) studied the role of glycyrrhizin as a potential treatment for chronic HCV infection. In Japan glycyrrhizin has been used for more than 20 years as a treatment of chronic hepatitis. In randomized control trials, glycyrrhizin induced a
significant reduction of serum ALT and improvement in liver histology. The mechanism by which glycyrrhizin improved liver function and histology were undefined.

Mondelli et al. (1999) reviewed clinical significance of HCV genotypes to see histological features of chronic liver disease and response to antiviral treatment. General consensus has been reached on the worldwide epidemiology and distribution of HCV types in certain risk categories (i.e. intravenous drug users), the association between genotype 1b and severe liver disease is still controversial. Although generalized use of genotyping was not presently recommended for clinical or epidemiological monitoring, several studies emphasized the importance of HCV genotyping as part of a therapeutic algorithm. This recommendation is based on overwhelming evidence in support of a correlation between genotype 1 and a poor response to interferon-a alone or in combination with ribavirin.

Zemel et al. (2001) studied cell transformation induced by HCV, NS3 serine protease. NS3 has oncogenic activity. When rat fibroblast (RF) were stably transfected with cDNA for the NS3 serine protease. The transfected cells grew rapidly and proliferated serum, lost contact inhibition and induced significant tumor formation in nude mice. These results suggested that the NS3 serine protease of HCV is involved in cell transformation and the ability to transform required an active enzyme.

Schulman et al. (2002) studied the effect of alpha-interferon plus ribavirin for 6 months or 12 months for the treatment of chronic hepatitis C in patients with bleeding disorders in Sweden. The prevalence of HCV genotype 1 was 67%. Overall, sustained viral response was achieved in 41%; 13 of 30 patients (43%) treated for 6 months vs. 12 of 31 patients (39%) treated for 12 months. The rate of sustained response was 22% in
those with HCV genotype 1 and 80% in other genotypes (100% in genotype 2), with no
difference between the treatment durations. It was further reported that six months of
therapy seems sufficient in the case of HCV genotype 2. For other genotypes, the
decision regarding duration of therapy has to be based on the tolerance of the individual
patient together with experiences from other studies.

Brinovec et al. (2002) found the efficacy of interferon alpha therapy in 80 HCV
patients in Slovenia. In 11 patients, the treatment was discontinued prematurely, after six
months, due to therapeutic failure. Complete response to therapy with disappearance of
HCV from the blood was observed in 34 patients (49%), while in 24 the response to
therapy was partial, i.e., the biochemical tests showed normalization of values but
viremia persisted. There was a significant relation between the therapeutic response and
those patients with the genotype 3 (p = 0.01). After three months of follow-up, complete
therapeutic response was still observed in 19 patients (28%), most of them with genotype
3. Despite persistent viremia there was no progression of liver inflammation in eight
partial responders, as evidenced by liver re-biopsy. Thus, it was confirmed that treatment
was justified in these patients.

Testino et al. (2002) demonstrated that interferon therapy did not prevent
hepatocellular carcinoma in HCV compensated cirrhosis. A randomized study with
interferon versus no therapy was conducted in a cohort of 122 HCV patients who had
undergone blood transfusion before 1980. HCV serotyping was determined by hepatitis C
virus serotyping 1-6 assay (Murex Biothec UK). HCV-RNA level was determined by
bDNA, (Chiron Corporation CA). Diagnosis of hepatocellular carcinoma was confirmed
with spiral-computed tomography. Fifty-nine patients (mean age: 55.3 +/- 7) received
interferon (3MU three times a week for 12 months), 8 stopped therapy for side effects
and 71 did not receive interferon (mean age: 56.8 +/- 8). In interferon treated patients an improvement in relation with worsening and death/orthotopic liver transplantation had been noted. The use of the interferon seemed to be scarcely useful when structural alterations of the cirrhotic kind showed up.

Durante et al. (2003) determined E2 and NS5A region variability in HCV genome during sequential treatment with two interferon-alpha preparations in patients who were either responder-relapsers or non-responders to treatment. In non-responders, the HVR-1 quasispecies broadened. Amino acids G406 and Q409, which represent a major viral epitopes, were highly conserved throughout treatment. Responder-relapse patients had a higher mutation frequency in NS5A than non-responders. Variability upstream of the ISDR was associated with treatment response. It was concluded that the pattern of HVR-1 quasispecies evolution correlated with the clinical response, and the conservation of specific amino acids might be useful for immune targeting in vivo. In responder-relapse patients, the initial HVR-1 evolution resembles that found in sustained responders. Variability within the entire NS5A, as opposed to a single region (ISDR), might have a role in influencing alpha-interferon treatment outcome.

Fujiyama et al. (2003) studied effectiveness of interferon therapy for chronic hepatitis C patients with low viral loads with genotype 2a or 2b. Furthermore, initial changes of HCV-RNA levels in early phase interferon therapy, and the number of pretreatment mutated clones at hyper-variable region-1 were determined in order to upgrade interferon therapy efficacy prediction rates. Patients with genotype 1b were treated for 26 weeks, while those with genotype 2a or 2b were treated for 16 weeks. Sustained response rates showed no difference in efficacy between the 2 groups (66.7% vs. 62.5%). Efficacy as HCV-RNA in early phase treatment showed no difference in
response rates between negative and positive groups at any time point from day 1. It was concluded that in a low viral-load group, the number of hyper-variable region-1 clones was a critical factor influencing interferon therapy efficacy. Thus, 16-week interferon therapy was effective and economical.

Isagulians et al. (2003) characterized the hosts in the earliest events of HCV infection before the on-set of adaptive immune response. Host met the replicating HCV with innate immune response in the form of pro-inflammatory cytokine production, activation of natural killer (NK), NKT and dendrite cells. Higher rates of HCV clearance were associated with white ethnicity and certain HLA haplo-types while Lower clearance rates were correlated with genetic immune deficiencies/disorders.

Pawlotsky et al. (2003) investigated efficacy of treatment of chronic HCV infection based on combination of pegylated interferon (IFN)-alpha and ribavirin. When successful, the treatment led to sustained HCV clearance, which in virtually all cases signified viral eradication. However, approximately 20% of patients with HCV genotype 2 or 3 infection, and 50% of patients with genotype 1 infection, failed to eradicate the virus. The risk of treatment failure was related to multiple factors, including the treatment schedule, adherence of therapy, host factors, and the severity of HCV-associated disease. Viral factors could also lead to true "HCV resistance". The mechanisms underlying this resistance were unknown, but indirect evidence suggested that chronic infection was associated with phenomena that protected HCV from the antiviral action of IFN-alpha and hinder the clearance of infected cells.

Thuluvath et al. (2003) evaluated the efficacy of a combination of interferon, ribavirin, and amantadine in patients with chronic hepatitis C who had previously failed
6-12 months of treatment with interferon and ribavirin. 23 patients were treated with a combination of interferon-alpha (2b) 3 million units subcutaneously three times per week, ribavirin 1000-1200 mg daily, and amantadine 100 mg twice daily for 6-12 months. At the end of treatment, the biochemical response was 47% and the virological response was 30%. However, the rate of sustained virological response was only 13% (3/23) it was concluded that triple therapy with interferon, ribavirin and amantadine resulted in a low sustained viral clearance in chronic hepatitis C patients who had previously failed interferon and ribavirin combination therapy.

Wu et al. (2003) reviewed the global prevalence of persistent HCV infection and the lack of a highly effective and well-tolerated antiviral therapy. HCV NS5B RNA-dependent RNA polymerase (RdRp), the centerpiece for viral replication, constituted a valid target for drug discovery. High genetic variation among the major HCV genotypes commands that any efficacious NS5B inhibitors have to be broadly active against NS5Bs from various genotypes. Rapid viral replication and its inherent genetic diversity would culminate drug resistance to any NS5B inhibitors. Therefore, iterative drug design and combination therapies of drugs that intervened the different steps in the HCV replicative cycle were needed to combat the viral infection.

Zhang, (2003) reviewed the efficacy and safety of pegylated interferons (peginterferons) in the treatment of chronic hepatitis C from 19 original articles (MEDLINE 1988 – 2001). Peg-interferon could effectively prolong the half-life of interferon and could be effectively administered conveniently as a once-a-week dose. The antiviral effect using peg-interferon was enhanced compared with that of standard interferon, but the frequency and severity of adverse events were typical of those associated with interferon-alpha.
**HCV Infection and Diabetes type-II:**

Since the HCV was identified, numerous epidemiological studies have reported a higher prevalence of type-2 diabetes mellitus (DM2) in subjects infected by HCV (Knobler et al., 1998). Patients with liver disease are known to have a higher prevalence of glucose intolerance, preliminary studies suggest that hepatitis C virus (HCV) infection may be an additional risk factor for the development of diabetes mellitus (Mason et al., 1999). It was found an association of diabetes mellitus and chronic HCV infection in 1,117 patients with chronic viral hepatitis. In that study after the exclusion of patients with conditions predisposing to hyperglycemia, diabetes was observed in 21% of HCV-infected patients compared with 12% of HBV-infected subjects (P = .004). HCV genotype 2a was observed in 29% of HCV-RNA-positive diabetic patients versus 3% of local HCV-infected controls (P < .005). The data suggested a relatively strong association between HCV infection and diabetes, because diabetics have an increased frequency of HCV infection, particularly with genotype 2a. Furthermore, it is possible that HCV infection may serve as an additional risk factor for the development of diabetes, beyond that attributable to chronic liver disease.

Mangia et al. 1998 studied HCV and diabetes mellitus in two hundred forty-seven patients with liver cirrhosis in Italy and gave evidence of negative association between the two diseases. One hundred fifty-seven (63.5%) of them were HCV positive, 38 (15.5%) HBV positive, 49 (19.8%) alcohol abusers, and three (1.2%) cryptogenic. Two control groups were also included. The first control group consisted of 138 patients with chronic hepatitis due to HCV infection (73.9%), HBV infection (15.9%), or alcohol abuse (10.2%) (Group 2). The second control group included 494 patients with an acute
osteoarticular trauma, age- and gender-matched with patients in-group 1 (group 3. Diabetes mellitus was present in 32.3%, 3.6%, and 9.7% of patients in groups 1, 2, and 3, respectively. When compared with controls (group 3), DM was significantly less frequent in-group 2 (p < 0.004) and significantly more frequent in group 1 (p < 0.001). The prevalence of DM was not different among patients with HCV, HBV infection, or alcohol abuse. In-group 3, the prevalence of DM appeared to increase steadily with age. Their findings disproved HCV infection as a trigger factor for DM, which should not be listed among the various extra hepatic manifestations of this viral infection.

Garrido et al. (2001) discussed hyperinsulinemia in cirrhotic patients infected with HCV with the aims to study the frequency of diabetes mellitus in cirrhotic patients with HCV infection, comparing it with that in cirrhotic patients without HCV infection and to investigate basal insulineemia values in both groups, as well as the possible factors causing insulinemia. It was found that percentage of diabetics in group I was 36% (18/50) compared with 18% (9/50) in-group II (p < 0.05) and basal insulinemia values were 23.5 microU/ml compared with 15.7 micro U/ml respectively (p < 0.05). In contrast, serum ferritin concentrations were much higher in patients of group I than in those of group II (137.7 vs. 87.6 ng/ml p < 0.05). Thus diabetes mellitus was more prevalent in patients with cirrhosis due to HCV than in those with cirrhosis due to other etiologic agents. Moreover, basal insulineemia values were higher in these patients, which could be explained by an increase in half insulin resistance associated with an increase in iron deposits.

Akbar et al. (2002) found prevalence of Type-2 diabetes in patients with HCV and HBV infection in Jeddah, Saudi Arabia with the objectives to determine the prevalence of type-2 diabetes mellitus (DM) in patients with HCV and HBV infections in
399 patients studied. 165 (41%) were anti-HCV positive and 234 (59%) were HBsAg positive. Type-2 diabetes was present in 35 of 165 (21.2%) patients with HCV infection, and 33 of 234 (14.1%) with HBV infection. 94% of anti-HCV-positive type-2 diabetes were older than 40 years and 6% were younger, while for non-diabetics the corresponding percentages were 55 and 45%, respectively. 76% of HBsAg-positive type-2 diabetics were older than 40 and 24% were younger, while the corresponding percentages for non-diabetics were 27 and 73%, respectively. Their findings indicated that type-2 diabetes was more common in patients with an HCV than with an HBV infection.

Qurshi et al. (2002) demonstrated that diabetes mellitus is equally frequent in chronic HCV and HBV infection in Pakistan. Of 400 patients with chronic liver disease 302 had HCV and 98 HBV infections. Diabetes was found in 24.5% HCV and 19.4% HBV related cases (not significant). Out of 410 healthy controls 18 were HCV and 17 HBV positive. Diabetes was found in only 1 (5.6%) HCV positive control and none of the HBV positive controls. Thus the diabetes was equally frequent in both HBV and HCV related disease but is significantly more in those with chronic liver disease than in controls. The pancreatic damage secondary to extra-hepatic viral replication appears to be the major cause but genetic factors also need to be explored.

