EARLY DIAGNOSIS OF LIVER DISEASES BY IDENTIFICATION AND CHARACTERISATION OF MARKER PROTEIN

By
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M.Sc (Chemistry)

A thesis submitted in partial fulfillment of the Requirement for the degree of Doctor of Philosophy (Ph.D)

In
CHEMISTRY

Institute of Chemistry, University of the Punjab, Pakistan.

(August, 2008)
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(August, 2008)
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DEDICATED TO

MY PARENTS, HUSBAND

Agha Shahid Khan

&

MY DAUGHTERS

Saleena Shahid

Anoushey Shahid
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>ALD</td>
<td>Alcoholic liver disease</td>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BCP</td>
<td>Bromocresol purple</td>
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<tr>
<td>Bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CLD</td>
<td>Chronic liver disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Kd</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo nucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase Polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
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ACKNOWLEDGMENTS

All appreciations and gratitude are for the most Gracious the most Merciful Almighty Allah Who guide me, gave me health, thoughts and opportunity to complete this study. Humblest obligations are also paid to the Holy Prophet Mohammad (Peace be upon Him) Who is forever a touch of guidance and knowledge for the entire humanity.

With the spirit of thought and deep sense of acknowledgement, I pray my sincere gratitude to my supervisor Dr. Irshad Khokhar, Professor of organic chemistry, Institute of Chemistry, University of the Punjab and co-supervisor Dr. Rukshan khurshid, Department of Biochemistry, Fatima Jinnah Medical college, Lahore for their absorbing attitude in procedural matters as well as their able guidance in the concerned complications.

I am deeply thankful to Dr. Jamil Anwar, Director, Institute of chemistry, University of the Punjab for allowing me the facilities required for research work. I am greatly obliged to my colleague Dr. Nasir Mahmood, School of Biological Sciences, University of the Punjab for his beneficial suggestions and comments during the study.
With the spirit of thought and deep sense of acknowledgement I pray my sincere gratitude to Mr. Faqir Mohammad Irfan and Mr. Ch. Muhammad Abid for their helping attitude all the time.

I am greatly obliged to my friend Tanzila Ishtiaq who encouraged and insisted me to start the PhD research project with great passion. I also thankful to Dr. Saleem Akhter for good wishes and friendly behavior towards me. I am greatly obliged Shahid Iqbal, scientific officer, Nimer for providing me help in HPLC. Special thanks are for my class fellows Dr. Rohi Mushtaq, Shafique Chaudhry and Moheet Khan for their love and affection for me. I am especially thankful to Col. Masood Bhai and Atiqa Baji for their alot of prayers and advices during my research work and compilation of the thesis. I am thankfull to Yasir Ch. for his support and co-operation. I am indebted for whatever I have learned due to the guidance and encouragement by Dr. Ajmal Niazi and Nasir Zaidi.

I offer my deepest and heartfelt obligation to my parents for their affection, devotion, sacrifice and unforgettable love they had for me and my elder sister Sabiha Sufi, elder brother Nasir Abbas and younger brothers Sufi Nasir and Sufi Azhar for their prayers and good wishes for me. I am also thankful to my nephews and nieces for their cooperation and best wishes. I am also thankful to my best friends Shaista Jabeen and Asma Abbas for their
moral encouragement. I also cannot forget the help and cooperation provided by my mother in law (late) without whom I could not imagine to complete this task.

As we know that the capabilities of a woman when she is wife depends on the qualities of her husband, I wish to extend my sincere tributes and appreciation to my husband who has always been a great source of encouragement moral support, sacrifice and inspiration for me and to my little kids Saleena and Anoushey for having lot of love and prayers. Last but not the least I want to express my gratitude for Zali Baji, who has been a source of great inspiration and spiritual guidance to me.

Fauzia Tabassum
December, 2008.
SUMMARY

According to the report of WHO (2002), 4 million deaths are due to liver diseases and at least 20% of these deaths are attributed to HCV infection while remaining percentage is due to other factors including alcoholism. In Pakistan unfortunately there is no registry which keeps the record of different diseases however according to WHO statistics in Pakistan 5 million people have infected by HCV alone and this figure may be increased significantly. There is no reported prevalence rate regarding the liver failure due to alcoholism or chronic liver disease either due to virus or any other factor.

Inflammation of liver can result from overuse of alcohol, reaction to certain medications or infection by viruses. There are several different viruses that cause hepatitis among which hepatitis C (HCV) is most frequent. Each of these viruses may produce similar symptoms and they can all infect and inflame the liver.

In recent research study the prevalence rate of liver inflammation due to HCV, alcoholism and CLD was found to be 23.33%, 20.0% and 13.33% after serum proteins analysis of suspected group. While involvement of HCV in alcoholism and CLD confirmed cases was found to be 23.44% and
26.66 % however in suspected category the percentages were found to be 16.66 % and 20.0 % respectively by RT-PCR. In serum of HCV infected patients 14 kda protein is most prominent while 60 kda and 30 kda proteins were at same level however in subjects belonging to alcoholic and CLD category both 30 kda and 60 kda proteins expressed predominantly. The 14 kda and 30 kda proteins were also subjected to in-vitro assay and in the presence of neem extract both proteins level was significantly declined.

As the percentage of liver diseases is going to increase in developing country Pakistan therefore the recent research project was carried out and has significance because it describes not only the specific serum proteins as marker proteins in different diseased states like HCV, liver damage by alcoholism and CLD but also provides information regarding the involvement of HCV in suspected as well as confirmed groups of these categories. The high percentage prevalence of HCV in all the three suspected and confirmed categories of disease states is a reflection of need to control and cure the HCV infection after proper early diagnosis either through serum proteins analysis or through molecular analysis by RT-PCR so that further liver damage could be prevented and health status of people of Pakistan can be improved.
INTRODUCTION

Inflammation of liver is called hepatitis. It can be possible by several factors including alcohol, reaction to certain medications or infection by viruses. There may be different viruses that cause hepatitis but HBV and HCV are most frequent viruses (Serag, 2002). The symptoms produced by these viruses are almost identical but they all infect and inflame the liver (Gumber and Chopra, 1995). 170 million individuals throughout the world have infected with HCV (Lauer and Walker, 2001). According to WHO estimates 4 million deaths were caused by HCV in which 796000 caused by fibrosis while liver cancer caused 616000 deaths and 20% of these total deaths were due to HCV infection (Serag, 2002; Lai et al., 2003).

The blood-borne infections transmission had been highlighted as an issue with transfusions since their earlier finding. Hepatitis or liver inflammation term was used by the development of modern techniques (Pinzani, 1999). B Surface antigen has particular significance in diagnostic of hepatitis infection in combination of antibody particularly raise against the antigen. The antibody used in screening tests for HBV.

First antigen test was reported in 1970 in Australia to screen blood donors throughout the country. Post transfusion jaundice can be controlled by routine testing of HCV and this strategy is being used throughout the world.
Post transfusion rate of hepatitis reduced to 20% in United States due to effective screening methods based on antigens a antibodies (Callewaert et al., 2004). Although with the screening methods HAV and HBV was controlled but people was still identified sub-clinical post-transfusion jaundice. This condition was different from hepatitis A or B.

In (NANBH) hepatitis first coined in 1975 for this condition but hepatitis C was not used at that time because it was thought that several causing agents may involve in this condition (Thiel et al., 1994). Later it was identified that causing agent is hepatitis C virus (Hayashi et al., 1994).

It is widely accepted that for chronic liver diseases including cirrhosis hepatocellular carcinoma, HBV and HCV are the leading causes. With the advancement in molecular biology some new viruses viz., Hepatitis G, TT and SEN were also identified as cause of liver disease (Serag and Mason, 1999). Hepatitis caused by viruses is public health problem and it is calculated that HCV has infected 170 million patients (Lauer and Walker, 2001).

HCV is major cause of hepatitis. Its genome is positive-stranded RNA and belongs the hepacivirus genus. It falls in retroviruses group and family in Flaviviridae. Virus attachment with cells leads to endocytosis which resulted in direct hepatic injury. According to studies carried out in Italy and Japan,
the prevalence rate of HCV in liver injury is 40% while the studies performed in UK and France do not agree with these results (Ryder and Beckingham, 2001).

Hepatitis C infection may be severe (acute), characterized by short term illness or may prolong for 6 months to 24 months (chronic). The symptomology phases are variable, in few patients it is asymptomatic with mild flue while in some persons (15-45%) the viral load in cleared by the persons themselves with in 4-8 weeks of infection depending on HCV virus genotype. In individuals with week immune system, HCV infection causes significant damage to liver (Deuffic et al., 1999).

In chronic HCV infection most people do not show any significant symptoms as a result for long period of time they even do not know that when they infected earlier. They may show general symptoms like fatigue, nausea, lethargy, abdominal discomfort and loss of apatite. The intensity of symptoms is variable. The important point for consideration is that in chronic HCV infection the viral load never cleared from the body unless proper treatment with interferon alpha (Poynard et al, 2000).

HCV disease mechanism indicates that this virus cause localized inflammation of liver instead of human immune system. Therefore mostly liver is get infected. Depending on person’s immune system, the virus may
attack again in a few years with symptoms or alternatively HCV infection may lead to even more serious condition resulting in cirrhosis and hepatocellular carcinoma (Poynard et al., 2001). In 20% patients, HCV may lead to cirrhosis of liver and this stage possibly can appear after at least 20 years of infection. End-stage liver disease may result from this cirrhosis. This cirrhosis caused by HCV will cause hepatocellular carcinoma (HCC) i.e. liver cancer and in advance stage HCC may lead to liver failure.

The clinical parameters describing abnormal liver function involve increased serum transaminase, cirrhosis in liver biopsy, high viral mass in blood serum, plus additional medical indications like Alcoholic liver disease (ALD), HIV infection, HCV infection and HBV infection (Gumber and Chopra, 1995).

The other parameters related to HCV associated chronic liver disease involve viral type, sex of the person with age, alcohol abuse and infection of the person with some other viruses (Serag et al., 2002). Liver cancers progression rates are variable however 10 % rate of progression in towards liver cirrhosis. The rates of progression towards cirrhosis are lower in people having chronic liver disease than in people infected with HCV (Pawlotsaky, 2002). The route of transmission in case of HCV involves injecting practices, use of drugs and frequent sexual activities. The majority of
infections i.e. up to 80% are due to unsafe injecting practices while another 5-10 % are attributed to blood transfusion products (prior to 1990) and remaining are due to blood to blood contact, tattooing and sexual contacts (Fontana and Lok, 2002). The patients with haemophilia are specifically at risk to acquire HCV infection (Hirata et al., 2001). In haemophilia patients transmission of HCV risk in high because fractionated products are pooled from blood of various blood donors. This evidence indicates the prevalence of hepatitis C in haemophilic patients (Higuchi and Gores, 2003).

In alcoholic beverages ethanol is used which may cause liver failure. The alcoholic hepatitis symptoms involve enlargement of liver, feeling tired and increased abnormal values of liver function.

The hepatitis due to alcohol can result in different clinical changes like elevation of liver test parameters, development of jaundice, which can result in liver failure if untreated. In sensitive cases the bilirubin amount is become enhanced. In this category the death rate is 50 % in short period.