Amarapurkar et al. (2002) studied chronic liver disease in diabetes mellitus patients in India in 53 patients of CLD with diabetes. Spectrum of liver disease in diabetic group were as follows: 56.6% cirrhosis, 15.1% chronic hepatitis, 22.6% fatty liver, 5.7% cirrhosis + hepatocellular carcinoma (HCC). The spectrum in control group was as follows, cirrhosis 46.1%, chronic hepatitis 36.5%, fatty liver 14.8%, cirrhosis and HCC 2.6%. Etiology of chronic liver disease in diabetic group was as follows: Non-
alcoholic steatohepatitis (NASH) with cirrhosis 11.3%, NASH 18.9%, cryptogenic cirrhosis 22.6%, HBV 17%, HCV 13.2%, alcohol 17%. Etiology of chronic liver disease in non-diabetic group was as follows: NASH with cirrhosis 1.7%, NASH 13.0%, cryptogenic cirrhosis 7.8%, HBV 30.43%, HCV 13%, alcohol 29.6% and autoimmune in 4.3%. Incidence of NASH with cirrhosis and cryptogenic cirrhosis were found to be statistically significantly high in diabetic group. Incidence of diabetes in cryptogenic cirrhosis was found to be 57% versus 30% in noncryptogenic cirrhosis. It was concluded that NASH, NASH with cirrhosis and cryptogenic cirrhosis are the major causes of chronic liver disease in patients with diabetes mellitus. Alcohol and viral causes were found to be important etiologies in non-diabetic control group. Diabetes mellitus is an important risk factor for chronic liver disease and progression of NASH to cirrhosis, which may present as cryptogenic cirrhosis.

Arao et al. 2003 also studied prevalence of diabetes mellitus in 866 Japanese patients infected chronically with HCV. The prevalence of HBV- and HCV-related cirrhosis was 32% and 33%, respectively. A case-control study was also conducted to determine the seroprevalence of HCV infection in a cohort of 459 diabetics. The prevalence of DM was higher in HCV-infected patients (20.9%; P < 0.02) than in HBV-infected subjects (11.9%). In the cirrhotic patients, DM was observed in 30.8% of the subjects with HCV compared with 11.8% of those with HBV (P < 0.01). Multivariate analysis revealed that the major independent variables associated with type II DM were male sex and cirrhosis). The relative odds of the development of DM were calculated to be 3.2 times higher in HCV-infected cirrhotic patients than in HBV-infected ones. In the case-control study of the diabetic cohort, 10.5% of patients were infected with HCV compared with 1.1% with HBV (P < 0.0001). The results indicated that HCV infection
was closely associated with DM, compared with HBV infection. Cirrhosis was an independent risk factor for DM.

Saxena et al. (2003) determined the susceptibility of the 196 patients with type-2 diabetes to HCV infection in a HD unit with high HCV prevalence at King Fahad Hospital and tertiary care center, in Hofuf, Saudi Arabia, from November 1995 to November 2000. The overall, HCV sero-prevalence of 41.3% (81/196) and annual seroconversion rate of 8.26% were observed. Anti-HCV positivity was associated with longer time on dialysis. Of the 196 patients 54 (27.5%) had type-2 diabetes mellitus and 142 (72.5%) were non-diabetics. Patients with type-2 diabetes recorded higher HCV prevalence (57.4% vs 35.2%), and annual seroconversion rates (11.48% vs 7.04%) after a shorter period on dialysis (32.6 vs 50.6 months), as compared to those of the non-diabetic group. A significantly higher HCV prevalence and annual seroconversion rate despite relatively shorter period on dialysis among patients with type-2 diabetes clearly point to the greater likelihood of their acquiring HCV infection even at an earlier stage than the non-diabetic patients, receiving treatment in a high prevalence HD unit.
MATERIALS AND METHODS
3. MATERIALS AND METHODS

A. Study Design:

The demographic data collected at the time of sampling was obtained by interviewing the patients and also from the clinical data of the patients with him/her or record placed with the hospital administration. Before carrying out this study, a standard questionnaire was prepared (see appendix I).

Patient details, including risk factors, age, duration of infection, racial origin, in certain cases serum albumin/globulin ratio (A/G ratio) and values of liver function tests (LFTs), complete blood count (CBC), platelet count, and liver ultrasound etc, were obtained where possible from the case notes of the respective clinician. Diabetic history of the diabetics such as age, sex, type of diabetes, liver infection/disease, treatment and complications if any, were also noted.

B. Collection of Blood Samples:

Blood samples of suspected HCV patients or chronic liver disease patients, HCV patients on combined therapy, HCV screening camp (general population) and diabetics (clinically diagnosed by the physicians) were collected at random, from National Hospital Faisalabad, Allah Rakhi Trust Hospital, Faisalabad, DHQ Hospital Faisalabad
and from the rural/urban area in and around Faisalabad city, directly referred to NIBGE for molecular diagnosis of HCV.

The samples of clinically diagnosed diabetics and non-diabetics (control) were also collected from the above-mentioned. Blood samples of known HCV positive and negative, were also collected for reference. After separating serum/plasma, the samples were stored at -40 °C till detailed analysis.

C. Biochemical and Serological Tests:

Hospital pathological laboratory conducted estimation of random and fasting blood glucose by using the commercially available kits.

Serum alanine aminotransferase (ALT) was tested with routine automated technique (upper limit of the normal, 40U/L). This test was performed at National hospital pathology laboratory Faisalabad, other standard clinical laboratories of the city where possible and at NIBGE too.

D. Procedure of the HCV Screening Kit (CCL).

Serum anti-HCV antibodies (anti-HCV) in the samples were determined using one-step cassette style anti-HCV Kit (IND Diagnostic Inc, Canada).

Procedure as per Manufacturer's protocol:
1. Blood sample was taken in a container without anticoagulant. Allowed the blood to clot and separated the serum and used it for HCV screening or stored at -40 °C till analysis.

2. *When we got ready to begin testing, brought the specimens to room temperature.*

3. Opened the sealed pouch by tearing along the notch and removed the test cassette from the pouch.

4. 0.2 ml (about 4 drops) serum sample was drawn with help of pipette and dispensed it into the sample well on the cassette.

5. *Waited for 10-20 minutes to develop the respective chromatographic bands if the given sample was anti-HCV positive and read the results*

**Result interpretation.**

1. The sample was said negative for anti-HCV, if only one colored band appeared on the control (C) region and no apparent band on the test (T) region.

2. The sample was said positive for anti-HCV, if in addition to a pink colored control (C) band, a distinct pink colored band appeared on the test (T) region.

**Note.** A total absence of bands in both regions was an indication of procedure error and/or the test reagent had been deteriorated.

**E. Anti-HCV Antibody ELISA Test.**
For the detection of antibodies to HCV antigens, ELISA Kit (Biokit, Spain) was used as per instructions from the manufacturer. The kit was purchased from the open market and stored at 4°C until use. An outline of the assay is as follows:

**Assay Procedure**

1) Typically 200μl of negative control (human serum negative for anti-HCV) was added to 3 wells and 200μl of positive control (serum positive for HCV antibodies) to 2 wells. Blank was left empty. 200μl of sample diluents (Tris buffer with stabilizing proteins) and 10μl of each sample were added to the specific well.

2) The plate was covered with adhesive seal, mixed gently by swirling and incubated at 37°C for 1 hour.

3) The adhesive plate cover was removed and discarded. The contents of the plate were aspirated and filled completely (approximately 300 μl) with the diluted washing solution (10x; phosphate buffer, 1% tween-20 and 0.01% thimersol). The process of aspiration and washing was repeated 5 more times. After the last washing, the micro-liter plate was blotted on absorbent tissue to remove any excess liquid from the wells.
4) 100 µl of diluted conjugate (rabbit anti-human IgG conjugated with peroxidase diluted in conjugate diluents as per kit manufacturer's protocol) was transferred into each well except the blank, covered the plate with the adhesive seal and incubated for 30 minutes at 37°C.

5) Adhesive plate cover was removed, aspirated and washed the micro plate as in step 3

6) 100µl of substrate-TMB solution {citrate buffer, hydrogen peroxide, 3, 3', 5, 5' tetramethylbenzidine (TMB) dissolved in dimethylsulphoxide (DMSO)} was added to each well and incubated uncovered for 30 minutes at room temperature.

7) The reaction was stopped by adding 100 µl of stopping solution (1N Sulphuric acid) and read the absorbance at 450nm

**Note.** The validity of the assay was determined as described below:

The presence or absence of anti-HCV antibodies in the samples analyzed was determined by relating the absorbance value of each sample to the cut-off value of the technique. This value was the mean value obtained for the negative control plus 0.300.

\[
\text{Cut-off} = NC_x + 0.300
\]
If the initial test result absorbance value was less than the calculated cut-off value the sample was considered non-reactive for HCV antibodies and if the values were equal to or greater than the cut-off, the sample was considered to be reactive or positive for HCV antibodies by the criteria of this test.

F. SERUM ALANINE-AMINOTRANSFERASE (ALT) ASSAY

Liqui-UV-Test.

Serum Glutamate-Pyruvate Test (SGPT) or Alanine-aminotransferase (ALT) level in the samples was determined using (Liqui-UV-kit, Germany).

Procedure as per Manufacturer's protocol:

2 ml from reagent R1 (0.18mM/L NADH, 15mM/L 2-Oxoglutarate) were added into enzyme reagent, R2 (L-Alanine 500 mM/L, TRIS buffer (pH 7.5) and LDH (1200 U/L) and mixed thoroughly. This working reagent is stable for 4 weeks at 2-8°C.

Then 1 ml of this working reagent was taken in a quartz cuvette having 1 cm path length and 200 μl of the serum or plasma was added/mixed well and read the absorbance after 1 minute (incubation at 25°C or 30°C) at 340 nm against air (decreasing absorbance). At the same time stopwatch was also started and read the
absorbance again exactly after 1 and 2 minutes. Temperature was kept constant for the duration of the test. Same assay was repeated for all the samples.

For absorbance changes per minute (ΔA/min) within 0.12-0.16 (340nm), only measurements from the first 2 minutes were used for calculation. SGPT activity in the sample was calculated using the following factor.

\[ \text{U/L (25°C, 30°C)} = \Delta \text{ A/min x 952} \]

G. MOLECULAR STUDIES:

i. Primers and Probes:

Synthetic oligonucleotide (Primers and Probes) used for reverse transcription; amplification and genotyping were derived from highly conserved 5'-untranslated region (UTR) of the HCV genome. Oligo-nucleotide probes and primers used in the study were either synthesized from Fermentas (MBI) Company after providing nucleotide sequence or synthesized at NIBGE on Gene Assembler Special (Pharmacia). All the probes and primers used in this study are listed in Appendix I.

ii. RNA Isolation:

All the reagents for RNA extraction were made in 0.1% Diethyl pyrocarbonate (DEPC) treated autoclaved distilled water.
RNA was extracted from the plasma according to Petrelli et al. (1994). 300 μl of denaturation solution (4.0M Guanidium isothiocyanate (sigma), 25mM Sodium citrate (pH 7.0), 0.5% sodium lauryl sarcosine, 700μl β-mercaptoethanol /0.1L solution) was added to 100 μl of sample (plasma/serum) in 1.5 ml Eppendorf tube. To this, 30 μl of 3M sodium acetate (NaOAc: pH 5.2), 400μl water saturated phenol and 80 μl of chloroform was added. The eppendorf tube was vortexed for 30 seconds at each step and kept on ice for 15-20 minutes. It was then centrifuged at 15000 rpm for 10-15 minutes.

The upper aqueous phase was transferred into another Eppendorf tube and 0.6 volume of iso-propanol was added to it. The tube was vortexed briefly and kept on -20 °C for 15-20 minutes or on ice for at least half an hour. It was then centrifuged at 4°C for 20-30 minutes. The supernatant was discarded and the pellet was washed twice with 70% ethanol and vacuum dried. The pellet was then stored by suspending in 70% ethanol at -70 °C until cDNA synthesis or dissolved in 10 μl sterile autoclaved distilled water (DEPC treated).

iii. cDNA SYNTHESIS:

cDNA was synthesized from all the RNA extracted in the previous step, by using reverse transcriptase enzyme following the protocol of Tisminetzky et al.(1994) with some modifications. Total reaction volume (20μl) contained, 10μl RNA sample, 1X first strand buffer (5X stock; 250mM Tris HCl pH 8.3, 375mM KCl, 15mM MgCl₂, 50mM DTT) 100μM dNTPs (dATP, dCTP, dGTP & dTTP), 20 pM of the antisense
primer (AS1: 5'-GTGCACGGTCTACGAGACCT-3') complementary -2 to -21 bp), 10U of ribonuclease inhibitor (10U/µl) and 50U of Molony Murine Leukemia Virus (M-MLV) reverse transcriptase (RT) enzyme. All these chemicals were purchased from GIBCO-BRL/Sigma Chemicals.

The reaction mixture was incubated at 42 °C for 1 hour in thermo-mixture (Eppendorf) incubator. After incubation, the samples were stored at -20°C till polymerase chain reaction (PCR) amplification.

iv. 1st Round of PCR (Regular PCR):

Regular PCR was run with a total reaction volume of 50µl containing 10µl synthesized sample cDNA as template DNA and with final concentration of each, 1mmol dNTPs. 1X PCR buffer, 50pmol of the both primers sense (S1: 5'-GCCATGGGCCTTAGTATGAGT-3') and antisense (AS1: 5'-GTGCACGGTCTACGAGACCT-3') complementary to the region flanking specific of the template DNA and 2U of Taq polymerase. The reaction volume was adjusted with sterile distilled water.

For PCR amplification one cycle of the temperature profile, 94°C for 2 minute only for first cycle, then 30 cycles with the temperature profile 94 °C for 30 seconds (denaturation of DNA), 55 °C for 30 seconds (annealing of primers), 72°C for 30 seconds (DNA amplification) and then at the end of 30 cycles, 4°C (soaking temperature) were used.
v. 2nd Round of RT-PCR (Nested PCR)

For nested PCR, 1-2 μl of the regular PCR product was used as template while internal primers both sense (S2: 5'-GTGCAGCCTCCAGGACCC-3') and antisense (AS2: 5'-CCGTGAGCGTTGTCGTTGGGATA-3') were employed (Tisminetzky et al., 1994) which were complementary to the amplified product of the regular PCR instead of external primers as were used in regular PCR. Others conditions were the same as in case of the regular PCR.

H. Analysis of PCR Products:

I) Agarose gel electrophoresis

The amplified PCR products of both regular and nested PCR were run on 1.5% agarose gel in 1X TBE {5X; 54.0g Trizma base, 27.5g Boric acid, 20ml EDTA (0.5M)} following (Davis et al., 1994 and Sambrook et al., 1989).

An adequate volume of electrophoresis buffer (1X TBE) was taken in the electrophoresis tank. Desired amount 1.5% agarose gel was prepared in 1X TBE by melting in microwave oven till boiling. It was then cooled to 55°C before pouring in the gel casting tray and the comb was inserted ensuring that no air bubbles were trapped in the gel.

After hardening of the gel at room temperature, comb was removed carefully. Gel caster containing gel was then placed in electrophoresis chamber containing
sufficient buffer. 10µl of the nested PCR products along with negative and positive controls were loaded on the gel in the respective wells after mixing with appropriate amount of 6X loading dye (40% sucrose and 0.01% bromophenol dye). Voltage was set to the desired level (typically 1-10v/cm of the gel). The movement of the dye in the gel indicated the migration of the DNA in the gel from the cathode to anode. Power supply was turned off when the dye had reached to a distance judged sufficient for separation of DNA fragments.

DNA fragments were visualized under ultraviolet light, which were illuminated by ethidium bromide dye (1% w/v), added in the gel.

1 Quality Control:

Several positive and negative controls were included in the PCR analysis to make HCV diagnosis authentic and reliable.

a. Negative plasma control:

In this control cDNA extracted from a known HCV-RNA negative sample was used in PCR at every time to confirm that amplifications were only in the cDNA of HCV positive samples.

b. No sample negative control:
In this control all the procedures for cDNA extraction were performed but no plasma sample was added. The control was used to find contamination if any, in the reagents & consumables used in cDNA extraction. No amplification both in regular and nested PCR products was detected of that control.

c. **Negative amplification control:**

All PCR reagents were added but no cDNA was used to confirm that the amplification is from target cDNA and PCR reaction mixture is free from any contaminating cDNA.

d. **Positive amplification control:**

10μg of cloned HCV cDNA was included in this control.

e. **Positive plasma control:**

This control was used to find the accuracy of the method used for the HCV cDNA extraction and subsequent amplification.

J. **Statistical Analysis:**

A general descriptive analysis was performed to compare demographic parameters. Pearson's Chi square test was used to see association between ELISA test and RT-PCR, their ALT level and RT-PCR.
K. HCV Genotyping:

i). HCV Genotyping Using Type Specific Primers:

HCV genotyping was tried following the Okamoto et al. 1992 method with some modifications:

1. 200ul sera was diluted with 1 ml of ice-cold RNA extraction solution (RNA-ZOL: GIBCO-BRL, Italy) and mixed thoroughly.

2. Then serum proteins were removed by phenol-chloroform extraction.

3. RNA was precipitated after the addition of 1 volume of ice-cold isopropanol and incubating on ice for 10-20 min.

4. After pellet formation by centrifugation in a micro centrifuge (Eppendorf), the RNA was washed with 70% ethanol, vacuum dried and then suspended in 50 ul of DEPC water.

5. cDNA was prepared as described above by adding only antisense primer for the core region of the HCV genome with final concentration of 50pmol was used and incubated at 37 °C for 60 minutes as described by Okamoto et al. 1992.

7. All the cDNA was amplified in the presence of both antisense and sense primers with final volume of 100 ul with 2.5U of Taq polymerase (Fermentas).
8. Two sequential amplifications were performed. For the first amplification, 35 cycles of 94 °C for 1 min, 55 °C for 1.5 min and 72°C for 2 min were while for the second amplification, 30 further cycles of 94 °C for one min, 60 °C for 1.5 min and 72°C for 2 min, were run.

9. 10 ul of first amplification was added to 90 ul of reaction mixture for second PCR containing 50 pmol of inner universal primer and 50 pmol of each of type specific inner anti-sense primers for first four genotype of HCV.

10. Finally, for the identification of HCV type-5 was carried out in separate tube with the addition of sense primer for the first PCR and two inner sense and anti-sense primers for this specific type with same PCR conditions as were in first case.

11. The products of the PCR were run on the 4% Agarose gel as was run in case of RT-RFLP analysis. The sequence of primers is shown in the Appendix-iv.

ii). **HCV Genotyping by Radioactive Probes:**

The amplified serum/plasma samples of the subjects were genotyped by dot-blot hybridization method (Tisminetzky et al. 1994) with genotype specific radio-labelled probes. In this experiment, nested PCR products (10 ul) of the HCV positive subjects in triplicates were denatured with 40ul solution containing 0.4 M NaOH and 25 mM EDTA. 50 ul of distilled water was added to the denatured sample and the tubes were incubated at 95°C for 10 minutes and then quenched on ice. The same denatured DNA
samples were dot-blotted onto three separate Hybond-N+ nylon membranes (Amersham, UK). The blotted membrane were either baked at 80°C for one hour in a vacuum oven (Cole Parmer, USA) or treated in Stratagene cross linker to fix the DNA.

Pre-hybridization was done in 5X SSPE, 5 X Den hart's solution (20 X SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA; 50 X Den hart's Solution: 1% Ficoll, 1% PVP, 1% BSA) at 1°C above Tm of the probes to be hybridized for 30 minutes. Genotype-specific oligonucleotide probes corresponding to HCV-1, 2 and 3 genotypes were end-labeled using T4 polynucleotide kinase and γ³²P·ATP according to Tisminetzky et al., 1994. The Tm (melting temperature) for the probe was determined according to Suggs et al., 1981.

After hybridization with specific HCV probes, the hybridization solution was collected back into 50 ml Falcon tubes and stored at -70°C. The membranes were washed in 200 ml of 2 X SSPE containing 0.2% SDS at room temperature while shaking, for 10 minutes and this step was repeated four times by replenishing the buffer each time. Finally, the membranes were washed in 5 X SSPE, 0.2 % SDS at temperature 2-4°C above the Tm of the probe. After the last wash, the membranes were air dried and exposed for autoradiography using X-ray film (Amersham) with intensifying screen at -70°C.

iii). **HCV Genotyping through PCR-RFLP analysis:**
HCV genotypes were also determined by restriction fragment length polymorphism (RFLP) of the nested polymerase chain reaction (N-PCR) products obtained from suspected patients, HCV patients taking therapy, from general population samples, diabetic and non-diabetic subjects as described by McOmish et al. (1994) with minor modifications. To differentiate HCV into its major genotypes, the amplicons or positive nested PCR products were restricted by three sets of restriction enzymes listed in table below.

The total reaction volume (30μl) contained following reagent in 1.5ml Eppendorf tube:

10μl nested-PCR product

17μl PCR water (double distilled deionized water)

3 μl buffer Y+/Tango™

5-10 units of each enzyme of a set (see in the table below)

The reaction mixture was incubated at 37°C for 3-4 hour on Eppendorf Thermo mixer-5436.

**Enzymes Used for HCV Genotyping by RFLP Analysis:**
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Set</td>
<td><em>Hae</em> III &amp; <em>Rsa</em> I</td>
</tr>
<tr>
<td>2. Set</td>
<td><em>Hinf</em> I &amp; <em>ScrF</em> I</td>
</tr>
<tr>
<td>3. Set</td>
<td><em>Hinf</em> I &amp; <em>Mva</em> I</td>
</tr>
</tbody>
</table>

**Analysis of DNA products by agarose gel electrophoresis:**

The nested PCR products or restricted PCR products were separated on 4% agarose gel in 1X TBE [5X stock TBE: 54.0g Trizma base, 2705g Boric acid, 20ml EDTA (0.5 M, pH 8.0)] following Davis and Sambrook (Davis et al. 1994 & Sambrook et al. 1989).

**Identification of HCV Genotypes:**

The nomenclature proposed by McOmish et al., 1993 & Simmonds et al., 1993 was used for the HCV genotypes identification based on PCR-RFLP analysis with some modifications as shown in the Appendix-III.
RESULTS
4. RESULTS

HCV is a major health problem all over the world causing liver related morbidity and mortality. This disease is highly prevalent in subjects with acute and chronic liver disease and associated hepatocellular carcinoma. The severity, clinical causes and risk factors related to acute and chronic disease are still poorly defined especially, in the area under study. This study was carried out to investigate (i) the prevalence of HCV, risk factors and severity of the disease in suspected HCV patients or chronic liver disease patients, in general population and in diabetics (ii) HCV genotypes prevalent in these categories of subjects (iii) to see the antiviral response in the known HCV cases from the local population of Faisalabad city as well as from the adjacent areas of city which are densely populated, largely industrial, highly polluted and populated city of Pakistan.

4. A. Selection of Subjects:

2000 samples from suspected HCV patients having some common symptoms of hepatitis such as fatigue, lethargy anorexia, pyrexia, body itching or history of jaundice, along with 300 samples from general population, 100 samples from the diabetics, 60 samples from non-diabetics as control and 97 samples from HCV patients taking combined (interferon + ribazole) therapy were recruited for the study from various localities of Faisalabad city as well as adjoining areas such Jhung, Gojra, Toba Tek Singh, Jaranwala and Chiniot during the years from January 2001 to December 2003. Brief description of the subjects of all categories along with mean ALT level is shown
Table-1: A brief description of subjects used for the study.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gender</th>
<th>No. of Subjects</th>
<th>Mean ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Suspected subjects</td>
<td>1073</td>
<td>927</td>
<td>2000</td>
</tr>
<tr>
<td>General population</td>
<td>232</td>
<td>68</td>
<td>300</td>
</tr>
<tr>
<td>(Screening Camp)</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Diabetic subjects</td>
<td>47</td>
<td>53</td>
<td>60</td>
</tr>
<tr>
<td>Control (Non-diabetics)</td>
<td>34</td>
<td>26</td>
<td>97</td>
</tr>
<tr>
<td>Therapy cases</td>
<td>56</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1442</td>
<td>1115</td>
<td>2557</td>
</tr>
</tbody>
</table>
Table-2: A general comparison of HCV-ELISA or anti-HCV test Versus HCV-PCR in all categories of samples.

<table>
<thead>
<tr>
<th>Category</th>
<th>No. of Subjects</th>
<th>Anti-HCV-antibody or ELISA positive No. (%)</th>
<th>RT-PCR positive No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV Suspected</td>
<td>2000</td>
<td>1812 (90.6)</td>
<td>1745 (87.25)</td>
</tr>
<tr>
<td><strong>General Population</strong></td>
<td>300</td>
<td>62 (20.6)</td>
<td>84 (28)</td>
</tr>
<tr>
<td>Diabetics</td>
<td>100</td>
<td>42 (42)</td>
<td>36 (36)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Diabetics</td>
<td>60</td>
<td>9 (15)</td>
<td>8 (13.4)</td>
</tr>
<tr>
<td>Therapy Cases</td>
<td>97</td>
<td>93 (95.87)</td>
<td>19 (19.6)</td>
</tr>
</tbody>
</table>
in the Table-1. Among the total subjects (2557) selected for the study, 1115 (43.6%) were females and 1442 (56.4%) were males.

4. B. General Comparison of Anti-HCV Test and RT-PCR.

A general comparison of anti-HCV test and RT-PCR is given in the Table-2. Out of 2000 suspected HCV subjects 1812 (90.6%) were positive by anti-HCV test while 1745 (87.25%) were found positive by RT-PCR test. Among 97 subjects on antiviral therapy, 93 (95.87%) were still anti-HCV positive while 19 (9.6%) were also RT-PCR positive. In HCV screening camp samples (n=300), taken from general population at random, 62 (20.6%) were anti-HCV positive while 84 (28%) subjects were RT-PCR positive. Among diabetic subjects, out of 100 samples 42% were anti-HCV positive and 36% were RT-PCR positive as compared to control group where out of 60 subjects selected for the study, 09 (15%) were found anti-HCV positive while 08 (13.4%) were RT-PCR positive for HCV infection.

4. 1. Suspected HCV Cases:

4. 1. 1. Risk factors:

A comprehensive history Performa was designed as shown in the appendix-1 indicating name, age, sex, address, various physical or clinical symptoms, family history, serological findings, liver function tests (LFTs) record, and treatment record if any, of the subjects were included in it. The subjects were also evaluated for other parameters such as disease duration, and treatment, previous hospital
admission/surgery, blood transfusion, complications as a result of HCV liver infection, history of diabetes, and exposure to risk factors.

As described in Fig. 1, out of 2000 subjects studied, (24%) subjects were exposed to single risk factor such as, blood transfusion, hospitalization, visit to Barber/Dentist, multiple pricks or surgical interventions. (36%) subjects were exposed to more than one risk factors whereas 30.5% patients never encountered any risk factor. 9.5 subjects had family history of HCV disease or deaths as result of this disease.

Epidemiological Findings:

2000 samples were collected from the years January 2001 to December 2003. As indicated in the Table-3 that among the 532 samples collected and analyzed during the first year of study, 493 (92.66%) were found anti-HCV positive by ELISA test while 493 were HCV-RNA positive by RT-PCR. In the year 2002, 702 samples were collected and analyzed and the data showed that 601 (88.46%) were anti-HCV positive while 587 (83.2) were HCV-RNA positive. 766 samples were further collected and analyzed during the year 2003 from the suspected HCV subjects and again very high HCV infection and incidence rate was observed as it was found that 698 (91.12%) were anti-HCV positive while 716 (93.47%) were RT-PCR positive.
Fig. 1: Risk factors involved in HCV transmission.
Table-3: Epidemiological data of the suspected HCV subjects.