The long terms alcohol intake many result in alcoholic hepatitis, which is different from cirrhosis. ALD and cirrhosis caused by alcoholic can also result in hepatitis. Long-term alcohol consumption is leading cause of liver cirrhosis instead of alcoholic hepatitis alone. Moreover patients who drink alcohol excess may have chance of hepatitis C. In western countries HCV as
well as alcohol intake raised the chances of cirrhosis and liver inflammation. Hepatitis caused by drugs intake also described in report which indicated that the disease is reflection of drug a patient is taking up.

The chronic liver disease leads to cirrhosis (Figure 1A, 1B) involving appearance of fibrosis and regenerative nodules ultimately resulting in loss of liver function. The liver cirrhosis may involves various factors e.g. alcoholism, HCV infection, ascites, liver transplant and encephalopathy.
Figure 1 (A). Normal Liver Histology

Figure 1 (B). Liver with Fibrosis
The word cirrhosis originated from Greek means orange-yellow color of inflamed liver. Rene Laennec in 1819 introduced the term ‘cirrhosis’ in his research work where he also described the stethoscope (Murphy, 1998). The improper functioning of liver can be diagnosed by jaundice, elevated ALT serum level, yellowing of eyes and skin due to increased accumulation of bilirubin (2-3 mg/dL) and urine of dark color may also be indication (Murphy, 1998). Cirrhosis resulting in fibrosis has many causes and in the same patient more than one cause is present. Alcoholism and hepatitis are most frequent causes in Western World. ALD progressing to cirrhosis/fibrosis results in 20 % patients who have heavy intake of alcohol from quite a long time. The extent of alcohol leading the liver cirrhosis in variable. In some men little drinking even 3 per day and in women even 2 in a day may cause cirrhosis. Alcohol damages the liver tissue by disturbing/blocking normal metabolic pathways of carbohydrates, proteins and fats with the development of clinical symptoms involving hepatomegaly, jaundice, fever and anorexia. The serum ALT and AST levels are raised in addition to hepatocyte necrosis, neutrophilic infiltration, mallory bodies and perivenular inflammation. The standard test for liver cirrhosis is liver biopsy, through a fine-needle approach. In histological terms liver cirrhosis may be macronodular, micronodular or combined, but with the development of serum markers the disease diagnosis can be further improved. If patients clinical data is positive then therer is not acute need of
liver biopsy. However in liver fibrosis there is further risk of liver damage so it should be avoided at maximum. The following blood tests are characteristic in diagnosis of altered liver function in case of cirrhosis.

- **Aminotransferases**: AST amount in serum is increased.

- **Alkaline phosphatase (AP)**: Its serum amount slightly increased.

- **Bilirubin**: It is amount increased in cirrhosis.

- **Albumin**: Its amount in serum declines as liver function disturbs with advancement of fibrosis.

- **Prothrombin**: Its retention in serum enhanced due to increase number of clotting factors synthesized in liver tissue.

- **Globulins**: This protein amount usually raised in blood.

- **Serum sodium**: Salt level usually increased.

- **Thrombocytopenia**: It usually results due to splenomegaly.

- White blood cells count and neutrophils level decreased.

- Defects due to abnormal level of coagulation factors due to liver disorder.

The albumin is most frequent protein of serum in human. The total serum protein test gives overall picture of serum proteins. The serum proteins electrophoresis is considered to be initial clinical test regarding evaluation of
numerous clinical conditions. In protein electrophoresis the proteins are separated depending on biochemical properties. Serum along protein loading dye is loaded in SDS-PAGE gel. The proteins are get separated based on their size and charge.

Serum protein electrophoresis in SDS-PAGE analysis is dominated by albumin as well as globulins. The globulins constitute very less part of total serum proteins. The relative amount of these serum proteins during electrophoresis is variable depending on physiological and disease state of the patient (Ravel, 1995).

The dominated serum proteins viz., albumin and globulins are synthesized in the liver. Albumin assists in making blood viscous. Moreover the albumin is get bound with drugs, tissues and growth factors therefore involved in tissue repairing and new tissue development. The globulin proteins may belong to alpha, beta and gamma classes. Globulins are also synthesized by body immune cells as well. The hemoglobin and albumin jointly made protein complexes and also involve in transportation of blood iron and resistance towards infections. By serum electrophoresis, different proteins can be resolved according to their charge and molecular weight.

Albumin is the indicator regarding the proper functioning of the liver as well as kidneys. Globulin raised levels may be reflection of blood diseases
including rare blood disorders like macroglobulinemia and myeloma. The normal values of serum proteins including albumin and globulin are indicated below (Table 1)

**TABLE 1**
**SERUM PROTEINS**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Normal Range</th>
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<tbody>
<tr>
<td>Total serum protein</td>
<td>5.1 – 9.5 (g/dL)</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>3.1 – 6.0 g/dL</td>
</tr>
<tr>
<td>Serum globulin</td>
<td>1.5 – 4.8 g/dL</td>
</tr>
<tr>
<td>Serum albumin/globulin</td>
<td>Higher than 1.3</td>
</tr>
</tbody>
</table>

The increased albumin, globulin levels may be reflection of hepatitis, alcoholism, hemolytic anemia, leukemia, lymphoma, macroglobulinemia, myeloma and tuberculosis. The decreased serum albumin amount may be reflection of malnutrition, kidney disease and some immune diseases (Ravel, 1995).

In addition to serum proteins electrophoresis there is also need to test the involvement of hepatitis C virus in CLD patients by different molecular tests including PCR. There are several reports indicating the involvement of HCV in different liver diseases ultimately resulted in HCC (hepatocellular carcinoma). Uncontrolled HCV infection resulted in liver cirrhosis, necrosis terminated in liver failure. Therefore HCV is main reason of HCC in European countries and in US. Data analyzed from different countries
strongly indicates HCV is major cause of hepatocellular carcinoma which is major reason of deaths world wide (Poynard, 2000; Gebo et al., 2002).

The laboratory tests are either serological or molecular tests (based on the presence of viral nucleic acid). The serological tests are mostly cheap therefore being used to detect antibodies in serum against viruses in viral infection and increases suppressed patients. The antibody titer against HCV virus antigen does not remain same in patients and with the time it declines. Therefore the spontaneous decline rate in short time in sometimes not accurately detectable with accuracy (Pawlotsky et al., 2000).

Enzyme immunoassays can be used to detect HCV antibodies specifically depending on the optimization and reliability of test protocol. The recent advanced third generation immunoassays can detect viral antibodies at very early stage of infection in 4-10 weeks. These assays contain virus core proteins. In HIV 1 patients and patients of kidney failure along HCV and HIV coinfection, the immunoassays may resulted in false negative results. This is due to reason that these patients have suppressed immune system. However with the advancement of immunoassays it was become possible to detect HCV presence in the patient’s serum samples with reliability (Pawlotsky et al., 2000; (Pawlotsky, 2002).
Molecular detection assays for HCV RNA have introduced with the advancement of molecular biology field. The quantitative PCR for HCV can detect 100 or even less molecules of viral nucleic acid. Qualitative PCR is most reliable test for detection of HCV nucleic acids (Pawlotsky, 2002; Dienstag, 2002). After determining the presence of virus the second step in the determine the genotype of the virus by qualitative PCR and this test in also essential in conditions even when serum ALT level is normal but other cause of liver disease are present like immune-suppressed patients, HIV co-infected patients and person with high alcohol consumption. ELISA test may also be used for detection of HCV genotypes but its reliability in still a matter of concern (Heydtmann et al., 2001).

Hepatitis C has further six genotypes. The knowledge of virus genotype and serotype is mostly utilized for treatment of disease and monitoring the viral load in the body during the treatment (Heydtmann et al., 2001; Poynard et al., 2000). The recovery rate is 88 % for genotypes 2 and 3 while for others the cure rate is about 48 % with interferon alpha treatment. Genotypes remain the same during the course of infection. The interferon is part of human defense system and produced naturally by virus-infected cells mostly. However interferon beta and alpha are produced in cells of vertebrates in response to bacteria, parasites and tumor cells. Interferons are
basically cytokines and type of glycoproteins. Interferons alpha resist the viral replication in the cell by assisting the immune system of the body.

In literature some plant extracts have also reported with antiviral properties like neem (*Azadirachta indica* A. Juss) is among one of them. Recently the biological and chemical properties of this chemical natural compound was reviewed (Biswas et al. 2002).

The recent research project was carried out to determine the serum proteins profile status in chronic liver disease patients with and without clinical history of alcoholism and hepatitis C by employing serum proteins electrophoresis (SDS-PAGE) as well as HPLC techniques. Involvement of HCV was also studied by doing RT-PCR of suspected patients of chronic liver disease. In-vitro assays were also conducted in order to determine the effect of neem leaves extract on targeted serum proteins.
REVIEW OF LITERATURE

A virus means poison or toxin. It is Latin and its noun is virus. Its size ranges from 20 – 30 nm. It is sub microscopic particle. Viruses only infect the host cell and easily replicate themselves. It cannot reproduce itself. The genetic material of virus is within the protective protein coat called “capsid”. They infect all eukaryotes and Prokaryotes including archea and bacteria. Virus that attacks on bacteria is termed bacteriophage. Shortly it is called phage. A person who studies the virus is called virologist and study of virus is called virology. HCV, HIV, influenza and rabies viruses cause several serious human diseases. Therapy of viral diseases are very difficult because viruses are resistant towards drugs. However, viral diseases can be prevented by the vaccines in the patients.

Viruses are organisms in between the life. Viruses mostly propagate in compatible hosts, a feature of living organisms. The history of viruses origin is still not clear. No single mechanism can explain the origin of viruses. By the molecular approaches only some hypothesis can be made because they donot fossilize well. To date research on microfossils by molecular biology tools may provide some evidence (Prescott 1993).
Virus genetic material is nucleic acid, which is not very big size. The viruses with few genes may have also small sequences of nucleic acids originated from living organisms.

There are viruses with large genome such as poxviruses, which may parasitize in host cells. Retrograde evolution or reverse evolution is a process known through which the non-functional genes are gradually get eliminated from the genome. The living cells bacteria and viruses can only reproduce in side host cells in parasitic life style (Prescott, 1993).

The viruses exhibit primitive type of replicating machinery because of simplicity and hypothetically it can be possible possible. However viroids, satellites and prions are other infectious particles.

1. Classification ofViruses

Viruses classification is difficult due to debate that viruses are living or non-living coupled with lack of adequate fossil data. Viruses are difficult to place in any life domain therefore classification of viruses began with family level. However domain “Aetota” has been described due to reason that Eubacteria, Archaea and Eukarya have proper cell structure. It is not possible that the families are classified into orders.

The chicken pox virus has Herpesviridae family while sub family is Herpevirinae and genus is Varicellovirus is an example of viral
classification. There is no order for it. The classification of virus is as follows:

Order --virales
Family --viridae
Sub family --virinae
Genus --virus
Species --virus

In order to maintain uniformity the “International Committee on Taxonomy of Viruses” introduced the classification system based on certain virus properties. Taxonomists for determining the order must consider nucleic acid for classification purpose. In addition to nucleic acids other properties can also be considered these are host type, immunological features, shape of capsid and disease.