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of Samples</th>
<th>Anti-HCV Positive No. (%Age)</th>
<th>RT-PCR Positive No. (%Age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>532</td>
<td>493 (92.66)</td>
<td>442 (83.08)</td>
</tr>
<tr>
<td>2002</td>
<td>702</td>
<td>621 (88.46)</td>
<td>587 (83.2)</td>
</tr>
<tr>
<td>2003</td>
<td>766</td>
<td>698 (91.12)</td>
<td>716 (93.47)</td>
</tr>
<tr>
<td>Total</td>
<td>2000</td>
<td>1812 (90.4)</td>
<td>1745 (87.25)</td>
</tr>
</tbody>
</table>
Table-4: Demographic and clinical data of the suspected HCV subjects.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Gender</th>
<th>Total Subjects</th>
<th>RT-PCR</th>
<th>Mean ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. (%)</td>
<td>Positive subjects No. (%)</td>
<td>level U/L</td>
</tr>
<tr>
<td>Years</td>
<td>M</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>62</td>
<td>56</td>
<td>158 (7.9)</td>
<td>102 (5.1)</td>
</tr>
<tr>
<td>21-30</td>
<td>322</td>
<td>213</td>
<td>535 (26.7)</td>
<td>476 (23.8)</td>
</tr>
<tr>
<td>31-40</td>
<td>374</td>
<td>350</td>
<td>724 (36.2)</td>
<td>690 (34.5)</td>
</tr>
<tr>
<td>41-50</td>
<td>206</td>
<td>183</td>
<td>389 (19.5)</td>
<td>326 (16.3)</td>
</tr>
<tr>
<td>51-60</td>
<td>72</td>
<td>65</td>
<td>137 (6.9)</td>
<td>109 (5.45)</td>
</tr>
<tr>
<td>61-75</td>
<td>37</td>
<td>20</td>
<td>57 (2.8)</td>
<td>42 (2.1)</td>
</tr>
<tr>
<td>Total</td>
<td>1073</td>
<td>927</td>
<td>2000 (100)</td>
<td>1745 (87.25)</td>
</tr>
</tbody>
</table>
4. 1. 2. Brief Demographics and its Relation with Molecular and Clinical Findings.

As shown in the Table-4, among HCV-RNA positive subjects, the prevalence of HCV infection was highest in 724 (36.2%) subjects in the age group of 31-40 years. In the age group of 21-30 years, the number HCV positive subjects were 535 (26.75%) whereas it was in 389 (19.45%) in the subjects of age group 41-50 years. Thus major HCV infection or incidence was noted in these three age groups. The age groups of 10-20 years and 51-60 years had almost similar infection rate which was 102 (5.1%) and 109 (5.45%) respectively while infection rate in 61-75 years age group had least infection rate where only 42 (2.1%) subjects were HCV-RNA positive.

The Table-4 also gave an over all picture of ALT level in the blood of the patients that is a major liver enzyme and is used as marker of liver infection or hepatitis. It was noted that mean level of ALT, although higher than normal value, did not vary significantly among various age groups and it ranged from 73 U/L to 95 U/L in age groups, from 10 years to 50 years of subjects. The mean level of ALT was 170 U/L in patients of age group of 51-60 years while 110 U/L in the age group of 61-75 years.

4. 1. 3. Anti-HCV ELISA Test.
All the 2000 samples selected for the study were tested by second generation Enzyme Linked Immunosorbent Assay (ELISA) kit (Biokit, Spain). The data obtained from these tests is shown in the Table-1 and Fig-2. 1812 (90.6%) subjects were found positive for anti-HCV antibodies while only 188 (9.4%) were negative by anti-HCV ELISA test.

4. 1. 4. Detection of HCV by RT-PCR

All the 2000 samples were analyzed for HCV-RNA detection by reverse transcriptase polymerase chain reaction (RT-PCR) by targeting 5’ untranslated region (5’UTR) of the virus genome. Regular PCR (R-PCR) was carried out, using both sense (S1) and antisense (AS1) external primers (see Materials and Methods for details). Then Nested PCR (N-PCR) was carried out by taking 1-2 μl of PCR products of regular PCR as template, using internal set of primers (S2& AS2). Nested PCR products indicated amplification in several samples as shown in the Fig. 3.

The results of nested PCR as shown in the Table-2, 3 and Fig. 3, indicated that 1745 (87.25%) subjects positive for Nested PCR as compared to anti-HCV ELISA test where 1812 (90.4%) samples were anti-HCV positive. A comparison of HCV ELISA test and RT-PCR is shown in the Fig-3 and association between them is shown in Table-5, which indicates that in 81.2 % cases, there was complete unanimity.
Fig. 2: Analysis of samples by agarose gel electrophoresis for HCV diagnosis after RT-PCR. Lane 1: DNA size marker, Lane 2: negative control, Lane 3: positive control, Lanes 4-14: specific RT-PCR products of HCV positive and HCV negative samples.
Fig. 3. A comparison of anti HCV ELISA test VS RT-PCR in suspected HCV cases.
Table- 5:  Statistical analysis by Chi Square test to see the association between anti-HCV ELISA with RT-PCR among suspected hepatitis patients.

<table>
<thead>
<tr>
<th>HCV ELISA Test</th>
<th>RT-PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>+ve</td>
<td>1624</td>
<td>188</td>
</tr>
<tr>
<td>Frequency</td>
<td>(1581)</td>
<td>(231)</td>
</tr>
<tr>
<td>-ve</td>
<td>121</td>
<td>67</td>
</tr>
<tr>
<td>Frequency</td>
<td>(164)</td>
<td>(24)</td>
</tr>
<tr>
<td>Total</td>
<td>1745</td>
<td>255</td>
</tr>
</tbody>
</table>

Figures within brackets are expected values.
Value of Chi-square = 97.5**
Thus association or linkage is indicated between the two parameters.
between RT-PCR analysis and HCV ELISA test. It was further noted that 9.4% HCV ELISA positive subjects were negative by RT-PCR. Similarly 6.5% RT-PCR positive cases were found HCV-ELISA negative. The value of Chi square test was 79.5** indicating significant association or correlation.

4.1.5. Alanine Aminotransferase (ALT) Test.

The results of ALT in suspected cases are given in the Tables-2, 3, 4, 6 & in Fig. 5. The data showed a wide range of activity from 10 units per liter (U/L) up to hundreds units per liter, when samples were spectrometerically assayed at 25°C. ALT level of all the samples studied, were noted and different level of ALT was observed in various categories of samples. It is mentioned here that the normal range of ALT level was 0-40U/L when samples were analyzed at 25°C.

1585 (79.25%) RT-PCR positive subjects, the level of ALT was higher than normal. The mean ALT level in all suspected cases was observed 61.45U/L that was almost one and half fold higher than the normal value on the average. In 1585 (79.25%) HCV-RNA positive subjects the value of mean ALT level was 97.6 U/L while in 160 (8%) RT-PCR positive subjects, the level of ALT was in upper normal limits (38.7 U/L). It was interesting to note that in 97(4.85%) HCV-RNA negative subjects, the level of ALT was similarly higher, although the mean ALT level (86.5U/L) was slightly in lower limits as compared to HCV-RNA positive patients. 158 (7.9%) subjects were also noted who were both HCV-RNA negative and also possess normal ALT value. The
value of Chi test was 456.6***, again indicating that highly significant association between these two parameters existed in suspected cases.

4.6. HCV GENOTYPING

4.6.1. Using Type Specific Primers:

Identification of major HCV genotypes was tried by using type specific primers from the core region of the HCV genome following by Okamoto et al 1992. We strictly followed the protocol and also did some modifications as were done in case of RT-PCR and RFLP analysis to get some results and to identify some HCV genotypes. But unfortunately all these trials resulted in total failure and could not get any amplification or identify any HCV genotype even trying again and again and also by changing the PCR conditions. We tried more than one hundred samples from the suspected HCV subjects for this purpose but at the end there was no result to present. So HCV genotyping was tried by PCR-RFLP analysis.

4.6.2. GENOTYPING OF THE NESTED-PCR PRODUCTS

a. By Dot Blot Hybridization.

When no results of HCV typing were obtained by using type specific primers, this method was tried on the availability of radiolabeled \( \gamma \) p\(^{-32} \) for some samples of the first year study in suspected subjects.

The genotyping of HCV was carried out for 120 HCV positive samples by dot blot hybridization using three different HCV genotype specific \( \gamma \) p\(^{-32} \) labeled
probes. Some results of HCV genotypes identified after autoradiography are shown in Fig. 5. The results summarized in the Fig. 8, have indicated that G1 probe presenting genotype-1 hybridized with 28 (23.3%) of the HCV positive samples while only 5 (4.16%) samples hybridized with G2 probe and 69 (57.5%) samples with G3 probe. 12 (10.0%) positive samples did not hybridize with any of the probes as such remained untyped. Mixed genotypes were also observed in 6 (5.0%) samples.

b. **By PCR-RFLP.**

Major HCV genotypes were also identified by Restriction Fragment Length Polymorphism (RFLP) analysis from the N-PCR products (see material and methods) by using three sets of restriction enzymes following McOmish *et al.* 1994. The agarose gel pattern of various HCV genotypes is indicated in Fig. 6, and 7 while epidemiological data of HCV genotypes by RFLP analysis is described in Fig. 9. According to RFLP analysis of 580 HCV positive samples, 54 (9.31%) were of genotype-1 while genotype-2 was found in only 12 (2.06%) samples and while genotype-3 was observed as major genotype as 490 (84.48%) samples were found to be of HCV genotype-3. There was no other genotype could be identified by this techniques while 24 (4.13%) samples showed different banding pattern on gel than the standards and thus remained unidentified.
4. A comparison of ALT level VS RT-PCR in suspected HCV cases
Table-6: Statistical analysis by Chi-square test to see the association between ALT level with RT-PCR among suspected HCV patients.

<table>
<thead>
<tr>
<th>ALT Level</th>
<th>RT-PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Normal</td>
<td>160</td>
<td>158</td>
</tr>
<tr>
<td>Frequency</td>
<td>(277)</td>
<td>(41)</td>
</tr>
<tr>
<td>High</td>
<td>1585</td>
<td>97</td>
</tr>
<tr>
<td>Frequency</td>
<td>(1468)</td>
<td>(214)</td>
</tr>
<tr>
<td>Total</td>
<td>1745</td>
<td>255</td>
</tr>
</tbody>
</table>

Figures within brackets are expected values.
Value of Chi-square= 456.6***
Thus association or linkage is highly significant.
Fig. 5: Genotyping of HCV by dot blot hybridization in suspected HCV samples with type specific $\gamma^p^{32}$ labeled probes. Same samples were blotted on the three membranes and hybridized with type specific probes.
Fig. 6: HCV genotyping by RT-RFLP analysis in suspected HCV subjects. Lanes 1-6: Restriction with Hae III & Rsa I, Lanes 7 & 15: 50bp DNA Size Marker, Lanes 8-13: Restriction with MvaI & Hinf I, Lanes 14 & 16-20: Restriction with ScrF I & Hinf I.
Fig. 7: HCV genotyping by RT-RFLP analysis in suspected HCV subjects indicating a typical HCV type-3 pattern on the 4% agarose gel. Lanes 1-6: Restriction with Hae III & Rsa I, Lanes 7 & 14: 50bp DNA Size Marker, Lanes 8-13: Restriction with ScrFI & HinfI, Lanes 15-20 Restriction with MvaI & HinfI.
Fig. 8. Prevalence of HCV genotypes in suspected HCV subjects determined by dot blot hybridization.
Fig. 9. Molecular epidemiology of HCV genotypes, determined by PCR-RFLP analysis in suspected HCV cases.
4. 2. THERAPY CASES:

Ninety-seven HCV patients who were on combined therapy (interferon and ribazole) were also recruited for the study in order to see the response rate of the therapy on various HCV genotypes prevalent in the region.

4. 2.1. Anti-HCV ELISA Test.

In case of HCV patients who were taking therapy for at least three months or completed their therapy for at least six months to one year, it was found that out of 97 samples selected for the study, 93 (95.85%) were still anti-HCV ELISA positive as indicated in the Fig. 10 and Table-7. Among them 60 subjects were under therapy for at least three months while 33 recently completed their therapy for at least six months to one year. Only 4 (4.15%) subjects were found HCV-ELISA negative who had completed their therapy about 4 years ago.

4. 2.2. RT-PCR Analysis.

All the 97 subjects who were still on therapy or completed therapy were tested by RT-PCR qualitatively to see the response rate of the combined therapy. It was found as indicated in the Fig. 10 and Table-7 that 19 (19.6%) patients were still remained RT-PCR positive. Among these 19 RT-PCR positive patients, 11 (57.89%) had completed their therapy more than one year ago but became again RT-PCR positive although they were PCR negative just after completion of therapy while 8 did not respond to the therapy and continually remained HCV-RNA positive during the treatment and even
after completion of therapy. 78 subjects became RT-PCR negative and among them, 32 subjects completed three months therapy while 46 subjects completed their duration of therapy and became RT-PCR negative for HCV-RNA detection.

It was noted that in a very high percentage (76.42%) of subjects, discrepant results between RT-PCR analysis and anti-HCV ELISA test was obtained. Actually very little association or non-significant co-relation was found in these tests in case of subjects who were on combined therapy. The value of Chi test was 1.4 and thus no association was observed in case of therapy subjects.

4. 2. 3. ALT Level Determination.

ALT level of the subjects taking therapy or who completed therapy was also measured. It was observed that among the 19 patients who were RT-PCR positive, 11 had high ALT (mean 156U/L) level while 8 had upper limits of the normal ALT (mean 40.6 U/L) value. It was also noted that only 2 persons out of 78 RT-PCR negative subjects still had high ALT level while 76 were both RT-PCR negative and also possess normal ALT level. The results of ALT level are shown in the Fig. 11 and Table-8. The value of Chi test 32.7**, again indicated association between RT-PCR and ALT level in even therapy cases also.

4. 2. 4. Genotyping by PCR-RFLP Analysis.

The molecular epidemiological data about the prevalence of HCV genotypes in therapy cases was done by PCR-RFLP only and presented in
Fig. 10. A comparison of Anti-HCV ELISA test VS RT-PCR in HCV subjects taking combined therapy.
Table-7: Statistical analysis y Chi square test to see the association between anti-HCV ELISA test and RT-PCR among HCV patients on combined therapy.

<table>
<thead>
<tr>
<th>HCV ELISA</th>
<th>RT-PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>19</td>
<td>74</td>
</tr>
<tr>
<td>Frequency</td>
<td>(18)</td>
<td>(75)</td>
</tr>
<tr>
<td>-ve</td>
<td>00</td>
<td>04</td>
</tr>
<tr>
<td>Frequency</td>
<td>(01)</td>
<td>(04)</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>78</td>
</tr>
</tbody>
</table>

Figures within brackets are expected values.
Value of Chi-square= 1.4 NS
Thus Independence is indicated between the two parameters.
**Fig. 11:** A comparison of ALT level VS RT-PCR in subjects taking combined therapy.
Table-8: Statistical analysis by Chi-square test to see the association between ALT level with RT-PCR test among HCV cases on combined therapy.

<table>
<thead>
<tr>
<th>ALT level</th>
<th>RT-PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ve</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>08</td>
</tr>
<tr>
<td>Frequency</td>
<td></td>
<td>(16)</td>
</tr>
<tr>
<td>High</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Frequency</td>
<td></td>
<td>(03)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>

Figures with in brackets are expected values.
Value of Chi-square= 32.7**
Thus association or linkage is indicated between the two parameters.
Fig. 12: Genotyping of HCV by RT-RFLP analysis indicating some samples of genotype-1 in subjects on therapy. Lanes 12 (upper) & 4 (lower); 50 bp DNA marker, Lanes 1-11; Restriction with *RsaI* & *HaeIII*, Lanes: 13-20 (upper) and 1-3 (lower); Restriction with *SrfI* & *HinFI*, Lanes 5-15 (lower); Restriction with *MvaI* & *HinFI*. 
Table-9: Prevalence of HCV genotypes in the subjects on antiviral therapy.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>GENOTYPES</th>
<th>NUMBER</th>
<th>%AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GENOTYPE-1</td>
<td>11</td>
<td>11.40</td>
</tr>
<tr>
<td>2</td>
<td>GENOTYPE-2</td>
<td>3</td>
<td>3.10</td>
</tr>
<tr>
<td>3</td>
<td>GENOTYPE-3</td>
<td>62</td>
<td>63.91</td>
</tr>
<tr>
<td>4</td>
<td>UNTYPED</td>
<td>21</td>
<td>21.60</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td>97</td>
<td>100</td>
</tr>
</tbody>
</table>
the Fig. 12 and Table-9. The results of RFLP analysis showed that out of 97 samples, 11 (11.40%) samples were of HCV genotype-1, while genotype-2 was found in only 3 (3.1%) samples. It was again observed that genotype-3 was the major genotype in therapy cases as well and 62 (63.91%) samples were found to be of genotype-3. No other genotype could be identified while significant number 21(21.64%) of samples remained unidentified.

4.2.5. **HCV Genotypes and Antiviral Response.**

As reported and discussed earlier that HCV genotypes were considered important in its treatment and management. From the data as mentioned in the Table-10, it is indicated that out of 97 therapy cases selected for the present study, 11 were of genotype-1 and the 7 became RT-PCR negative during treatment or after the completion of treatment and response rate of the antiviral treatment was 63.63%. Only 3 subjects were of genotype-2 and response rate in these subjects was 66.6% while 62 subjects were found to be of HCV genotype-3. In HCV type-3 subjects, the response rate of antiviral treatment was observed highest and it was 85.48%.

There were 21 subjects who could not be genotyped by PCR-RFLP analysis but interestingly response rate of antiviral treatment in these subjects was also good and it was 76.19%. It is mentioned here that all this data of anti-viral response were taken after the completion of 3 months to 1-year treatment and from the subjects who were retested for HCV-RNA detection after the follow-up of treatment from one year to four years although the number of such subjects was very small.
Table-10: HCV genotypes and response to combined (interferon alpha + ribazole) therapy.

<table>
<thead>
<tr>
<th>HCV GENOTYPES</th>
<th>PCR+VE</th>
<th>PCR-VE</th>
<th>TOTAL</th>
<th>% RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1</td>
<td>4</td>
<td>7</td>
<td>11</td>
<td>63.63</td>
</tr>
<tr>
<td>G-2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>66.6</td>
</tr>
<tr>
<td>G-3</td>
<td>9</td>
<td>53</td>
<td>62</td>
<td>85.48</td>
</tr>
<tr>
<td>UNTYPED</td>
<td>5</td>
<td>16</td>
<td>21</td>
<td>76.19</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>19</strong></td>
<td><strong>78</strong></td>
<td><strong>97</strong></td>
<td></td>
</tr>
</tbody>
</table>
3. GENERAL POPULATION

4. 3. 1. Demographic and Clinical Data of the Subjects:

For this investigation, Allah Rakkhi Hospital and Hepatitis Research Group in Health Biotechnology Division at NIBGE organized HCV screening camp, in and around Faisalabad city during the first six months of the year 2002. A prevalent HCV infection case was defined as a person who attended the screening camp and was positive with One Step cassette Style for Anti-HCV test.

The description of the subjects is presented in Table-11 along with the mean ALT levels. Three hundred subjects attended the HCV screening camps in duration of six months. Of these 232 (77 %) were male and 68 (23%) were female. The average age was 32±28 years. Most of the subjects had no signs of any disease, weakness, weakness, nausea, pyrexia, lethargy or jaundice. Only 26 (8.7%) subjects had some clinical symptoms of HCV infection. There was no case of intravenous drug abuse or haemodialysis and only five (1.66%) persons had the history of blood transfusion.

4. 3. 2. Prevalence of HCV Infection:

4. 3. 2. 1. Anti-HCV Screening:

The prevalence of HCV infection in general population was estimated by One Step cassette Style ANTI-HCV test. (See Fig. 13). 62 subjects were positive whereas 238 subjects were negative. (Tables-3& 13), thus the
Table-11: Demographic and clinical data of the samples from general population (screening camp).

<table>
<thead>
<tr>
<th>Age groups Years</th>
<th>Gender M/F</th>
<th>Total Subjects</th>
<th>No of HCV -RNA positive subjects</th>
<th>Mean ALT level U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. (%Age)</td>
<td>No. (%Age)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>10/4</td>
<td>14 (4.7)</td>
<td>4 (1.33)</td>
<td>43±36</td>
</tr>
<tr>
<td>21-30</td>
<td>94/32</td>
<td>126 (4.2)</td>
<td>31 (10.3)</td>
<td>64±54</td>
</tr>
<tr>
<td>31-40</td>
<td>102/21</td>
<td>123 (40.9)</td>
<td>35 (11.66)</td>
<td>81±76</td>
</tr>
<tr>
<td>41-50</td>
<td>15/6</td>
<td>21 (0.7)</td>
<td>10 (3.3)</td>
<td>99±45</td>
</tr>
<tr>
<td>51-60</td>
<td>11/5</td>
<td>16 (5.3)</td>
<td>4 (1.33)</td>
<td>110±95</td>
</tr>
<tr>
<td>Total</td>
<td>232/68</td>
<td>300 (100)</td>
<td>84 (28%)</td>
<td>103±63.3</td>
</tr>
</tbody>
</table>
prevalence of HCV infection by Anti-HCV screening in general population was 20.6%.

4.3.2.2. **Molecular Test Screening:**

HCV-RNA was isolated and cDNA was prepared (See Materials and Methods) from blood of all the subjects. These samples were subjected to two rounds of PCR amplification using specific UTR primers, to investigate the prevalence of HCV infection in the randomly selected population. A two hundred and ten (210) base-pair DNA fragment from 5'UTR of HCV genome which is highly conserved, was amplified in only 84 subjects (See Fig.15 & Table-13). Hence, the prevalence of HCV infection in general population by molecular method was 28%. The detailed prevalence in subjects with respect to age is presented in Table-11.

4.3.2.3. **ALT Levels and Molecular Test:**

A comparative analysis of ALT level and PCR test is presented in Fig. 13. Among 300 cases tested, 36 (12%) subjects positive for HCV by RT-PCR had higher ALT level (mean 68.4U/L). A significant percentage 16%(n=48) of subjects from general population, which were positive for HCV by RT-PCR, positive for HCV by RT-PCR had normal value of ALT (mean 36.4U/L) level. Interestingly 22(7.4%) subjects had higher ALT Level but were RT-PCR negative. Value of Chi-square test by statistical analysis was 42.7** as indicated in Table-12.
Fig. 13: Anti-HCV antibodies test by HCV screening kit (CCI). Lanes 1 & 2: Anti-HCV negative samples, Lanes 3, 4 & 6: Anti-HCV +ve samples, Lanes 5 & 7: weakly anti-HCV positive samples.
Fig. 14: A comparison of plasma ALT level VS RT-PCR in samples taken from general population.
Table-12: Statistical analysis by Chi square test to compare ALT level with RT-PCR analysis in general population samples.

<table>
<thead>
<tr>
<th>ALT Level</th>
<th>RT-PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>194</td>
</tr>
<tr>
<td>Frequency</td>
<td>(68)</td>
<td>(174)</td>
</tr>
<tr>
<td>High</td>
<td>36</td>
<td>22</td>
</tr>
<tr>
<td>Frequency</td>
<td>(16)</td>
<td>(42)</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>216</td>
</tr>
</tbody>
</table>

Figures within brackets are expected values.
Value of Chi-square= 42.7**
Thus association or linkage is indicated between the two parameters.
Fig. 15. A comparison of anti-HCV test VS RT-PCR in samples taken from general population.
Table-13: Statistical analysis by Chi Square test to compare anti-HCV antibodies test with RT-PCR among the general population samples taken from HCV screening camp.

<table>
<thead>
<tr>
<th>Anti-HCV Test</th>
<th>RT-PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>+ve Frequency</td>
<td>53</td>
<td>09</td>
</tr>
<tr>
<td>(17)</td>
<td>(45)</td>
<td></td>
</tr>
<tr>
<td>-ve Frequency</td>
<td>31</td>
<td>207</td>
</tr>
<tr>
<td>(67)</td>
<td>(171)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>216</td>
</tr>
</tbody>
</table>

Figures within brackets are expected values.

Value of Chi-square= 42.7**
Thus association or linkage is indicated between the two parameters

4.3.3. Relation between Anti-HCV and Molecular Tests.
A comparative study between anti-HCV screening test and RT-PCR is shown in the Fig. 15 and Table-13. As mentioned above that 62 (20.8%) samples were anti-HCV positive while 84 (28%) were RT-PCR positive. The comparative analysis of both these tests showed that 53 (17.66%) cases were both anti-HCV and RT-PCR positive. Similarly 207 (69%) samples were negative both by Anti-HCV test and RT-PCR analysis. It was interesting to note that in 31 (10.33%) cases, samples were RT-PCR positive but negative by anti-HCV screening test. Similarly in 09 (3%) cases, the subjects were positive by anti-HCV test but they were negative by RT-PCR test.

4.3.4. HCV Genotyping by RT-PCR-RFLP Analysis.

All the positive HCV-HCV samples were subjected to HCV genotyping by RT-PCR-RFLP method (See materials and methods). The results of PCR-RFLP analysis indicated (Table-14 & Fig. 16) that genotype-3 was found to be a major genotype. RFLP analysis showed that 8 (9.52%) subjects belonged to HCV-genotype-1. HCV-genotype-2 was found in only 2 (2.38%) subjects while 64 (76.19%) samples out of all 84 RT-PCR positive samples analyzed were of genotype-3. No other genotype could be identified by this technique while 10 (11.90%) samples could not be classified and as such remained untyped.
Fig. 16: HCV genotypes by PCR-RFLP analysis in general population indicating specific pattern of type-1, untyped and HCV type-3 on the 4% agarose gel. Lanes 1-6: Restriction with *Hae* III & *Rsa* I, Lanes 7 & 14: 50bp DNA Size Marker, Lanes 8-13: Restriction with *SstI* & *HinII*, Lanes 15-20 Restriction with *MvaI* & *HinII*. 
Table-14: Molecular epidemiology of HCV genotypes in general population samples (HCV screening camp).

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>GENOTYPES</th>
<th>NUMBER</th>
<th>%AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GENOTYPE-1</td>
<td>8</td>
<td>9.52</td>
</tr>
<tr>
<td>2</td>
<td>GENOTYPE-2</td>
<td>2</td>
<td>2.38</td>
</tr>
<tr>
<td>3</td>
<td>GENOTYPE-3</td>
<td>64</td>
<td>76.19</td>
</tr>
<tr>
<td>4</td>
<td>UNTYPED</td>
<td>10</td>
<td>11.90</td>
</tr>
<tr>
<td>5</td>
<td>TOTAL</td>
<td>84</td>
<td>100</td>
</tr>
</tbody>
</table>
4.4. **HCV STUDIES IN THE DIABETIC SUBJECTS**

4.4.1. **Diabetic and Non-Diabetic Subjects:**

A debate in the world is still going on whether any association existed between HCV patients and the onset of diabetes or vice versa. To add a little more in this debate, blood samples from 100 clinically diagnosed diabetics subjects were collected at random along with 60 samples from control group that were clinically non-diabetics and anti-HCV negative from above mentioned hospitals.

4.4.2. **Types of Diabetes:**

On the basis of clinical findings, biochemical analysis and WHO criteria, diabetic patients were categorized into type-1 and type-2 diabetes. 95 diabetic (95%) patients were diagnosed to have type-2 diabetes while only 5 (5%) patients were classified as having type-1 diabetes.

![Bar chart showing percentage of type-I and type-II diabetes patients](chart.png)

**Fig. 17:** Types of diabetes in the subjects studied for HCV infection
4.4.3. **Family History of Diabetics:**

Analysis of the patient data collected in the present study indicated that 56 (56%) diabetics had family history of diabetes, while 44 (44%) diabetic patients had no family history of diabetes. This shows that 56% subjects were genetically susceptible to acquire diabetes. In this category, the subjects were diagnosed to have diabetes between 35-45 years. Most of the diabetics were diagnosed to have diabetes between 31-50 years of age.