The biologist David Baltimore described the classification system on the basis of viruses mode of replication and genome type and divided the viruses into seven groups. By this mode we can easily distinguish the viruses. In modern virus classification ICTV system and Baltimore system of classification are used jointly (Presscott 1993).
2. Virus structure
A virus is a complete particle and acts as gene transporter and also termed as virion. It is made up of a protein coat called capsid plus nucleic acid. Viral genes encode capsid protein. Capsid morphology can be used for viruses classification. The promoters are also involved in capsid formation. Inside the capsid, viral nucleic acid is present. Proteins, which are closely connected with nucleic acid, are nucleoproteins and viral coat proteins along nucleic acids constitute nucleocapsid.

3. Virus genome
RNA and DNA constitute viral genome. It is very rare that virus has both DNA and RNA at the same time however DNA core with several mRNA segments are exceptionally possessing by cytomegalo virus. Single stranded RNA is preset in plant virus while double stranded DNA be located in bacteriophage. There are also some viruses having abnormal nucleotides. They have hydroxymethylcylosine instead of cytosine as a normal part of their genome (PressCott, 1993).

4. Genomic diversity among viruses
4.1 Shape
Polyomaviruses viral genomes may be circular and adenoviruses are linear respectively. The shape of the genome is not relevant to the type of the nucleic acid. Several genes may encode for diverse protein. may code for
one protein. The brome mosaic virus describes that its not essential that all viral genome fragments may be in only one virus. (Presscott, 1993)

4.2 The viral genomic DNA strands
Viral genome can be single or double stranded. The single strand of nucleic acid corresponds to unpaired bases while in double stranded nucleic acids two bases are joined to each other. However some Hepadnaviridae nucleic acids are of mixed type i.e partially single stranded and partially double stranded.

4.3 Sense nature of nucleic acids
The sense strands of viral nucleic acids may be plus strand or positive sense or these may be minus strand with sense which is negative. The nature of sense depends that whether it is complementary to viral RNA encoded from structural genes. The positive sense RNA can only translates to protein so negative sense strand must convert to positive sense strand before translation by RNA polymerases. DNA nomenclature is also identical to viral RNA nomenclature.

4.4 Size of genome
The size of the genome of different viruses is variable due to sequence and number of bases in the nucleic acids. It was observed that the smaller genome encode $10^6$ daltons proteins and the largest genome encodes about $10^8$ daltons (Presscott, 1993).
Due to higher error rate the viral genomes especially RNA viral genomes are quite smaller than DNA viruses genomes in order to minimize the chances of error. When the RNA replicates it result in a maximum upper size limit. It was observed that when it is not within the scope, which causes too many errors in the genome and when it replicates. The viruses having DNA as genome have comaparatively less error rate (Flinth et al., 2004).

4.5 Replication

Virus is submicroscopic organism. It does not grow through cell division by itself. It uses the metabolic machinery of the host cell and produces its essential components. Viruses division may or may not result in the destruction of the host cell (Presscott 1993). Virus not essentially able to kill the host cell but can resut in significant alteration in cell morphology and metabolism and collectively this whole sequences of events is called cytopathic effect of the viruses.

Virions transmit often via body fluid or by direct contact or through a vector. Viruses float free in water if the environment is aqueous. For virus reproduction in lytic cycle the characteristic of virulent phages such as T4 phage host cells will be induced by the virus to begin manufacturing the necessary protein. The viruses replication depends essentially on the type of host cell and its factors mostly proteins (Hourigan, 1999).
Viruses cause the cells to lyse when new capsid assembled and it released the virus particles.

By the process known as exocylosis virus donot completely lyse the cells by attaching the membrane of the cell. They take cell membranes for the synthesis of coat of viruses. In lysogenic cycle cells are remain intact earlier essentially and viral genome is get integrated in the host cell. The virus which enters in lysogenic phase will not always remain in such state and environmental condition allows it continue to survive (Ortiz, 2002).

In lysogeny, the host cell remains intact resulting in the spreading of viruses in the vicinity of healthy cells. The hepatitis involves the inflammation and injury of cells in the liver tissues. In some conditions the cells limits its own growth, healing on its own and it move forward resulting in the improvement of liver tissue. In chronic state of hepatitis, virus continues to exist longer and while in acute cases the disease terminates in short time with severe symptoms. Viruses of hepatitis group known to be a group of viruses are the cause of most liver damages. Toxins like alcohol and autoimmune proteins, which is relating to the immune response against substance normally present in the body (Hourigan, 1999; Ortiz, 2002).

In this stage the patient relating to the stage in development of a disease before the symptoms are observed. When the disease make worse the liver
functions that consider as part of other things, to examine for the presence of infection of harmful substances, the state of being controlled of blood composition and production of bile (A digestive juice secreted by the liver and stored in the gall bladder, aids in the digestion of fat) to help digestion.

5. Acute hepatitis

A patient with hepatitis along mild symptoms donot require intensive treatment but in case of serious conditions and significant damage of liver only liver transplant is possible solution. In young people due to active immune system, the viral hepatitis remains suppress however in aged persons with weak immune system the disease may produce even more serious condition (Brain and Ma, online text book of virology).

The initial features of acute hepatitis are not specific and patient have flue like symptoms, which are almost common in all. The other common symptoms include vomiting, feeling sick, joint pains, fever, headache and diarrhea. Loss of appetite is another common symptom. Jaundice along abdominal pain with dark urine are some other specified symptoms of the hepatitis disease and common indications of liver disorder. The percentage of clinical symptoms in most cases is 33 % jaundice, 10 % hepatomegaly and 5 % splenomegaly and lymphadenopathy (Ryder and Beckingham, 2001).
6. Chronic hepatitis

Patients majority will remain symptom less or mildly indicative of a disease in chronic hepatitis. The clear appearance is only being shown by the blood tests that indicate abnormality. Characteristics and symptoms may be related to the cause of hepatitis and the extent of liver damage. In review it was experience that symptoms of acute hepatitis jaundice can be a late aspect and may indicate big damage.

There are also other characteristics including abdominal enlargement of liver or spleen, fluid retention that is ascites and having low-grade fever. If disease was not cured then results in severe damage of liver that is the disease cirrhosis and leads to the weight loss, causing mental injury (injure the under lying soft tissues) and bleeding inclinations, lung scarring, acne, the thyroid grands having inflamation or irritation and in women kidneys may also inflamed in autoimmune hepatitis (Hourigan, 1999; Ortiz, 2002).

6.1 Types of hepatitis:

Most of the patients have acute hepatitis due to viral infection. Not only hepatitis viruses but also many other viruses like cytomegalovirurs, Epstein-Barr virus produce related related symptoms. The types of hepatitis viruses are as follows:
6.2 Hepatitis A (Infectious jaundice)

Infectious jaundice or hepatitis A, is a type of picornavirus. The route of transmitted is from feces. Contaminated food is also responsible for its infection in humans. It has short cycle but in some cases it has characterized long suffering (chromic stage) once a patient have hepatitis A. After virus entry in the body, antibodies are produced by immune system against virus.

Patient is usually recommended to avoid alcohol and take full water. Jaundice can be spread through personal contact by drinking contaminated water and by taking of raw seafood. This is usually occurs in third world countries. If we strictly avoid the peeled, unhygienic and raw foods it can be controlled. When the patient feels illness the infection can prolong from 15 to 45 days. It was observed that approximately 15 % of the cases might experience infection symptoms again with in six months to one year after diagnosis of infection.

6.3 Hepatitis B

Acute and chronic hepatitis is caused by hepadnavirus. After an initial infection 15% of individuals develop hepatitis due to weak immune response. Described routes of transmission may include sexual activity, blood transfusions, body fluids like secretions, mother to baby, syringes,
razors etc. In contrast to proven routes of transmission still in almost half of the cases the exact ways of transmission are still not clear.

The virus can be transmitted through intravenous drug use, needles, used razor blades and through open wounds. As a form of prevention the In different countries Needle-replace programme has coordinated. In US by prevention measures, HBV infection has significantly declined. After entry in the body, the body produces anti HBV antibodies which are not sufficient to clear the virus from the body because virus protects itself by integrating in host cell genome (Poynard, 2001).

Vaccination for HBV is good strategy but still this infection results in 500,000 to 1,200,000 deaths worldwide each year. In some parts of world its prevalence is quite high like in Asian countries where it is a big killer. In chronic hepatitis B infection FDA approved treatment options available are: Pegylated interferon, alpha-Interferon, adefovir and lanivudine. 45 % response rate of treatment can be achieved on treatment (Soriano, 2002).

6.4 Hepatitis C
Hepatitis C belongs to Flavivirus. Transmitted mainly through sexual activity and blood transfusion. HCV may results in the highs and most decisive point cirrhosis. Hepatitis C can remain in the body having no symptoms of illness or disease for 10 – 20 years. In 2007 researchers of
University of Washington announced vaccine production but still this work in progress and no vaccine is available for Hepatitis C (Poynard, 2001; Soriano, 2002).

Cases with hepatitis C having a tendency to life threatening hepatitis they fall victim to an illness. Cirrhosis of liver is stimulated by the HCV. Antiviral drug ribavirin and interferon can greatly reduce the viral load in the body. The genetic constitution of the virus can only find out the rate of reaction to this treatment strategy. Important thing is that the genotype1 of HCV is more drug resistant than other types. The unique receptor proteins include CD81 and SR-B1 and cladin-1 which acts as co-receptor (Bissell, 1999; Benhamou, 1999; Poynard, 2000).

6.5 Hepatitis E

The symptoms of hepatitis E are comparable to hepatitis A but in short time it becomes severe i.e., fulminant form especially in childrens and pregnant women in sub continent.

6.6 Hepatitis F

HFV (Hepatitis F virus) is a type of hypothetical virus and several suppositions were made during 1990 but still there is not report get validated and confirmed.
6.7 Hepatitis G

Hepatitis G is also another type of hepatitis has been identified. Its route of transmission is sexual intercourse and blood. Its site of replication is still controversial and it is considered that it is mainly replicated in the liver (Mario et al., 2003).

7. Alcoholic Hepatitis

Alcoholic beverages are also cause of hepatitis due to ethanol in them. Frequent intake of ethanol is responsible of alcoholic hepatitis. In this hepatitis body metabolism suffered, hepatomegaly, increase of liver blood test values and development of fluid (ascites) in the abdomen results. In Alcoholic hepatitis the liver enzymes levels are become elevated due to severe liver inflammation. The infection starts from mild symptoms and result in even more serious condition resulting in end stage liver disorder which is end stage resulting in the liver cancer (HCC). In persons infection is usually characterised by dull consciousness or the elevation of bilirubin levels and elevated level of clotting factor (prothrombin time). The mortality rate is up to 50 % in both categories i.e elevated bilirubin level or increased level of clotting factors (Serag, 2002).

Frequent alcoholic consumption results in hepatitis, which is different from the liver cirrhosis. Alcoholic hepatitis is found to exist in patients with
ALD and at advance stages it results in liver fibrosis. Frequent and long term alcohol intake causes liver cirrhosis. In western countries cirrhosis is due to hepatitis C and alcohol intake mainly (Paradis, 1996; Mathurin, 1998).

“It was reported that 170 million people world-wide are chronically infected by HCV (Lauer and Waller, 2001). According to the WHO Report of 2002 (WHO Report, 2001), chronic liver diseases were responsible for 1-4 million deaths, including 796,000, which ultimately leads to HCC.” The HCV is reason of 20 % of these deaths (Serag, 2002).

7.1 Epidemiology

Chronic hepatitis C virus infection has a great importance in causing liver disorders (WHO Report; Serag, 2002).