![Graph](Image)

**Fig. 18:** Mode of acquiring diabetes in the subjects selected for detection of HCV.
4.4.3. Demographics and Clinical Findings.

Brief demographic data along mean ALT level and RT-PCR results are shown in the Table-15. In diabetic cohort, there were 59 (59%) females and 41 (41%) males that fall in the age group range from 15-74 years. In non-diabetic group, there were 32 (53.33%) males and 28 (46.67%) females and were in the age group ranged from 10-73 years. It is also shown in the Table-15 that HCV infection was more prevalent between 31-50 years of age as compared to other age groups while higher mean ALT level was observed in subjects of age groups ranging from 51-75 years.

4.4.4. Anti-HCV Screening and RT-PCR Testing:

HCV-RNA detection in the diabetic subjects is shown in the Fig. 19. In case of diabetic subjects, out of 100 samples analyzed, 36 (36%) were RT-PCR positive while 15% subjects were RT-PCR positive out of total 60 taken as non-diabetics control. It was further noted that (42%) diabetics were positive by ELISA test. Among them, 31% subjects were positive both by ELISA and RT-PCR tests as indicated in the Fig. 20. Similarly 53% subjects were negative by both tests. In 16% cases contrary results were obtained between RT-PCR and ELISA testing and 11 (11%) RT-PCR negative subjects were seen anti-HCV positive while 5(5%) anti-HCV negative subjects were found to be RT-PCR positive for HCV.
Table-15: Demographic and clinical data of the diabetic subjects

<table>
<thead>
<tr>
<th>Age groups Years</th>
<th>Gender</th>
<th>Total Subjects</th>
<th>No of HCV-RNA positive subjects</th>
<th>Mean ALT level U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>No.</td>
<td>(%)</td>
</tr>
<tr>
<td>10-20</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>(9)</td>
</tr>
<tr>
<td>21-30</td>
<td>7</td>
<td>7</td>
<td>14</td>
<td>(14)</td>
</tr>
<tr>
<td>31-40</td>
<td>12</td>
<td>15</td>
<td>27</td>
<td>(27)</td>
</tr>
<tr>
<td>41-50</td>
<td>10</td>
<td>21</td>
<td>31</td>
<td>(31)</td>
</tr>
<tr>
<td>51-60</td>
<td>8</td>
<td>7</td>
<td>15</td>
<td>(15)</td>
</tr>
<tr>
<td>61-75</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>(4)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>59</td>
<td>100</td>
<td>(100)</td>
</tr>
</tbody>
</table>
Fig. 19: Analysis of samples by agarose gel electrophoresis for HCV diagnosis after RT-PCR in diabetic subjects. Lane 1: DNA size marker, Lanes 2 & 3: negative control, Lanes 4 & 5: positive control, Lanes 7, 8, 10 & 12-14: specific RT-PCR products of HCV positive samples. Lanes 6, 9, 11 & 15 HCV negative samples.
Fig. 20: A comparison of RT-PCR analysis and anti-HCV test among diabetic subjects.
DISCUSSION
5. DISCUSSION

Hepatitis C Virus (HCV) is often called silent killer as in most of the cases no signs or symptoms of the disease appear and patients come to know only when the disease is in advance stages. The second shortcoming in this regard is that presently in most of the developing countries facilities for its correct diagnosis are not proper and enough. Mostly the people are screened by anti-HCV antibody screening or by anti-HCV ELISA test for HCV infection but the problems or chances of error in this test are more as HCV is present in the blood at extremely low level (Li et al., 1995) and the routine serological tests are not so sensitive to detect HCV in the samples tested. Thus the detection of HCV more accurately is done by molecular methods and PCR based detection in this regard is very important, as it is more sensitive and specific technique than the serological methods.

HCV is a typical example of a disease in which direct detection of the virus is essential for a correct diagnosis. In contrast to other available in vitro assays, RT-PCR has more potential for its diagnosis because it offers a definitive identification of HCV (Albadalejo et al., 1998).

As HCV is present in the blood at a very low level, so the routine serological tests are not so sensitive that might be detected HCV, in all the samples tested. Moreover difficulties in virus isolation techniques and problems in antigen detection assays have made nucleic acid-based amplification, the method of choice for the direct identification of HCV. Indeed by RT-PCR, it is possible to assess the status of the infection, to detect viral replication in seropositive patients and to diagnose the infection
in immune-compromised patients and also during the window period that precedes seroconversion (Alter, 1995 & Tedeschi et al., 1995).

The results obtained during this study have been discussed in the following section under respective topics.

a. Chronic Liver Disease Patients

Samples recruited for this study, were taken from chronic liver disease patients or suspected HCV patients referred to NIBGE from various clinicians/clinical labs from all over the Faisalabad Division for diagnosis and molecular testing of HCV infection from year 2001 to year 2004. When these samples were checked by Enzyme Linked Immunosorbent Assay (ELISA) test, as indicated in the Fig. 3, it was found that 90.6% (n=1845) subjects were anti-HCV positive. It is mentioned here that most of these patients were already diagnosed some abnormality with their livers as they had abnormal live function tests (LFTs) especially, ALT and GOT levels were higher, or had clinical symptoms similar to hepatitis like fatigue, upper abdominal pain, nausea, jaundice, itching, pyrexia and lack of appetite etc. Many of the patients were already positive by anti-HCV ELISA, tested by commercially available kits by various clinical labs of the city so it was not so surprising that such a high number of samples was anti-HCV positive. Also most of the patients of this category were of chronic liver disease patients, they might had developed antibodies against HCV infection so they became anti-HCV ELISA positive. A similar study was already conducted in Hazara Division of Pakistan by Khan et al., (2003) in which high frequency of hepatitis seropositivity among chronic liver patients was reported but the percentage of HCV seropositive patients were less 25% (40.8%) than in this study where it is 90.6%, thus indicating much higher occurrence of chronic HCV patients in this area of Pakistan.
When these samples were analyzed by polymerase chain reaction (PCR), again high percentage (87.25%) of patients was found to be HCV-RNA positive that showed an indication of continued infection/active infection or persistent infection. In case of PCR, the number of HCV positive samples was 3.6% (n=67) less as compared to anti-HCV ELISA test.

A comparison of RT-PCR and anti-HCV ELISA tests in these subjects (Fig. 3 and Table-5) indicated that although this difference was not so significant, it showed that these patients might had past HCV infection but resolved or cleared the infection with the help of their strong immune system as reported earlier by Cohen, (1999), thus became anti-HCV positive. A similar study has already been done by Caudai et al., (1998) in which they had evaluated the concordance between viremia and antibody testing in HCV diagnosis. In that study, 682 blood samples collected from patients with known or suspected HCV infection were tested and an overall concordance between serological and PCR results was found to be 77% while in this study, even more similar type of concordance between these two tests was found which was 81.2%. Thus in this study, even more co-relation or association was observed between anti-HCV ELISA test and RT-PCR analysis.

This co-relation or association was also confirmed by statistical analysis of the data by chi square test (P= 0.05) that showed highly significant association between anti-HCV ELISA testing and RT-PCR analysis in these subjects. In that study, 5% HCV antibody negative subjects were found to be HCV-RNA positive and 18% antibody HCV positive cases were found to be HCV-RNA negative. This study also indicates similar trend but only in the first case, as 6.05% HCV antibody negative samples were found to be HCV-RNA positive. But contrary to their results in second case where 18% antibody HCV positive subject were reported to be HCV-RNA negative, in our case,
only 9.4% HCV antibody positive subjects were found to be HCV-RNA negative as shown in the Fig. 3. It thus indicates increasing specificity of the both the techniques in diagnosis of HCV infection especially, in suspected HCV cases.

The reason where the discrepancy between the antibody positive and HCV-RNA negative cases existed, might be that HCV may be present in peripheral blood mononuclear cells (PBMC) in those cases and not in the serum/plasma as has been reported earlier by Caudai et al., (1998) who detected HCV-RNA in peripheral blood mononuclear cells (PBMC) in 10.5% out of 38 plasma viremia-negative but HCV-seropositive subjects indicating that HCV-RNA might be persisted in PBMC and could begin viral replication again. The detection of HCV-RNA in PBMC in anti-HCV-positive subjects without viremia could reduce false-negative results of HCV-RNA testing by RT-PCR in serum or plasma.

Demographic data of the chronic liver patients as described in the Table-4 indicates that no case of HCV infection was detected below age of 10 years. Only 5.1% cases of HCV patients were found in the age group of 10-20 years. Higher prevalence or infection rate was observed in the age groups of 21 to 40 years. This might be happened due to the reason that such individuals were more prone to risk factors of getting HCV infection such as visits to Barbers/Dentists or routine injections with contaminated syringes which is not uncommon practice in the area.

During the study, it was also noted that most of the HCV suspected cases, which were HCV positive by ELISA or RT-PCR, also had higher level of ALT enzyme as indicated in the Table-6 and Fig. 4. There was generally good correlation between the HCV positive samples and higher ALT level. But some discordant results were noted where ALT level was found higher even in the absence of HCV detection. These
contradictions might be due to liver inflammation by some other reasons such as the use of some medicine/drug or viral infections other than HCV.

Some patients were also found who had normal ALT level, although HCV-RNA was detected or vice versa. These findings were according to the some previous findings that HCV infected subjects might have either intermittent episodes of overt disease, characterized by alternating periods of high ALT activity and quiescent periods in which activities were normal or infected persons might have, raised ALT values throughout the course of chronic hepatitis (Petrelli et al., 1994). It was also demonstrated earlier that individuals who were HCV positive with serum ALT level 1.5 times more than the upper limits of normal could have histologically advanced liver disease and they could respond to interferon therapy (Van Thiel et al., 1995).

According to another study, approximately one third of patients with chronic HCV infection had normal ALT levels with chronic HCV infection including 75 patients with persistently normal ALT and 200 patients with abnormal ALT. (Jamal et al., 2000). Chronic hepatitis C patients with persistently normal ALT have low-activity grade and stage on liver biopsy. In these patients the HCV-RNA level was lower as compared to patients with abnormal ALT level, which may explain the slower fibrosis progression rate.

It was also noted from the clinical data of the patients that 102 (5.1%) were found to be positive both by HBV and HCV, thus mixed or co-infection was indicated and it has already been reported that mixed or co-existence of two viruses in the same patients were not uncommon and such interaction was found to play important role in fulminate hepatitis and development of hepatocellular carcinoma and cyto-pathic effect is enhanced during co-infection (Alberti et al., 1995). In a similar study, chronic liver disease (CLD) patients admitted to Military Hospital, Rawalpindi in years 1999-2000,
were included and the data indicated that 7.2% out of 97 samples were co-infected with both of HBV and HCV while 35.1% were anti-HCV and anti-HBc positive (Bukhtiar, et al., 2003). We found from the clinical data of these patients that the disease was not more severe in many co-infected patients than with having only HCV infection, which is interesting.

b. Therapy Cases:

In case of HCV patients who were taking recommended doses of interferon alpha injections (3MU) thrice a week and 1000-1200 mgs of ribazole per day as combined therapy for at least three months (n=60) or completed their therapy for at least six months to one year (n=37), it was noted that out of all 97 samples tested, 93 (95.85%) were still anti-HCV ELISA positive (see results). They were actually those persons who were still under therapy for at least three months (n=60) or recently completed their therapy for at least six months to one year (n=33). Only 4 (4.15%) persons could able to be both anti-HCV ELISA as well as RT-PCR negative, who had completed their therapy 3-4 years ago and also had taken aggressive therapy (4.5 MU interferon injections daily for first 4-6 weeks along with ribazole and then the standard therapy for the remaining eleven months of their treatment.

It was further noted from the data of these subjects that 19 (19.6%) were still RT-PCR positive. Among these 19 PCR positive patients, 11(57.89%) had completed their therapy more than one year ago but became again RT-PCR positive, although they were PCR negative just after completion of therapy. These were actually relapse cases and relapsers are not uncommon in HCV disease. Eight subjects did not respond to the therapy altogether and continually remained HCV-RNA positive during the treatment and even after completion of therapy. They were those cases that were resistant to the combined therapy and are called non-responders. Among 78 (80.41%) RT-PCR
negative individuals, 41% (n=32) became HCV-RNA negative after three months of combined therapy, thus showing good response to therapy while 59% (n=46) became HCV-RNA negative at the end or completion of 6-12 months therapy. Overall 57 (58.76. 3%) subjects remained HCV-RNA negative even after the completion of therapy and follow up of one year thus showing sustained response. The results of therapy cases showed relatively good response to combined therapy as compared to some findings where only 17% cases showed sustained response with combined therapy (Garson et al., 1995).

From the comparison of anti-HCV and RT-PCR results of these subjects, very interesting data were obtained which might be of great clinical importance. It was found that most of the patients on continued therapy or who completed their therapy regime were still anti-HCV positive even they were no more RT-PCR positive. In other words they have no more any Viremia. Thus these subjects were very important to observe response rate or to monitor the sustained response of the therapy. As reported earlier that seroreversion may be observed in three circumstances: spontaneously, induced by therapy, and in conjunction with human immunodeficiency virus infection (Lefrere et al., 1997). In these cases long-term follow-up of seroreversion might indicate whether they have definitively eradicated HCV from their systems.

As anti-HCV ELISA in most of such cases remained positive and thus gave false results to see or monitor therapy response so it is very necessary and imperative that PCR should be performed in such situation to avoid any ambiguity and to see response rate as well or to check the sustained response of the combined therapy. Earlier in a longitudinal study to see the markers of HCV infection in a cohort of 178 multi-transfused patients that were followed over 8-years to establish well-documented
cases of partial or full seroreversion, it was noted that among 30 HCV-infected subjects, 5 had partial or full seroreversion (Lefrere et al., 1997).