Now in the form of interferon, viremia can be declined effectively in 60 % of cases result in cirrhosis control. In Western countries mortality rate due to hepatitis C is increasing because of not proper treatment as well as diagnosis (Deuffic et al 1999; Alter et al., 1999).

The accurate and early detection can often lead to a better cure of disease. By examining resemblances with other hepatitis viruses, the main causes of the spread of HCV is through blood mixing and blood products, and using of contaminated syringes. Now the most common risk factor is
intravenous drug use. The sexually active individuals at risk of HCV due to exchange of secretions and involvement of other viral infections like herpes simplex virus infection (Alter et al., 1999).

7.2 Natural History

In Europe and the USA hepatitis C can cause liver failure, digestive tract hemorrhage, cirrhosis. Liver failure due to hepatitis results in transplantation of liver (Deuffic et al. 1999). HCC is mainly due to HCV and results in significant mortality rate (Deuffic et al. 1999; Serag 2002).

Liver fibrosis may result in cirrhosis which may require almost 20-30 years depending on immune system of individual (Poynard, 1997, Mathurin, 1998; Poynard 2000). For the development of fibrous connective tissues in the liver several factors involved like sex, age, alcohol intake, HIV infection as well as lower CD4 count (Paradis, 1996; Mathurin, 1998; Bissell, 1999; Benhamou, 1999; Poynard, 2000; Poynard, 2001; Soriano, 2002). Liver fibrosis may also caused by overweight and diabetes (Hourigan, 1999; Ortiz, 2002).

7.3 Genomic typing and serotype

HCV constitutes six sub-types and its proper diagnosis depends on molecular and serological tests (Zeuzem, 2000; Manns, 2001). The minimum viral load and treatment duration has also crucial role in control of
The interferon alpha and ribavirin combinational treatment resulted in 88% cure in case of 2, 3 while 48% for other genotypes (Fried, 2002; Hadziyannis, et al., 2002). During the infection viral genotype does not change therefore there is no need to test it again. The serotyping although cost effective but not preferred on genotyping because of high prevalence of false positive results, moreover for accurate determination of virus subtype genotyping is essential. The fibrosis has no direct relation with genotype according to few workers (Datz et al., 1999).

### 7.4 Pathophysiology

The HCV virus causing chronic hepatitis C does not directly destroys the hepatic cells. Instead virus induces the natural immune system of the body resulting in fibrosis control but not complete viral eradication (Heydtmann, 2001). In quantitative terms, CD8 and CD4 T-cell responses are not enough to control acute stage HCV control (Wedemeyer et al., 2002). The patients with weak immune response in acute phase may not show any symptom and are likely to become chronic carriers. In chronic carriers CD8 T cells produce interferons and lytic activity, therefore interferon injections are recommended for the treatment of the disease. Therefore interferon and
ribavirin a type of blessing due to potential high antiviral response using natural human body defense system (Soriano, 2002).

### 7.5 Clinical diagnostic tests

The laboratory tests diagnosis of virus are molecular tests as well as serological tests primarily for antibodies of viral particles. Cheaper initial diagnosis is based on anti HCV antibody detection is cheaper way of diagnosis. By applying the blood screening assays the risk of transfusion related diseases could be reduced. After infection the antibodies usually persists in the body of patients who recover after severe viremia (Takaki et al., 2000).

Enzyme linked immunoassays are also used to detect HCV antibodies in patients serum. This test is reliable than simple precipitation test based on interaction of antibody with antigen. The viral antigens can detect antibodies at quite early stage i.e with in 1-2 months. Immuno-suppressed individuals have low antibody response for particular antigen of the virus. The low immune response can be possible in several types of conditions viz., HCV, HIV (Pawlotsky, 2000). The individuals, who are become vaccinated against HBV, are found to be positive in ELISA tests due to prevalence of virus specific antigen in their serum (Pawlotsky, 2000).
Molecular detection assays for HCV have also been described. PCR technique can detect very less viral molecules i.e below one hundred particles. HCV nucleic acid detection is more specific test for diagnosis (Pawlotsky, 2000; Pawlotsky, 2002). PCR test is suggested when apparently there is no clinical signs of infection appear especially in people at high-risk level. The PCR based strategy is successful due to reason that it does not depends on the hosts immune response i.e antibody titer but linked directly to viral load in the body. Recently, ELISA also used as serological test to determine virus in the body (Pawlotsky, 2000; Pawlotsky, 2002). Some times virus stays in liver and disturbs liver function resulting in abnormal liver functioning (Castillo, 2003).

7.6 Determination of HCV nucleic acid in serum

Quantitative PCR is used to determine viral load in the body. Standardised international units are used to clinically define relevant HCV RNA loads. (Pawlotsky, 2000; Pawlotsky, 2002). Viral load estimations are critical because they result in estimation of virus in quantitative terms (Pawlotsky, 2002). The patients with persistent high viremia must given prolong treatment with interferon alpha 2b so that chances of relapse could be reduced. However in immunosuppressive diseases like AIDS antibody titer
enhanced not due to cirrhosis advancement but due to weak immune response (Pawlotsky, 2000; Pawlotsky, 2002).

### 7.7 Liver biopsy

Liver biopsy results in diagnosis of disease in histological terms (Dienstag, 2002). Staging of liver disease can be identified by this technique. It is helpful in accurate determining the actual cause of liver. The accurate histological examination of liver is quite essential to determine the staging of liver fibrosis and to determine the treatment duration. Even in individually with persistently normal serum transaminases, lesions on liver should be examined histologically so that liver inflammation towards advanced could be checked (Alberti, 2002).

The liver biopsy is considered to be the gold standard however still it poses serious limitations. The main limitations associated with liver biopsy include sampling disorders and errors which constitute 20 % of overall cases (Maharaj, 1986). The variability % for 15-mm sample is up to 55 % in most cases (Bedossa, 2003). The incidence of biopsy disturbance may result mortality rate up to 1 % (Poynard et al., 2000). The importance of liver biopsy will be declined greatly because of availability of new serum markers (Afdhal et al., 2003).
7.8 Treatment

Against hepatitis C virus there is still no vaccine available due to its diverse subtypes. In previous few years due to better prevention programs and improved laboratory tests, HCV has controlled significantly. The treatment of hepatitis involves correct diagnosis, controlling secondary complications and reducing its spread in general public (Corrao and Arico, 1998). Some other factors like metabolic disorders (overweight, diabetes, steatosis) should also be controlled to avoid the severity of disease (Hickman et al., 2002).

“Several main treatment regimens have been assessed in large trials, the first of which (standard interferon regimen monotherapy with three injections three times a week) was approved in 1990 and the last (combination of ribavirin and pegylated interferon) in 2002.” The interferon alfa (alfa 2a or 2b) 3 MIU thrice per week for 1-2 years results in its control. A combination of standard interferon (3 MIU three times a week) and ribavirin (1000-1200 daily doses for 2 years is quite useful treatment strategy (Lindsay, 2001; Zeuzem, 2000) however ribavirin in combination with pegylated interferon alos showed good results (Zeuzem, 2000). The pegylated interferon
monotherapy and monotherapy with simple interferon also produce effective results (Lindsay, 2001).

Uptill now two pegylated species of interferons have also been approved for treatment of HCV, several carcinomas and even in HIV treatment. The pegylated interferon as well as normal form of interferon also produce significant results (Manns, 2001). The pegylated interferon alpha 2a also produces significant results and its molecular mass is approx equal to 40 kda as described earlier (Fried, 2002). There is not much difference in the two form of interferons alpha. In a multicentre study (Jaeckel, et al., 2001) up to 98 % patients had recovered with normal liver function. This indicates that high doses of the drugs are useful in better controlling of the disease with improved percentage cure response with controlled viremia; the treatment can be adjusted accordingly.

### 7.9 Liver fibrosis

Damage of liver may result ot cirrhosis of liver (Friedman, 2003). The main causes of liver fibrosis result from alcohol, HCV infection and environmental factors. The liver damage can be intensified by the accumulation of ECM protein. The ECM proteins result in the accumulation of fibrous tissue in liver, which ultimately transformed into nodules, which
can regenerate. This accumulation of fibrous tissue may result in the development of necrotic tissue, which has capability to transform to other parts of liver tissue (Gines, 2004).

Liver fibrosis is essentially irreversible process (Schaffner, 1968). However fibrosis in one point of view may control the further damage to liver tissue (Albanis, 2001). In 1970 studies suggest that fibrosis is irreversible process (Soyer, 1976). In 1980 with the studies on hepatic stellate cells (HSCs) suggest that these produce collagen in hepatic tissue (Friedman, 1985). Experimental evidences suggested various fibrogenic inducing substances. In addition to HSCs (Geerts, 2001; Friedman et al., 1992; Rockey, 1992; Pinzani, 1989; Ramadori and Saile, 2004; Poynard et al. 2000; Poynard, 1997).

Fibrosis is also controlled by genetic and environmental factors (Bataller, 2003). In 1990 with the discovery of view that fibrosis can be reversed, scientists started to look the antifibrotic drugs (Hammel et al., 2001). Companies started to look antifibrotic drugs but still the best approach is to remove the cause (Bataller and Brenner, 2001). Antioxidants and Renin-angiotensin provided good results in animal models regarding antifibrotic properties. But animal models are not sufficient but also clinical trials are
also required. Drug induced hepatitis is also a major cause of altered liver function (Brunt, 2004). Ludwig, 1980, first described the accumulation of fats in liver cells. This accumulation of fat containing liver cells ultimately leads to HCC. Although extensive research is being carried out on liver fibrosis, but still there is need to diagnose the exact causing agents and possibilities to control this disorder specifically and accurately by antifibrotic drugs.

7.9.1 Natural history and diagnosis of liver fibrosis

Fibrosis leads to cirrhosis may results in morbidity and mortality (Poynard et al., 2000). Cirrhosis may appear after several years i.e. 15-20 years with some complications including encephalopathy, ascites, hepatic failure and improper function of kidneys. Decompensated cirrhosis condition is assigned to more disturbed situation of liver cirrhosis and to save the life of patient transplantation is mostly recommended (Davis, 2003). Hepatocellular carcinoma is end stage of liver cirrhosis. Liver fibrosis can be experimentally studied by using different animal and cell based models and several factors intensify the situation including alcohol, viral infections and drugs (Berenguer et al., 2003) The liver fibrosis is complexed by chromosomal coupled with environmental factors (Friedman, 2003). In CLD
several genetic factors are involved. In other research studies the involvement
of genetic factors was not properly validated so there is need to do more
research regarding the possibility of genetic factors in CLD disease (Bataller,
2003). The liver fibrosis results in regeneration of liver tissue (Friedman,
2003). In severe liver injury the parenchymatous cells are greatly damaged
and HCV is possibly involved into it. ECM proteins are get accumulated in
inflammatory response of liver. In liver tissue the fibrosis results in
limitizing the further damage of liver tissue. The distribution of liver injury
depends on the nature of liver damage which occurs and initially at portal
tracts, however in ALD pericentral regions are mostly infected. Fibrosis
involves in the development of fibrotic tissue which results in the cirrhosis
of the hepatic tissue (Pinzani, 1999).

The following signs and symptoms may occur in the presence of cirrhosis or
as a result of the complications of cirrhosis. Many are nonspecific and may
occur in other diseases and do not necessarily point to cirrhosis. Likewise,
the absence of any does not rule out the possibility of cirrhosis.

- Nail changes.

The nails are curved to either at 180 degrees or these may be bending
from the ends resulting in club shaping.
• “Hypertrophic osteoarthritis. Chronic proliferative periostitis of the long bones that can cause considerable pain.

• Dupuytren's contracture. Thickening and shortening of palmar fascia that leads to flexion deformities of the fingers.” Thought to be due to fibroblastic proliferation and disorderly collagen deposition. It is relatively common (33% of patients).

• “Gynecomastia. Benign proliferation of glandular tissue of male breasts presenting with a rubbery or firm mass extending concentrically from the nipples. This is due to increased estradiol and can occur up to 66% of patients.”

• “Hypogonadism. Manifested as impotence, infertility, loss of sexual drive, and testicular atrophy due to primary gonadal injury or suppression of hypothalamic or pituitary function.”

• “Liver size. Can be enlarged, normal, or shrunken.

• Splenomegaly. Due to congestion of the red pulp as a result of portal hypertension.

• Ascites. Accumulation of fluid in the peritoneal cavity giving rise to flank dullness (needs about 1500 mL to detect flank dullness).”

• “Caput medusa. In portal hypertension, the umbilical vein may open. Blood from the portal venous system may be shunted through the
periumbilical veins into the umbilical vein and ultimately to the abdominal wall veins, manifesting as caput medusa”.

- “Cruveilhier-Baumgarten murmur. Venous hum heard in epigastric region due to collateral connections between portal system and the remnant of the umbilical vein in portal hypertension”.
- “Jaundice. Yellow discoloring of the skin, eye, and mucus membranes due to increased bilirubin (at least 2-3 mg/dL or 30 mmol/L). Urine may also appear dark.”
- Other. Weakness, fatigue, anorexia, weight loss.”

7.9.2 Complications of liver fibrosis

As the disease progresses, complications may develop. In some people, these may be the first signs of the disease.

- “Bruising and bleeding due to decreased production of coagulation factors.
- Jaundice due to decreased processing of bilirubin.
- Itching (pruritus) due to bile products deposited in the skin.”
“Hepatic encephalopathy - the liver does not clear ammonia and related nitrogenous substances from the blood, which are carried to the brain, affecting cerebral functioning: neglect of personal appearance, unresponsiveness, forgetfulness, trouble concentrating.”

“Sensitivity to medication due to decreased metabolism of the active compounds.

Hepatocellular carcinoma is primary liver cancer, a frequent complication of cirrhosis. It has a high mortality rate.”

“Portal hypertension - blood normally carried from the intestines and spleen through the portal vein flows more slowly and the pressure increases; this leads to the following complications:”

- “Ascites - fluid leaks through the vasculature into the abdominal cavity.
- Esophageal varices - collateral portal blood flow through vessels in the stomach and esophagus.”
- “Problems in other organs.
- Cirrhosis can cause immune system dysfunction, leading to infection. Signs and symptoms of infection may be aspecific are more difficult.”
Fluid in the abdomen (ascites) may become infected with bacteria normally present in the intestines (spontaneous bacterial peritonitis).”

“Hepatorenal syndrome - insufficient blood supply to the kidneys, causing acute renal failure. This complication has a very high mortality (over 50%).”

“Hepatopulmonary syndrome - blood bypassing the normal lung circulation (shunting), leading to cyanosis and dyspnea (shortness of breath), characteristically worse on sitting up. Rodriguez-Roisin R (2004)”.

“Portopulmonary hypertension - increased blood pressure over the lungs as a consequence of portal hypertension.[2] (Rodriguez-Roisin, 2004).”

7.9.3 Liver cirrhosis and its causes

The uncontrolled liver fibrosis is transformed into liver cirrhosis. The liver “cirrhosis has many possible causes and sometimes more than one cause is present in the same patient. In world, the chronic alcoholism and hepatitis C are the most common causes of liver cirrhosis”.
- Alcoholic liver disease (ALD). This liver disease caused by frequent alcohol intake results in development of cirrhosis in drinkers is of 15% for quite long duration i.e from more than 10 years. The sensitivity towards alcohol is variable and results in cirrhosis (3-4 drinks a day in men and 2-3 in some women). Alcohol seems to block the normal metabolism of protein, fats, and carbohydrates in liver thus injure the hepatic tissue. Patients with alcoholic hepatitis develop various symptoms including hepatomegaly fever, “jaundice, and anorexia. AST and ALT are both elevated but less than 300 IU/L in most liver diseases. Liver biopsy may show neutrophilic infiltration, hepatocyte necrosis and mallory bodies.”

- “Chronic hepatitis C. Infection with this virus causes inflammation of and low grade damage to the liver that over several decades can lead to cirrhosis. It can be diagnosed with serologic assays that detect hepatitis C antibody” or molecular tests which determine the viral RNA.

- Chronic hepatitis B. “The liver cirrhosis by hepatitis B virus is most common worldwide, especially South-East Asia, but it is less common in the United State and Europe.” Untreated HBV infection causes initial liver inflammation which leads to cirrhosis depending on the
individual immune system. In some reports it was also indicated that Hepatitis D coinfection with hepatitis B accelerates cirrhosis. The chronic hepatitis B virus antigens can be diagnosed with in 3-4 months of infection. The serological and molecular tests based on protein and HBV DNA are used to disease diagnosis and treatment strategy.

- Non-alcoholic steatohepatitis (NASH). In this disease fats accumulation in liver tissue results in scars development on liver tissue. This type of hepatitis linked with obesity, diabetes, protein malnutrition and “treatment with corticosteroid medications. This disease is almost identical to that of alcoholic liver disease but patient does not have an alcohol history. The liver tissue biopsy is recommended for diagnosis”.

- Primary cirrhosis. It may be asymptomatic or with secondary complications including pruritus, non-jaundice skin hyperpigmentation with hepatomegaly and fatigue. The serum proteins profile is elevated especially alkaline phosphatase, “cholesterol and bilirubin. The authenticated standard diagnosis is antimitochondrial antibodies with liver biopsy as confirmation if showing florid bile duct lesions. It is frequent in women”.
• “Autoimmune hepatitis. This disease is caused by hyperactivation of immune system” resulting in damage to the liver causing inflammation earlier which is transformed into cirrhosis. The protein profile includes “elevations in serum globulins, especially gamma globulins. The treatment with prednisone and azathioprine drug is beneficial”.

• Hereditary hemochromatosis. This disease is prevalent “with family history of diabetes mellitus, cirrhosis, skin hyperpigmentation and cardiomyopathy, all due to signs of iron overload.” “Labs serological tests show transferrin saturation of > 60 % and ferritin > 300 ng/mL”. In this disease HFE mutations is quite common.

7.9.4 Diagnosis of liver cirrhosis

“The diagnosis of cirrhosis is a liver biopsy, through a fine-needle approach mostly. Histologically cirrhosis can be classified as micronodular, macronodular, or mixed, but this classification has been abandoned since it is nonspecific to the etiology, it may change as the disease progresses” and “serological markers are much more specific. However, a biopsy is not necessary if the molecular, radiologic and clinical data suggests cirrhosis”.

Moreover, regarding liver biopsy there remains always risk of damaging the liver tissue which may further complicate the situation cirrhosis cases.

### 7.9.5 Lab findings

The following findings usually observed in liver cirrhosis:

- **Bilirubin**: Its level increases in disturbed liver function.
- **Albumin**: Its level either may be significantly increased or decreased in response to altered liver function.
- **Prothrombin time**: Its level is mostly up regulated.
- **Globulins**: Its level is also upregulated in hepatic disorders.
- **Aminotransferases**: These are increased in blood. Sometimes even normal levels found in liver diseases.
- **Alkaline phosphatase**: Its level is mostly not much enhanced in blood in response to disturbed liver function.
- **Serum sodium**: Its level increases due to altered liver metabolism.
- **Thrombocytopenia**: it is due to congestive splenomegaly.
- **Leukopenia and neutropenia**: due to splenomegaly mostly.
7.9.6 Endoscopy

Gastroscopy i.e “endoscopic examination of the esophagus, stomach and duodenum is usually carried out in patients with prevalent cirrhosis”. If local lesions are found then prophylactic local therapy (sclerotherapy) and beta blocker treatment.

7.9.7 Pathology of liver cirrhosis

Initially the “liver may be enlarged, but with progression of the cirrhosis, its size becomes reduce due to the appearance of dead tissue.” The surface of the tissue is changed from smooth to irregular, its consistency is become firm due to development of fibers and the color is often turned yellow due to accumulation of proteins. Depending on the size of the nodules these classified into three macroscopic types: micronodular (under 3 mm), macronodular increased size from 3mm.

In microscopic examination the liver nodules and fibres are observed, growing irregularly and some dead cells could also be seen. The histological normal structure of liver tissue is disturbed. Fibrous network mostly surrounds the dead tissue along lymphocytes and macrophages. If bile tissue is involved then cirrhosis is termed as biliary cirrhosis resulting in damaging of biliary ducts and accumulation of dead tissue.
7.9.8 Pathophysiology of liver cirrhosis

The liver is central organ to synthesize different types of proteins including albumin, clotting factors and complement. It may also involve in detoxification of nitrogenous wastes and storage of essential vitamins like “vitamin A. In addition, it is central point in the metabolism of lipids and carbohydrates. The cirrhosis is followed by hepatitis and fatty liver (steatosis) mostly. If the cause is removed at this stage, the changes are still fully reversible”.

In pathophysiological terms liver cirrhosis involves the establishment of dead tissue that replaces parenchyma tissue which blocks the portal flow of blood through the organ and disturbing normal function. Stellate cell “normally stores vitamin A also involves in development of cirrhosis. Damage to the hepatic parenchyma leads to activation of the stellate cell, which becomes contractile (called myofibroblast) and obstructs blood flow in the circulation”. TGF-β₁ is also secreted by this cell (Iredale, 2003).

The nodules made up of fibrous tissue gradually spread throughout the entire hepatic tissue, leading to blockage of arteries and reduced supply of blood to liver tissue. The reduced size of spleen was observed. Due to
development of nodules and septa the blood flow to portal system is restricted resulting in more secondary complications of cirrhosis.

7.9.9 Treatment of liver cirrhosis

“The liver damage due to cirrhosis cannot be reversed, but treatment could stop further complication and its progression.” The monitoring of the patient is required generally. The potential liver damaging substances like alcohol use should be discouraged. A healthy diet is encouraged to cope with cirrhosis problem because it is energy requiring process and body needs more energy to fight against it. Salt intake should be restricted as in cirrhosis accumulation of salts (especially sodium) was observed. High-protein food increases the nitrogen balance, therefore protein diet should be controlled.

Treatment of liver cirrhosis is possible if causes are eliminated to prevent secondary complications:

- Elimination of causes: If causing agents of disease are controlled then disease can be controlled as well as cured. In hepatitis caused by alcohol, so if alcohol intake will be controlled then disease can be treated. Hepatitis-related cirrhosis can be controlled by taking proper
medicines in time such as interferon injections along ribavirin drug at appropriate doses.

- Preventing complications. Antibiotics if used in time then secondary infections can be controlled, and various medications can help with itching. For liver portal hypertension, the propranol is frequently used.

If complication uncontrolled these become too much elevated and cannot be controlled then liver ceases its functioning as a result “a liver transplant is quite essential. Survival from liver transplantation has been improving over the last 20 years and is now around 90 %, depending largely on the severity of disease and other medical problems in the recipient.” Transplantation necessitates the use of drugs viz., ciclosporin or tacrolimus drugs which have some draw backs as well.