It has also been indicated earlier that any HCV antibody-positive immune-competent patient with no detectable serum HCV-RNA and normal Alanine aminotransferase values and whose serial samples showed a progressive decrease in the level of HCV antibodies present might be considered as having a resolved infection (Lanotte et al., 1998). Thus from the study of HCV treated cases as shown by Fig.10 and statistical Table-7, it is clear that actually very little or non-significant association existed between anti-HCV ELISA test and RT-PCR test in subjects who were on combined therapy. From the study it can thus be inferred that these subjects should be monitored only by HCV-RNA determination for HCV infection and not by anti-HCV antibody testing that might give inaccurate picture about patient condition, recovery rate or about the response of the therapy.

From the data in the Fig. 11 and Table-8, it was noted that generally ALT determination during therapy or after completion of therapy was another important parameter to see the sustained response of combined therapy as ALT level became normal in patients who responded to therapy and significant association (P<0.05) existed between normal ALT level and RT-PCR negative cases. But at the same time, it was also noted that some patients had normal ALT level after completion of therapy and continuously retained normal ALT level even they became HCV-RNA positive after the follow up of one year or so. It is thus very important that the these individuals should only be tested for HCV-RNA for at least 2 years after the completion of therapy at every 6 months interval and not relayed on ALT testing only for monitoring of the sustained response of the therapy. It has been reported earlier that HCV infection monitored by the level of Alanine aminotransferase (ALT) was not always correlated
with disease progression or the response of HCV infection to therapy and HCV-RNA measurement was a better mean of determining and monitoring HCV infection than either ALT level or histopathological characteristics and might provide insight into hepatic injury caused by HCV infection even without an invasive liver biopsy (Choi et al., 1999). Thus our study again emphasized the importance and necessity of the RT-PCR in HCV detection and diagnosis, especially for monitoring therapy response and follow up studies.

c. General Population.

Three hundreds samples were taken from general population of the area in order to get an idea about the prevalence/incidence of HCV infection in general population during a period of six months and the data are presented in Fig. 14 & 15 and Tables-2, 11 & 12. It was found that 62 (20.6%) subjects were positive by anti-HCV screening while in case of RT-PCR 84 (28%) subjects were positive. From the results, it is thus clear that in RT-PCR, even very recent cases of HCV infection can also be detected that are not yet seroconverted or are still anti-HCV antibody negative. These findings once again emphasized the superiority of molecular testing over routine serological testing for early detection or diagnosis of HCV infection especially for safer blood transfusion. RT-PCR was performed to check HCV-RNA in recently infected cases or in resolved cases that could not be checked or screened by anti-HCV test alone. From comparative data of anti-HCV test with RT-PCR, it was noted that in 13% cases, discrepant results were found between RT-PCR and anti-HCV test.

Anti-HCV screening test and anti-HCV-ELISA test for the detection of antibody to HCV (anti-HCV) have been evolved since 1990 and third or even forth-version of such assays is now available that detects anti-HCV in almost 95% cases of HCV infection (De-Medina et al., 1995) and is the only screening test in most clinical
laboratories of the world, which is not so specific and sensitive especially, for early diagnosis of HCV infection and also in immune-suppressed patients as reported before that their efficacy is still limited because these assay did not detect anti-HCV antibodies in all infected persons. Also these assays did not distinguish between onset of acute illness and seroconversion due to 2-12 months of window period of seronegativity after acute infection and occasionally gives false antibody reaction (Caldwell et al., 1993). These assays also give false results in persons who have cleared/ recovered from HCV infection and in patients on antiviral therapy as we discussed earlier in therapy cases.

Anti-HCV antibody test data of this study was concordant to another report in which 18.4% of healthy donors from Egyptian peoples residing in Saudi Arabia were found to be positive by ELISA (Fathalla et al., 1994) hence indicating hot areas of high prevalence of HCV infection in the general population but the data was discordant to another study preformed in Italian population where overall confirmed anti-HCV prevalence was only 2.4% (Kondili et al., 2002).

RT-PCR results of these subjects indicated even more alarming situation as higher (28.2%) number of HCV-RNA positive subjects were found, indicating new or recent cases of infection, which were yet to be seroconverted. One possible explanation of such high prevalence of HCV detected by RT-PCR, might be that they were either very recently infected by HCV and specific antibodies were not yet formed enough to be detectable by the ELISA test or they are asymptomatic carriers of HCV RNA, as has been reported earlier by Cortorti et al., (1995). The cases, which were negative by anti-HCV ELISA and positive with RT-PCR, also confirmed the previous findings that viral sequence could be found in individual whose anti-HCV test was negative (Garson et al., 1990 & Weiner et al., 1990). In ELISA test, anti-HCV antibodies are detected but the appearance of antibodies in the blood after the onset of hepatitis can be delayed from 3
to 52 weeks (Farci et al., 1991). Moreover HCV infections are associated with low viral titer, which may account for low antigenic stimulus. This suggests the necessity for the screening of volunteer blood donors through molecular means.

It is however mentioned here that although detection of HCV-RNA was believed to be the most reliable method to diagnose HCV infection (Centrevas et al., 1990), a pit fall of nested PCR was that it was prone to contamination (Aslazadeh et al., 1996), giving false positive or false negative results. Thus the possibility of cross contamination cannot be avoided in this study too, although in order to maintain quality assurance, all possible measures were taken to avoid any cross contamination by the use of both positive and negative controls (see materials and methods) at every step.

The reasons of high incidence of HCV in the general population of this highly populated area consisting of mostly uneducated people might be (i) lack of awareness about risk factors involved in its transmission/spread (ii) malpractice among medical community such as reuse of syringes, and use of un-sterilized or not properly sterilized medical instruments especially by Dentists and lastly but not leastly (iii) reuse of contaminated razors by the barbers etc. Yet another important reason for higher rate of HCV carriers might be that presently, facility for HCV screening in most Blood Transfusion Centers of the country is not up to the standard, thus a major source of transmission of HCV infection. To draw a clear-cut picture about the prevalence of HCV infection in general population of Pakistan, a number of screening programs of HCV infection detection are needed. Some unknown vector yet to be identified may also be involved in its spread to the healthy population. Being a big industrial center of the country, general hygienic conditions and environmental pollution are also major problems of the locality that might also contribute in spread of HCV infection in the general population. Mosquito might also be a strong candidate in this respect and study
of its involvement in HCV spread/transmission is needed to be focused more comprehensively.

During this study, it was noted once again that most of the individuals, who were HCV positive by both ELISA and PCR, also had higher level of ALT similarly as was seen in case of suspected or chronic HCV patients. The only difference noted here was that range of mean ALT level in general population was relatively less as compared to suspected cases and high percentage of subjects had normal ALT level even they were RT-PCR positive. Possible explanation of normal ALT level in such cases might be attributed that these subjects might had infected from HCV very recently and no substantial damage to their livers was occurred yet or they might be the cases of healthy carrier state of HCV infection as discussed earlier that HCV associated infected persons might have either intermittent flare-ups of overt disease, characterized by alternating periods of high ALT activity and quiescent periods in which ALT level was normal (Petrelli et al., 1994).

Conflicting data concerning the presence of histologically confirmed liver damage indicated that either invariable association between the detectable HCV viremia and liver injury (histological evidence of liver disease in the absence of raised ALT level values) existed (Alberti et al., 1992) or that detection of persistently high levels of viremia was possible even in the absence of overt liver disease (Brillanti et al., 1993).

d. HCV and Diabetes:

Prevalence of HCV infection in the Diabetics type-II was also studied to see any suggested relation between HCV infection and diabetes as conflicting reports are found in the literature weather if there any association exists between the two diseases or not. From this study as shown in Table-15 and Fig. 20, it is indicated that 36% (n=36)
diabetic subjects out of 100 studied, were HCV-RNA positive as compared to the control non-diabetic cohort where only 15% persons out of 60 were found to be HCV-RNA positive. These results indicate a positive association between HCV infection and diabetes mellitus type-II, although the number of samples was small.

In comparison between anti-HCV ELISA test and RT-PCR in diabetic patients, here again, it was found almost similar trend between the two tests as was seen in case of suspected cases or general population cases. Many researchers have already reported the prevalence of HCV infection in diabetics or vice versa. For example Mangia et al., (1998) studied HCV and diabetes mellitus in 247 patients with liver cirrhosis in Italy and gave evidence of negative association between the two diseases. Their findings disproved HCV infection as a trigger factor for DM, which should not be listed among the various extra hepatic manifestations of this viral infection.

In some reports the percentage of HCV infection was less as compared to our study. For example 11.5% incidence of HCV infection in diabetic patients was indicated by Sim et al., (1996) while Mason et al., (1999) reported presence of 4.2% HCV infection in a diabetic cohort and suggested that the diabetogenic action was worthy of further investigation.

Contrary to above reports, Saxena et al., (2003) determined the susceptibility of the 196 patients with type-2 diabetes to HCV infection at King Fahad Hospital and tertiary care center, in Hofuf, Saudi Arabia and found that patients with type-2 diabetes recorded higher HCV prevalence (57.4%) as compared to those of the non-diabetic group where it was (35.2%). The results of present study also indicate almost similar trend of HCV infection when we compare our data of diabetic subjects with non-diabetics controls and it was 36% in case of diabetics as compared to 13.4% in controls. In other reports, it was indicated that type-II diabetes was present in 35 (21.2%) patients
with HCV infection (Akbar et al., 2002) while Qurshi et al., (2002) and Aroa et al., (2003) have also shown similar association between the two diseases and found that diabetes mellitus was equally frequent in both chronic HCV and HBV infection. In first case, 400 patients with chronic liver disease, 302 had HCV and 98 HBV infections and the diabetes was found in 24.5% HCV and 19.4% HBV related cases while in second case the prevalence of HBV- and HCV-related cirrhosis were 32% and 33%, respectively.

These findings and other reports supported this study that HCV infection was high in diabetic subjects. HCV appeared to cause pancreatic damage and was among the various extra hepatic manifestations of this virus. It thus might be resulted in development and cause of diabetes type-II disease but genetic factors also need to be explored in order to get a clear-cut picture of this relation or association between these two diseases.

d. Genotyping Study

After confirmation of HCV infection with RT-PCR, it was important to know the existing HCV genotypes of the positive samples in the three categories of subjects studied i.e., HCV suspected subjects, Therapy cases and in general population samples. The data of HCV genotypes have been presented in Fig. 5, 6, 7, 8, 12 & while in Table-9 & 14. HCV genotypes are considered important in progression of the disease and also in its management and treatment. Some HCV genotypes are thought to be sensitive against the anti-viral therapy and have good response rate while others are considered resistant to combined therapy and conflicting opinions are found in this regard.

There is great diversity in HCV genotypes around the world and geographical localization of some genotypes are associated with special routes of infection (Bastie et
al., 1995; Green et al., 1995). There is some evidence that infection with various HCV genotypes is associated with different courses of disease and response to therapy (Dusheiko et al., 1994) and there was significantly good therapeutic outcome in patients with genotype-3 but at the same time, it was found that the association between genotype-1b and severe liver disease was still controversial (Mondelli et al., 1999). These differences have clinical significance for patient treatment and in understanding the pathogenesis of HCV infection (Mellor et al., 1995).

Genotypes of the samples were determined by dot blot hybridization using allele's specific probes in some of the chronic disease patients only while rest of the samples were genotyped by restriction fragment length polymorphism (RFLP) analysis of the RT-PCR products of the HCV-RNA positive subjects. Determination of HCV genotypes was also tried by using type specific primers from the core region of the HCV genome following Okamoto et al., 1992 but unfortunately we did not get any results, although it was tried several times both with exactly same methodology as well as with some modification. A possible reason for the failure might be that core region of HCV genome is not as conserved as its 5'UTR for PCR amplification. It was also reported earlier by Toniutto et al., 1996 that Okamoto's method was much less efficient than the 5'UTR assay for determination and identification of HCV genotypes. So it was quite possible that primers we used might be not complimentary enough that we could get any DNA amplification that was detectable on the gel for the identification of any HCV genotype.

The results indicated that out of 120 positive samples of chronic disease patients analyzed, by dot blot hybridization for genotyping following Tismintisky et al., (1994), 69 (57.5%) were of genotype-3 (G3) while 5 (4.1%) were of genotype-2 (G2) and 28 (23.3%) were of genotpe-1 (G1). Mixed infection with two or more genotypes was also
noted in 6 (4.55) subjects. 14 (11.66%) samples did not hybridize with any of the 3 probes used and remained untypable. These subjects might have had infection with other known genotype(s) or with some new genotype yet to be identified.

Genotyping results of present study are not concordant with some early findings in which it was reported that genotype-1a and 1b (G1) were the prevalent genotypes (Gerotto et al., 1994, Zeuzem et al., 1995, Bernier et al., 1996, Gayowski et al., 1997, Zali et al., 2000, Molin et al., 2002, Schulman et al., 2002 & Djebbi et al., 2003) in some parts of the world including Canada, USA, Iran and Tunisia etc. These findings were also contradicted to other data in which HCV genotype-2 (G2) was found more common than HCV-1 (Ravaggi et al., 1994, Chan et al., 1995, Soetjipto et al., 1996 & Re et al., 2003 and Tismintzky et al., (1994)) in some other parts of the world like Indonesia, China, Argentina and Italy. But the results of the present study indicated similar trend as has been reported earlier by Apichartpiyawul et al., 1994, where HCV-3 (G3) was found more prevalent genotype (43%; 3a 30% & 3b 13%), followed by HCV-1 (34%; 1a 21% & 1b 13%) and also by Silini et al., 1995 who reported HCV type-1 and type-3 were the major genotypes equally prevalent in Italy.