In patients with previously stable cirrhosis, the enhancement of disease may occurs due to different causes, such as constipation, infection due to microrganisms, frequent alcohol intake, extensive non-specific medication, bleeding from hepatic tissue. It may proceed to further any complication as listed above. Patients with decompensated cirrhosis required intensive treatment therefore require admission to hospital in order to monitor diferent physiological parameters.
7.10 Epidemiology of liver cirrhosis

“The liver cirrhosis and chronic liver disease were the 10th leading cause of death for men and the 12th for women in the United States in 2001, killing about 27,000 people each year also the treatment cost of cirrhosis in terms of hospital costs, human suffering and less productivity is high (Sorensen, 2003).”

“Little is known on modulators of cirrhosis risk. Studies have recently suggested that coffee consumption may protect against cirrhosis, especially alcoholic cirrhosis (Klatsky, 2006)”.

8. INTERFERONS

Interferons are produced by natural immune cells of body in response to different foreign agents like viruses and bacteria. Interferons are glycoproteins also known as cytokines. The viral replication is mostly inhibited by interferons.
8.1 Types of interferon

Interferons have three major classes

Interferon class I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor that consists of IFNAR1 and IFNAR2 chains.

Interferon class II: IFNGR seconcdary receptor.

Interferon class type III: These interferons attach to IL10R2 (also called as well as CRF2-12.

The results described that interferon is part of several other signalling pathways however, the JAK-STAT signaling pathway is the best-characterised and is considered to be the main pathway involves in the activation of interferons.

8.2 Sources and functions of interferons

Interferons contain collective properties like viral inhibition, anticancerous properties with macrophage and natural killer lymphocyte activation. These interferons also result in major histocompatibility complex activation against invading microbes coupled with T cells. Generally the interferon induction
depends on type of inducers usually microbes (viruses and bacteria). The cytokines with interleukins, TNF along CSF are produced in the body in response to several antigens and the other secretory proteins rarely pass the placenta and the blood-brain barrier.

The p53 activity in increased by interferons. It results in increased production of tumor inhibiting proteins. Apoptosis is stimulated as a result of this and transcriptional level are also increased. However cells invaded by viruses show more apoptosis. P53 induced by virus results in its differential behaviour than it usually does. In response to IFNs some proteins are get decreased like p21 which is involved in stability of the cell. In lab studies it was found that normal cells show greater apoptotic activity than cells devoid of p53 (Takaoka et al., 2003).

Interferons have show efficacy in certain carcinoma p53 induction has significant role. Interferons are natural protein drugs which can replace chemotherapy drugs that activate p53 (Takaoka et al., 2003).

In hepatitis C patients treatment with interferon results in better blood parameters. The provision of interferon after infection can result in better control of disease and significant reduction in the virimia. In 2001 FDA approved pegylated interferon in which PEG molecules are get attached with
interferon. Similarly pegylated form of interferon-alpha-2a get approval in 2002. By using these species of interferon the treatment rate could be enhanced to 75%.

Intranasal administration of interferons in very low doses is effective to treat viral respiratory diseases such as cold and flu in Europe and Russia. Interferon mechanism of such action is not well understood but it is thought that doses must be larger by several orders of magnitude to have any effect on the virus. Recently by these claims, most European scientists are suspicious in these claims (http://www.pathobiologics.org).

9. Serum proteins

Serum proteins analysis is essential in response to different diseases. Many specialists suggest the serum protein analysis as a way of early diagnosis by the interpretation of results can be possible by qualified molecular biologist or biochemist.

Electrophoresis is technique to resolve serum proteins based on their size and charges which may produce due to R groups of amino acids which constitute the protein. Serum after making certain dilution is loaded in SDS-polyacrylamide gel wells and under certain volatge the proteins are get resolved on SDS-PAGE gel (Jacoby and Cole, 2000).
In electrophoresis proteins are loaded on different physical locations on the gel called wells (Ravel, 1995). The proteins are stained in different staining solutions containing bromophenol blue as a result the resolved protein bands are get stained and can be easily visible on the gel. Florescent dyes are also used to label the proteins so that these can be visualized under UV light. Albumins and globulins constitute the major pattern of serum proteins electrophoresis. In normal conditions albumins are produce predominately in blood serum but in case of abnormal functioning of liver this protein level is increased even much. Globulins comprise not much fraction of liver proteins (Ravel, 1995).

9.1 ALBUMIN

The albumin band on SDS-polyacrylamide gel is mostly dominated in serum proteins under some hepatic disorders. Due to physiological disorders some proteins level is raised as in indication of serum markers. Different factors results in decreased albumin in serum and these include liver diseases, hormone therapy, malnutrition, renal loss. Plasma serum proteins profile are usually altered in response to different factors including trauma, burns, acute, necrosis, inflammation, infarction, chemical injury and malignancy (Ravel, 1995).
The recent research project was carried out to determine the serum proteins profile status in chronic liver disease patients with and without clinical history of alcoholism and hepatitis C by employing serum proteins electrophoresis (SDS-PAGE) as well as HPLC techniques. Involvement of HCV was also studied by doing RT-PCR of suspected patients of chronic liver disease. In-vitro assays were also conducted in order to determine the effect of neem leaves extract on targeted serum proteins.
MATERIALS AND METHODS

1. SAMPLES COLLECTION
The blood samples of different categories viz., HCV patients, HCC patients and CLD patients were obtained from different hospitals viz., Sir Ganga Ram hospital, Sheikh zaid hospital and Services hospital in Lahore. Thirty samples of each category were collected for further biochemical and molecular analysis. The 30 samples of suspected individuals of above each three categories were also obtained from different localities in the vicinity of Lahore city. The volume of blood for each sample was 10 ml and it was taken in EDTA containing tube.

2. BIOCHEMICAL TESTS
The blood samples of different categories were subjected to different biochemical tests in order to determine any biochemical change in patient’s serum. The following biochemical tests were performed for this purpose: Serum bilirubin, serum total protein, albumin, alanine transferase, alkaline phosphatase were estimated by standard kit method (Randox, United Kingtom). Serum sodium and potassium were estimated by flame photometer.
2.1 Determination of serum bilirubin by Evelyn Malloy method (Magos, 1960).

**Principle:**

Bilirubin and the diazo reagent form an azobilirubin complex, which can be measured colorimetrically. The color of the azobilirubin varies with pH.

It is based on the following principle that (1) conjugated (directed) bilirubin is water soluble and therefore will react with diazo reagent in a water solution, and (2) Un conjugated (indirect) bilirubin is not water soluble, therefore, alcohol is necessary to put the un conjugated bilirubin in solution. So that it can react in the diazo reaction.

**Reagents:**

- **Diazo A:** 5g of sulphanilic acid and 15 ml of concentrated HCL dissolve in distilled water and make volume up to one litre.

- **Diazo B:** 0.5g of sodium nitrite dissolve in 100 ml of distilled water, keep it in refrigerator.

Freshly prepared by taking 5 ml of diazo A and 0.15 ml of diazo B.
**Procedure**

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Test (total)</th>
<th>Test (conjugated)</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>Diaz reagent</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>2.5 ml</td>
<td></td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>2.5 ml</td>
<td></td>
</tr>
</tbody>
</table>

Mix and keep in dark for 30 min and read the absorbance at 540 nm against distilled water.

Calculation: \[
\frac{\text{Abs of sample} \times \text{Conc of standard}}{\text{Abs of standard}}
\]

### 2.2 Determination of serum alkaline phosphatase by method of Wiwanitkit (2001).

**Principle:**

A kinetic method uses p-nitrophenyl phosphate (PNPP) as a substrate. PNPP does not absorb at the wavelength chosen to read the test (405 nm).

Alkaline phosphatase cleaves phosphate; free p-nitrophenol (PNP) is formed and converted to a yellow color at an alkaline pH. The optimum pH for alkaline phosphatase is about 10.0; the rate of formation of the yellow color is used to measure enzyme activity.
### ALP

\[
\text{PNPP + H}_2\text{O} \rightarrow \text{PNP + inorganic phosphate} \quad \text{Mg}^{2+}
\]

**Regents:**

1. 0.5N NaOH: 2.0g NaOH/100 ml distilled water
2. 0.5N NaHCO\(_3\): 4.2g NaHCO\(_3\)/100 ml distilled water
3. Amino antipyrine: 0.6g %
   
   0.6g Amino antipyrin dissolved in distilled water and make upto 100 ml. Store in brown bottle.
4. Potassium Ferricyanide: 2.4%
   
   2.4g potassium ferricyanide dissolved in distilled water and make volume upto 100 ml. Store in brown bottle.
5. Buffer: pH 10.2
   
   6.36g of anhydrous sodium carbonate and 3.36g of sodium bicarbonate dissolve in distilled water and make volume one litre. Keep it at 4\(^\circ\)C.
6. Substrate:
   
   Dissolve 2.18g disodium phenyl phosphate in one litre of distilled water. Boil the solution quickly to kill any organism, cool immediately and preserve with 4.0 chloroform. Keep it at 4\(^{0}\)C.
**Procedure**

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Substrate</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Incubate for 3 min at 37°C

<table>
<thead>
<tr>
<th>Serum</th>
<th>0.1 ml</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td></td>
<td>0.1 ml</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td></td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Incubate for 15 min at 37°C and take it out

<table>
<thead>
<tr>
<th>0.5N NaOH</th>
<th>0.8 ml</th>
<th>0.8 ml</th>
<th>0.8 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5N NaHCO3</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>Amino antipyrine</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Potassium Ferricynide</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Mix and read the absorbance at 510 nm against blank within 5 min.

Calculation:
Abs of sample $\times$ Conc of standard
Abs of standard
2.3 Determination of alanine amino transferase by colorimetric method (Teitz, 1976).

Principle:
The transaminase (glutamic pyruvic transaminase) catalyses the transfer of the amino group from $\alpha$-amino acid to $\alpha$-ketoacids in the following reaction.

dl-alanine + $\alpha$-ketoglutarate $\leftrightarrow$ Glutamate + Pyruvate

Pyruvate formed, reats with 2,4 – dinitrophenylhydrazine yielding, in an alkaline medium, a colored compound which can be measured at 505 nm.

Reagents:-
1- Phosphate buffer. pH 7.4

   Add 17.64g di potassium hydrogen phosphate (dehydrate), the potassium di-hydrogen phosphate 2.177g along benzoic acid 1g in dissolved in 0.9 litre water adjusted pH to 7.4 and added chloroform 10 ml. Mixed the contents and adjusted total volume to 1 litre.

2- GPT Substrate:-

   Dissolve 146 mg $\alpha$-oxoglutaric acid and 8.9g dl-alanine in 100 ml distilled water. Adjust to pH 7.4 by adding sodium hydroxide 4% drop wise (about 2.5 ml). Dilute to 500 ml with phosphate buffer. Store in refrigerator.
3- Dinitrophenyl hydrazine:- (DNPH) (1N HCl 100 ml → 9.9 ml concentrated HCl upto 100 ml with D/W)

Dissolve 0.2 g DNPH in 1000 ml 1N HCl. Stored in polyethylene bottle in a refrigerator.

4- Sodium hydroxide (0.4N or 1.6%)

Note: -1st make the concentrate of GPT substrate upto 100 ml water pH 7.4.

Working solution:- Dilute 20 ml of this concentrate with 80 ml phosphate buffer (pH 7.4) final volume upto 100 ml.

**Procedure**

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>50 ul</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td>50 ul</td>
<td></td>
</tr>
</tbody>
</table>

- Incubate at 37°C for 30 min

| DNPH      | 0.25 ml | 0.25 ml | 0.25 ml |

- Keep for 20 min at room temp

| NaOH 0.4N | 2.5 ml | 2.5 ml | 2.5 ml |

Mix and read the absorbance at 505 nm against blank using the apparatus of spectrophotometer (Spectronic 21).
2.4 Determination of Serum Calcium by Colorimetric method (Barnett et al., 1973).

Reaction Principle
Complex with O-Cresolphthalein complexone in an alkaline medium

Sample Material
Serum heparinized plasma or urine. Dilute urine in 1+1 in 0.9% NaCl.
Multiply by 2

Reagents

<table>
<thead>
<tr>
<th>Contents</th>
<th>Initials concentration of solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Standard Calcium</td>
<td>2.5 mmol/l (10 mg/dl)</td>
</tr>
<tr>
<td>2. Buffer</td>
<td></td>
</tr>
<tr>
<td>2-Amino-2-methyl-propan-1-ol</td>
<td>3.5 mol/l, pH 10.7</td>
</tr>
<tr>
<td>3. Chromogen</td>
<td></td>
</tr>
<tr>
<td>O-Cresolphthalein complexone</td>
<td>0.16 mmol/l</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>6.89 mmol/l</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>60 mmol/l</td>
</tr>
<tr>
<td>4. EDTA</td>
<td>150 mmol/l</td>
</tr>
</tbody>
</table>

Calculation
Concentration × 2.50 (mmol/l) = \(\frac{\text{Asample}}{\text{Astandard}}\)
Concentration (mg/dl) = \(\frac{\text{Asample} \times 10.0}{\text{Astandard}}\)

Normal Values
Serum 2.02 – 2.60 mmol/l (8.10 -10.4 mg/dl)
Urine 2.5-6.2 mmol/ 24 hrs (100-249 mg/24 hrs)
2.5 Estimation of Serum Albumin by Bromocresol Purple Method (Lasky et al., 1985)

**Principle**

Serum albumin quantitation by 5,5-dibromo-o-cresolsulphonphthalein (bromocresol purple, BCP). The albumin and BCP complex absorbs light at 600 nm.

**Reagent**

- Sodium acetate buffer: 0.07 mol/l, pH 5.2
- Bromocresol purple: 0.06 mmol/l
- Brij 35
- Preservative

**Procedure**

- Wavelength: 600 nm
- Cuvette: 1 cm light path
- Temperature: 20-25 °C

<table>
<thead>
<tr>
<th></th>
<th>Reagent Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>10 µl</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Standard</td>
<td>--------</td>
<td>10 µl</td>
<td>------</td>
</tr>
<tr>
<td>Sample</td>
<td>--------</td>
<td>--------</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
Mixed and incubated at 20-25 °C for 2 minutes. Measured the absorbance of the sample (A sample) and of the standard (A standard) against the reagent blank.

Calculation

\[
\text{Albumin concentration} = \frac{\text{A sample}}{\text{A standard}} \times \text{concentration of standard}
\]

Normal values in Serum

Adults: 34- 50 g/l.

2.6 Estimation of Serum Total Protein By Biuret Method (Okutucu et al., 2007).

Principle

Peptide bond reacts with cupric ions resulting in color complex formation.

Reagents

<table>
<thead>
<tr>
<th>Biuret reagent mixture</th>
<th>Concentartion of solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide</td>
<td>100 mmol/l</td>
</tr>
<tr>
<td>Na-K-tartrate</td>
<td>16 mmol/l</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>15 mmol/l</td>
</tr>
<tr>
<td>Cupric sulphate</td>
<td>6 mmol/l</td>
</tr>
</tbody>
</table>
Procedure

Wavelength 546 nm

Cuvette 1 cm light path

Temperature 20-25 °C

<table>
<thead>
<tr>
<th></th>
<th>Reagent Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>0.02 ml</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>Standard</td>
<td>--------</td>
<td>0.02 ml</td>
<td>------</td>
</tr>
<tr>
<td>Serum</td>
<td>--------</td>
<td>--------</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>Biuret reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Mixed and incubated for 30 minutes at 20-25 °C. Measured the absorbance of the sample (A sample) and of the standard (A standard) against the reagent blank.

Total Protein Conc. = A sample/A standard x Standard concentration

2.7 Estimation of Serum Potassium (Teitz, 1976)

Principle

Sodium tetraphenylboron reacts with potassium ions in a protein free alkaline medium to produce a turbid suspension of K-tetraphenylboron. The amount of turbidity produced is proportional to the Potassium concentration.
Potassium Assay

Wavelength 578 nm

Temperature 20-25 °C

<table>
<thead>
<tr>
<th>Standard</th>
<th>Macro Sample</th>
<th>Standard</th>
<th>Micro Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working</td>
<td>2000 µl</td>
<td>2000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>-----</td>
<td>100 µl</td>
<td>----</td>
</tr>
<tr>
<td>Supernatant</td>
<td>-----</td>
<td>200 µl</td>
<td>----</td>
</tr>
</tbody>
</table>

Mixed carefully and allowed to stand for at least 5 minutes. Measured the absorbance of standard and sample against reagent blank.

Potassium concentration (mmol/l) = A Sample/A standard x 5

Normal Values

Serum: 3.6-5.5 mmol/l

2.8 Estimation of Serum Sodium (Teitz, 1983)

Principle

β-galactosidase acts on ONPG (O-nitrophenyl) as substrate in the presence of sodium. The break down of substrate results in decline of OD at 405 nm.
**Reagent**

**Contents**

i) Buffers and enzyme

- Tris buffer: 450 mmol/l, pH 9.0
- Cryptand: 5.4 mmol/l
- β-galactosidase: 1 U/ml

ii) Diluent and substrate

- Tris buffer: 15 mM, pH 9.5
- O-nitrophenyl galactoside: 5.6 mM

**Procedure**

- Wavelength: 405 nm
- Temperature: 37 °C

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>40 µl</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Standard</td>
<td>------</td>
<td>40 µl</td>
<td>------</td>
</tr>
<tr>
<td>Sample</td>
<td>------</td>
<td>------</td>
<td>40 µl</td>
</tr>
<tr>
<td>Buffers and enzyme</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

Mixed and incubated for 5 minutes at 37 °C, then added:

- Diluent and substrate: 400 µl
Mixed and incubated at 37 °C for 1 minute. Read absorbance at 405 nm.

Normal values

136-146 mmol/l (313-336 mg/dl).

3. SDS-PAGE GEL ELECTROPHORESIS

The serum samples of patients of different categories including HCV, HCC and CLD were subjected to Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE). The serum concentration was estimated by taking OD at 280 nm after 1/10 dilution in 30 mM Tris-Cl (pH 8.5). Buffer was set as blank. Generally 1 OD = 1 mg/ml of protein. The amount of protein loaded per well is 100 µg and the samples were subjected to mild heating at 40 °C after mixing in protein loading dye before loading in wells. The 10 % SDS-PAGE gel was used to estimate the serum proteins profile of different categories of the patients. The 10 ml 5 % “stacking gel contained dH₂O 5.65 ml, 30 % acrylamide mix 1.65 ml, 1.0 M Tris pH 6.8 2.5 ml, 10 % SDS, 0.1 ml 10 % ammonium persulfate, 0.1 ml TEMED 0.004 ml)” and “resolving gel 10 ml of 10 % (dH₂O 4.0 ml, 30% acrylamide mix 3.3 ml, 1.5M Tris pH 8.8 2.5 ml, 10 % SDS 0.1 ml, 10% ammonium persulfate 0.1 ml, TEMED, 0.004 ml)” collectively comprised SDS-PAGE gel. The applied voltage was set at 100 volts and gel was allowed to run for two hours. The 10 X SDS-gel Running buffer composition involves (Trizma base 30.3 g, Glycine 140 g,
SDS 10 g) and making total volume 1 litre with distill water.

After gel running, the gel was placed in staining gel solution (0.50 g Coomassie Blue, 200 mL absolute ethanol and 200 mL water) for 1 hour and then subjected to destaining solution (400 mL absolute ethanol, 100 mL acetic acid) till protein bands were become appeared. The gel was photographed by using gel documentation system (Dolphin, USA).

4. HPLC ANALYSIS OF PATIENTS SERUM

Principle:
The high performance liquid chromatography (HPLC) is a type of column chromatography in which molecules in liquid phase are passed through solid phase under some pressure (1000-3000) Ib inch$^{-2}$ and separated on the basis of charge or size. In this study the HPLC was carried out using hydrophobic resin (Octadecyl silane, C-18) as a stationary phase and our serum was mixed in mobile phase using acetonitril and methanol as mobile phase. The protein was eluted from the column by applying gradient of acetonitril and protein was eluted on the basis of hydrphobicity. The elution profile was monitored by taking absorbance at 280 nm.

Reagents:
Triflouro acid (Analytical grade)
Methanol (Analytical grade)
Acetonitril (HPLC grade).
**Procedure:**

The HPLC analysis was carried out for representative samples for each category i.e normal, HCV and alcoholic patients. For serum analysis of patients, 5 ml blood was kept undisturbed for 15 min at 37 °C. The pellet down bottom of culture tube while serum was proceed further. The serum was diluted in 1:100 ratio with HPLC grade distilled water and then absolute methanol was also added as 20 % (v/v) ratio.

The 500 microlitres sample was injected in HPLC injecting pocket. Before loading the sample the HPLC column (octadecyl silane silica resin, C-8) was equilibrated with 100 ml of 0.005 % TFA (Trifloro Acetic Acid). The size of the column is 25cm length and 4 mm width. The flow rate was adjusted to 0.75 ml/ min and the applied pressure was 1000-3000 Ib/cm³. The sample was loaded when the base line absorbance value was equal to zero. After loading the sample the column washed with TFA (0.0005 %). The elution was carried by applying mixture of 0.005 % TFA and 100 % absolute methanol and gradient was set from 0.00-100 % methanol. Over a variable period of time different proteins were eluted and their peaks were observed in the chromatogram. The proteins absorbance was recorded at 280 nm. The serum was also subjected to SDS gel electrophoresis.
5. RT-PCR ANALYSIS FOR HCV

The reverse transcriptase PCR was performed to find out the possibility of HCV in various categories of the patients viz., suspected, CLD and HCV confirmed patients. Trizol reagent was used to isolate RNA from blood. For RT-PCR the resulting fragment is of 296 bp region of noncoding region of virus genome which is highly conserved. The primers used were described in (Table 2). The RT-PCR reaction mixture contains RT-PCR Buffer 1X, MgCl₂ 1.5 mM, 100 pmol each of HCVRT-R and HCVRT-F primers (Table 2). RT-PCR carried out at 62°C for 15 min plus 40 cycles of 94°C for 0.20 min, 57°C for 1 min, and 72°C for 0.45 min. The PCR product (296 bp) was resolved on 1.5 % agarose gel.