The findings of present study about the prevalence HCV genotypes have also showed very close concordance with another study done recently in our neighboring country India where a high prevalence of genotype 3 (>76%) and very low prevalence of genotype 2 (<2%), was documented in 153 samples representing different regions of the country (Das et al., 2002). However, in that study genotype-3a was found to be the highest (50%) with a decreased frequency of approximately 25% of 3b, approximately 14% 1b and approximately 10% 1a.

When the samples were genotyped by RFLP analysis, even more similar trend was noted in all the three categories of subjects studied for this said purpose. In
suspected HCV cases 75.5% subjects were of genotype-3, in case of patients taking combined therapy, it was prevalent in 62 (63.91%) subjects while in general population cohort, it was noted in 78.6 % samples. Thus from the results of the data presented here, it is clear that HCV type-3 was the most prevalent genotype in the area. Earlier no or very little data about the HCV infection/incidence and particularly about its genotypes common in the specified area are available. Thus the knowledge of HCV genotypes present in Pakistan which is just like India might be helpful in its management or treatment by the Clinicians of the area under study as it has relatively high incidence rate of HCV infection. It might also be a sign of some relief as it is considered but yet to be concluded that HCV type-3 was more responsive or sensitive to anti-viral therapy.

A preliminary study to see the response rate of the combined therapy against HCV genotypes was also done. Although the results in this regard were very preliminary and no final conclusion can be drawn and needed more vast follow-up studies for longer period but generally it was noted good response rate of the anti-viral therapy in all the three HCV genotypes (type-1, type-2 and type-3) along with significant number of untyped subjects when we compare our results with other studies as indicated in the Table-10 but again it was noted here that HCV genotype-3 seemed to be relatively more responsive to the therapy than other types.

Although earlier in a very brief study it was reported about the existence of HCV-3 in Pakistan by Mellor et al., 1996 but the sample size (3 samples) was very small, taken from Karachi which is present in the Sindh Province of Pakistan while in present study, much more samples were included that were taken from the Faisalabad region present in the Punjab Province nearly 1000 Kilometer away from Karachi. From the findings of the study, it can therefore be inferred, that HCV-3 was the major genotype prevalent in Pakistan These findings have also some positive points as we see
in some earlier reports by various researcher that, among HCV genotypes, HCV-1b (following Simmonds et al., 1993) was associated with more severe liver disease (Nousbaum, et al., 1993) and appeared to be less responsive to interferon therapy than HCV-2 and HCV-3 (Chemello et al., 1994). The patients with HCV type-2 respond to therapy much better than those with type-1 (Yshioka et al., 1992; Kanai, et al., 1992) but the infection with HCV type-3 was even more sensitive to interferon (IFN), than HCV type-2 (Tisminetzky et al., 1994).

The reasons for the better response to therapy in these patients are unclear but might be related to the specific inherited differences in population of the various countries. They also belonged from a different ethnic background and appeared to have had a different source of HCV infection in comparison to patients from the other areas of the world. Moreover as it was reported earlier in receptor binding assays that E2 of HCV3a genotype consistently failed to bind CD81 which is a putative cell receptor for HCV and thus might have better response to interferon therapy as compared to HCV genotype-1 (Shaw et al., 2003). In another report even better response to antiviral therapy has been indicated by Schulman et al., (2002) in which it was shown 80% sustained response in cases of patients having infection with HCV genotypes other than type-1. In case of type-2, rate of sustained response was 100% but in case of type-1 poor sustained response was noted where it was only 22%.

It is important to note that in therapy cases, over all 58.7 % patients showed sustained response after the period of more than one year of completion of therapy that is a little bit satisfactory and encouraging because some previous findings indicated less than 20 % cases retained sustained response with this therapy (Garson et al., 1995).
Conclusions and Recommendations:

The findings of the study are concluded here with the remarks that the region under study is lying among the hot area of the world where the HCV infection rate is higher that is very alarming and demands immediate attention of the concerned authorities to take maximum proper preventive measures to stop its spread and transmission to the general population of the country but at the same time of some satisfaction as HCV genotype-3 is the most prevalent genotype found here that might be managed relatively easy as compared to other genotypes. Genotyping knowledge might also be helpful in development of its community-based vaccine in future. It is also suggested that the screening of blood for transfusion should be made compulsory, making blood transfusion safer for the recipients and avoiding of risk factors involved in its transmission to the healthy population.

It was further found that molecular diagnosis of HCV by RT-PCR is more superior specific and sensitive method than serological testing especially for its early diagnosis, when HCV specific antibodies yet to be formed and also in Immuno-compromised patients, where anti-HCV antibodies, if some formed, are below to detectable level. This test is also recommended for the patients on antiviral therapy to check responsiveness of the therapy because anti-HCV ELISA would be positive in these subjects even after the completion of therapy and clearing its infection.

At the last, it was also concluded that HCV infection might be involved in the development of diabetes mellitus type-II by infecting pancreatic cells, which is needed to be studied more in detail.
SUMMARY
6. Summary

Hepatitis C virus (HCV) is a major health problem worldwide and almost more than 3% population of the world is infected with HCV. HCV is present in the blood at extremely low level (Li et al., 1995) and the routine serological tests are not so sensitive to detect HCV in the samples tested. Thus the detection of HCV more accurately is done by molecular methods and PCR based detection in this regard is important, as it is more sensitive and specific.

Present study was taken in order to find the incidence/prevalence of HCV infection and its genotypes in the local population of this area and also to find if any relationship existed between HCV infections and clinically diagnosed diabetic mellitus type-II cases.

For this purpose, 2000 samples at random, from suspected HCV patients having some common symptoms of hepatitis were recruited for this study from various localities of Faisalabad region such as Jhing, Gojra, Toba Tek Singh, Jaranwala and Chiniot, along with 300 samples from general population, 100 samples from the Diabetics, 60 samples from non-Diabetics as control and 97 samples from HCV patients taking combined (interferon + ribazole) therapy.

The results indicated that among the suspected HCV cases, 90.6% subjects were found to be anti-HCV ELISA positive. When these samples were retested through RT-PCR, 87.25% samples were found to be HCV-RNA positive, indicating a difference 3.35% between these two tests. The samples that were ELISA positive but PCR negative
may be an indication of past infection or resolved cases still ELISA positive. It was further found that HCV infection was more prevalent in the persons of age groups of 31 years to 50 years which are more prone to risk factors involved in its transmission.

In case of subjects who were on combined therapy (interferon+Ribazol) for at least 3 months to 1 year, out of 97 samples, 94 (95.87%) were found still anti-HCV positive while only 19.58% subjects were found RT-PCR positive, indicating importance and superiority of molecular testing over serological methods especially, in therapy cases, for monitoring response rate of the medicine.

In case of general population, out of 300 subjects tested, 20.6% were anti-HCV positive while 28% were RT-PCR positive, indicating higher prevalence of HCV infection in general population of the area. In general population samples, higher number of RT-PCR positive samples were found than diagnosed by anti-HCV testing, indicating new or very recent cases of infection, which were not yet seroconverted. It is also an alarming and dangerous situation, required urgent attention as more cases from general population are getting fresh HCV infection.

One hundred diabetic subjects were also tested along with 60 non-diabetics and anti-HCV negative as control. Here again higher prevalence of HCV was found in diabetics as compared to the control group, indicating positive relation between HCV infection and diabetes mellitus type-II disease.

Alanine aminotransferase (ALT), a marker of liver injury/damage was also checked for all the categories of the subjects tested. Generally good relation between RT-PCR positive subjects with higher ALT level was noted, although in certain case, important contradictions or discrepancies were noted where ALT level were higher, even in the absence of HCV-RNA or vice versa.
HCV genotypes are considered important in its management or treatment. So HCV genotypes were also determined in some (n=120) of the HCV-RNA positive subjects by dot blot hybridization in suspected cases only while through PCR-RFLP analysis in 580 suspected cases, 84 HCV-RNA positive subjects from general population and 97 subjects on combined therapy before the initiation of therapy. It was noted that HCV genotype-3 was the most prevalent genotype in the area. It is a sign of some relief as considered that it is more responsive or sensitive against the anti-viral therapy.

Response rate of the therapy against HCV genotypes was also checked. Although the results in this regard are very preliminary and no final conclusion can be drawn from the data and needed more follow-up studies for longer period but generally good response rate of the therapy in all the three HCV genotypes (type-1, type-2 and type-3) along with significant number of un-typed samples was noted. HCV genotyp-3 seemed to be relatively more responsive to the therapy than other types prevalent in the area.

So it may be concluded from the study that the area seemed to be very hot area as far as HCV incidence/prevalence is considered and its increasing infection to the general population is alarming. It requires urgent attention of the concerned authorities to take preventive measures. RT-PCR is very important for early diagnosis of HCV infection as well as for monitoring antiviral therapy response. HCV genotype-3 is the most prevalent type found in the area and relatively higher anti-viral response with combined therapy was noted in subjects infected with this type. Lastly relatively more incidence of HCV infection was noted in clinically known cases of diabetic mellitus-II than control.
REFERENCES
7. REFERENCES


Alter, H. j. 1995. To C and not to C: these are the questions. Blood 85: 1681-1691.


Overby, L. R. 1993. Hepatitis C: looking at a virus that hasn't been seen. GUT 34: 6-9.


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APPENDIXES
APPENDIX-I

HISTORY PERFORMA FOR HCV STUDIES

1. Labs Name: ___________________________ Dated: __________

Serial No.: ________ Sample: Serum/Plasma

2. Patient’s Data:

Name: __________________________

Age: _____ Sex: Male/Female. Blood Group: _____________

e. Address: ___________________________________________________________________
..........................................................................................................................

f. Profession: ________________ g. Phone No: ____________ h. Martial Status: ________

i. Education: ________________ j. Socio-economic status: ________________

l. Referred by: __________________________

3. Presenting Complaint/symptoms:

____________________________________________________________________________
..........................................................................................................................
..........................................................................................................................

4. Past History:

i. Jaundice: Yes/No ii. Dialysis: Yes/No iii. Blood transfusion: Yes/No

iv. IV Drug abuse: Yes/No v. H/O Surgery: Yes/No vi. H/O Hospitalization: Yes/No


Visit to Dentist/Barber: Yes/No.

x. Any other: ___________________________________________________________________

..........................................................................................................................
5. Family History:-

HBV/HCV or other type of hepatitis in the family: ______________________

i. Relationship with patient: _________________________________

ii. Any other important information: ___________________________

6. History of any other Infection or Disease: _______________________

7. Investigations: Dated: ____________

i. SGPT/ALT: ________________

ii. SGOT: ________________


iv. vi. P. T.: ______

8. HBsAg: Positive/Negative; Cutoff Value. ________________

9. Anti-HCV TEST: By ELISA OR Screening Test; Positive. ________ Negative. ______

10. Liver Biopsy or Liver ultrasound: Yes/No

11. Blood C/P:

12. 13. Current therapy status; if any: _____________________________

14. HCV PCR Diagnostics:

a. Qualitative PCR Result: Positive or Negative

b. Genotyping, if already done: 1, 2, 3, 4, 5, 6.

c. Quantitative PCR Value, if: _________________________________

Result of HCV-PCR from NIBGE: Positive/ Negative
APPENDIX-II

A) Primers used for cDNA synthesis and amplification of target sequences of HCV from 5'-UTR of its genome.

<table>
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<tr>
<th>Primers</th>
<th>Round</th>
<th>Sequence (5'----3')</th>
<th>5'position</th>
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<td>Outer Anti-sense primer 01</td>
<td>5'GTGCACGGTCTACGAG ACCT 3'</td>
<td>-02 to -21</td>
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<td>Inner Sense primer 02</td>
<td>5'GTGCAGCCTCCAGGAC CC 3'</td>
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<tr>
<td>Inner Anti-sense primer 02</td>
<td>5'GGCAGCTCGGAAGCA CCCTAT 3'</td>
<td>-27 to -46</td>
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B). Genotype specific $\gamma^{32}$ P labeled probes used for HCV genotyping:

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<td>5'-CGCTCAATGCCTGGAGAT-3'</td>
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<td>G2</td>
<td>5'-CACTCTATGGCGGGGCAT-3'</td>
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<td>5'-CGCTCAATACCCAGAAAT-3'</td>
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APPENDIX-III

HCV genotyping by RFLP

A) HaeIII-RsaI HCV

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B) ScrFI-HinFL

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C) Mval-HinFL

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APPENDIX-IV

Primers sequences for HCV genotyping by using type specific primers.

1. For cDNA synthesis and first PCR amplification.
   1. Antisense outer primer: (5'-ATGTACCCCATGAGGTCCGC-3')
   2. Sense outer primer: (5'-CGCGCGACTAGGAAGACTTC-3')

2. For second PCR amplification and identification of HCV 1st four types.

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<th>Primers</th>
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<tr>
<td>Tyep-1 specific antisense:</td>
<td>(5'-TGCCTTGGGGATAGGCTGAC-3')</td>
<td>57bp</td>
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<td>Tyep-2 specific antisense:</td>
<td>(5'-GAGCCCATCCTGCCCACCCCA-3')</td>
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<td>Tyep-3 specific antisense:</td>
<td>(5'-CCAAGAGGGACCGGAACTC-3')</td>
<td>174bp</td>
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<td>Tyep-4 specific antisense:</td>
<td>(5'-ACCCTCGTTTCCGTACAGAG-3')</td>
<td>123bp</td>
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<tr>
<td>Tyep-5 specific sense:</td>
<td>(5'-CGCGCGAGCGGTAAAACCTC-3') for first PCR</td>
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<td>Tyep-5 specific antisense:</td>
<td>(5'-GCTGAGCCCCAGGACCGGTCT-3')</td>
<td>88bp</td>
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<td>Type-5 specific sense:</td>
<td>(5'-CGTAAAAACTTCTGAACGGTC-3')</td>
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