Table 2. Primer Sequences

"HCVRT-F 5'-CTGTGAGGAACACTACTGTCTTC-3'
HCVRT-R 5'-GGTCACGGTCTACGAGACCT-3'
HCVIN-F 5'-CAGAAAGCGTCTAGCCATGGCGTT-3''
HCVIN-R 5'-CCCTATCAGGCAGTACCACAA-3'

5.1 RT-PCR of non encoding region of HCV.

Internal primers (HCVIN-F, HCVIN-R) were used for nested PCR to further confirm the test. The parental PCR product was diluted ten times and hundred times by water and 0.001 ml of each was used in PCR. The nested
PCR reaction mixture contained PCR reaction buffer 1X, MgCl₂ 2.0 mM, dNTPs 0.2 mM, HCVIN-F and HCVIN-R primers 100 pmoles each (Table 2), 0.001 ml PCR product, and 2.5 units of taq polymerase in a reaction volume of 50 microlitre. The nested CR was carried out for total 30 reactions including 95°C for 0.5 min, 60°C for 0.45 min, and 72°C for 1.5 mins. The total PCR product i.e 50 microlitres was loaded on 2 % agarose gel for detection of amplification.

6. EFFECT OF NEEM EXTRACT AND INTERFERON ALPHA 2B ON SERUM PROTEINS

The ten neem leaves were washed in water and were boiled in 200ml autoclaved water after cutting into small pieces. The boiling was continued till water was reduced to half in volume. This neem extract was then filtered through 0.2 µm amicon filter and subjected to incubation with serum (100 µg) of HCV patients at 37°C for one hour under aseptic conditions. The total volume of incubation mixture was adjusted to 100 µl. The incubation of same amount of serum was also performed with interferon alpha2b (100 µg) under same conditions. After incubation 20 µl of protein containing incubation mixture was loaded on 10 % SDS-PAGE in order to determine the effect of interferon alpha 2 and neem on serum proteins of HCV patients.
7. PROTEIN PURIFICATION FROM SDS POLYACRYLAMIDE GEL

The 14 Kda and 30 Kda protein bands were cut from SDS-polyacrylamide gel by fine surgical razor. These bands were placed in sterilized eppendorfs separately containing 30 mM Tris-Cl (pH 8.0) 50 µl and subjected to fine mincing in the same eppendorf with the help of microtip. The mixture was centrifuged at 13000 rpm for 1.5 minutes and supernatant was saved at –80 °C for further biochemical analysis.

8. EFFECT OF NEEM EXTRACT AND INTERFERON ALPHA 2B ON 14 KDA AND 30 KDA PURIFIED PROTEINS

The 14 Kda and 30 Kda purified proteins (50 µg each) were subjected to incubation with 50 µl neem extract. The same purified proteins were also incubated with 50 µl interferon alpha 2 (50 µg) and total volume was adjusted to 100 µl. The mixture was incubated at 37 °C for 1 hour along controls containing both proteins alone with out neem and interferon alpha2. The samples were loaded on 10 % SDS polyacrylamide gel in order to determine the effect of interferon alpha2 and neem extract on specified purified proteins.
RESULTS

1. Biochemical analysis of serum samples

The blood samples of confirmed hepatitis C and CLD patients were collected from different hospitals viz. Ganga Ram Hospital, Sheikh Zayed Hospital and Services Hospital of Lahore from patients belonging to different localities of Punjab while samples from suspected individuals of above two classes and alcoholism were collected from vicinity of Lahore on the basis of patient history. All the samples were categorized in three groups containing 30 samples each and different biochemical tests were applied on them (Table 1). For each category positive confirmed samples were also used as control and for each category 30 samples were used as positive control. The percentage of positive cases among hepatitis C suspected individuals was found to be 23.33 % (Table 1) on the basis of different biochemical tests results. These biochemical tests were performed in biochemistry laboratory of Ganga Ram hospital, Lahore. The values of different biochemical tests for suspected positive individuals was represented the same range as indicated by hepatitis C confirmed patients (Table 1). However the values of biochemical tests of negative cases of suspected hepatitis C individuals were quite similar to normal individuals and there was observed no significant change (Table 1).
Table 1. Biochemical analysis of serum samples from suspected as well as control subjects

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th>Normal (30)</th>
<th>Hepatitis C suspected (30)</th>
<th>Alcoholism suspected (30)</th>
<th>CLD suspected (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive cases (7)</td>
<td>Positive cases (%)</td>
<td>Positive cases (6)</td>
<td>Positive cases (%)</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>6.3-7.9</td>
<td>9.0-12.0</td>
<td>23.33</td>
<td>9.85-10.50</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>34-50</td>
<td>46-55</td>
<td>37-51</td>
<td>39-57</td>
</tr>
<tr>
<td>Serum bilirubin (mg/dl)</td>
<td>Up to 1.1</td>
<td>0.9-1.2</td>
<td>0.8-1.5</td>
<td>0.10-1.3</td>
</tr>
<tr>
<td>Serum alanine transferase (U/L)</td>
<td>Up to 40</td>
<td>51-72</td>
<td>48-95</td>
<td>43-74</td>
</tr>
<tr>
<td>Serum alkaline phosphatase (U/L)</td>
<td>39-117</td>
<td>125-245</td>
<td>140-210</td>
<td>121-230</td>
</tr>
<tr>
<td>Serum calcium (mg/dl)</td>
<td>8.10-10.4</td>
<td>9.1-12.0</td>
<td>8.6-11.0</td>
<td>8.9-12.5</td>
</tr>
<tr>
<td>Serum potassium (mmol/L)</td>
<td>3.6-5.5</td>
<td>5.0-7.0</td>
<td>5.2-9.0</td>
<td>5.0-7.5</td>
</tr>
<tr>
<td>Serum sodium (mg/dl)</td>
<td>313-336</td>
<td>345-390</td>
<td>330-410</td>
<td>338-410</td>
</tr>
</tbody>
</table>
2. Identification of specific serum proteins as marker proteins

The specific marker proteins were identified in different categories of the subjects by applying SDS-PAGE analysis. In total thirty samples of each category of positive confirmed individuals, each category represented specific banding pattern and 60-70 kda and 14 kda proteins were dominated (Table 2, Figures 1-7). In suspected individuals also thirty samples of each category were subjected to SDS-PAGE analysis and in hepatitis C category 20 %, alcoholism category 10 % and in CLD category 16.66 % cases have same serum protein profile as in case of positive control category (Table 2, Figures 1-7). The 60-70 kda protein was more prominent in cases of alcoholism and CLD subjects while 14 kda protein was dominated in hepatitis C cases. However in hepatitis patients 60-70 kda protein was also expressed but expression was not quite high as in case of alcoholism and CLD cases (Table 2, Figure 2). The 30 kda protein was uniformly expressed in all groups except normal group where instead of 30 kda protein 18 kda protein is prominent. In normal subjects only 60-70 kda protein was expressed but its expression level is less as compare to expression level in alcoholic and CLD patients (Table 2, Figure 1-7).

The specific protein patterns in different cases viz. chronic liver disease (CLD), hepatitis and normal subjects were also observed on HPLC (Fig. 8-
indicating the variation in serum profile of patients with reference to normal subjects. The major peaks again correspond to 60-70 kda protein, 30 kda protein and 14 kda protein in HPLC analysis (Fig. 8-10). In CLD category 60 kda and 30 kda proteins peaks are more dominant while in HCV category 14 kda protein peak is more dominant however 60 and 30 kda proteins are expressed with no significant difference (Fig. 8-10).

**Table. 2** Identification of specific marker proteins in different categories of individuals.

<table>
<thead>
<tr>
<th></th>
<th>Hepatitis C (30)</th>
<th>Alcoholism (30)</th>
<th>CLD (30)</th>
<th>Normal (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Confirmed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60-70 kda (+++)</td>
<td>60-70 kda</td>
<td>60-70 kda (+++)</td>
<td>60-70 kda (+++)</td>
<td></td>
</tr>
<tr>
<td>30 kda (+++)</td>
<td>(++)</td>
<td>(++)</td>
<td>(++)</td>
<td></td>
</tr>
<tr>
<td>14 kda (+++)</td>
<td>30 kda (+++)</td>
<td>30 kda (+++)</td>
<td>14 kda (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 kda (+)</td>
<td>14 kda (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Suspected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(on the basis of biochemical tests)</td>
<td>6/30 (20 %)</td>
<td>3/30 (10 %)</td>
<td>5/30 (16.66 %)</td>
<td></td>
</tr>
</tbody>
</table>

**Key:**  
+++ = Very dark band.  
++ = Light dark band.  
+ = Very dim band in few samples only.
3. Isolation of specific proteins for in-vitro assay
The 30 kda protein as well as 14 kda protein was specifically purified from SDS-PAGE gel for further characterization in in-vitro assay. The purification quality of protein was checked by doing SDS-PAGE of purified proteins again. For in-vitro bioassay filter sterilized neem extract as well as purified interferon alpha 2b was used against purified proteins. The purified proteins (50 µg) each were incubated along fixed amount of neem (50 µl concentrated from 1 g of leaf tissue) and interferon alpha 2b protein (50 µg) at 37 °C for 3 hours and there was observed some variation in 30 and 14 kda band intensities specifically in case of neem extract (Figure 6, 7) however there was observed no variation in case of interferon alpha 2b case (Figure 6, 7). This indicates that neem extract has some enzymatic/chemical activity as a result of which 30 and 14 kda proteins were partially degraded.

4. RT-PCR analysis for the detection of HCV
The reverse transcriptase PCR was performed on obtained blood samples of various categories of the patients viz., CLD, alcoholism and HCV patients of both suspected and confirmed groups (Table 3; Figure 11, 12). The highest percentage prevalence of HCV in confirmed category was observed in CLD group (26.66 %) however in alcoholic group 23.44 % percentage was observed. In suspected category the highest percentage prevalence was
observed in HCV group (23.33 %) while lowest percentage was found to be in CLD group (20.0 %) however in alcoholic subjects percentage is quite low i.e. 16.66 % (Table 3; Figure 11, 12).

**Table. 3** Detection of HCV in different categories of subjects through RT-PCR.

<table>
<thead>
<tr>
<th></th>
<th>Hepatitis C (30)</th>
<th>Alcoholism (30)</th>
<th>CLD (30)</th>
<th>Normal (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Confirmed</strong></td>
<td>30/30 (100 %)</td>
<td>7/30 (23.44 %)</td>
<td>8/30 (26.66 %)</td>
<td>0/30 (0.00 %)</td>
</tr>
<tr>
<td><strong>Suspected</strong></td>
<td>7/30 (23.33 %)</td>
<td>5/30 (16.66 %)</td>
<td>6/30 (20.0 %)</td>
<td></td>
</tr>
<tr>
<td>(on the basis of biochemical tests)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: SDS-PAGE analysis of normal individuals serum samples.

Key: 1-11 = Individuals serum samples.  
M = Protein marker.
Figure 2: SDS-PAGE analysis of serum samples from HCV subjects.

Key: 1-6, 9-11 = HCV suspected subjects.
    7, 8 = HCV confirmed controls.
Figure 3: SDS-PAGE analysis of serum samples from CLD subjects.

Key: 1-3, 5-7, 9-11 = CLD suspected subjects.
4,8 = CLD confirmed controls.
Figure 4: SDS-PAGE analysis of serum samples from ALD patients.

Key: 2, 3 = ALD confirmed patients.
1, 4-11 = ALD suspected subjects.
Figure 5: SDS-PAGE analysis of patients serum samples against nim extract and IFN alpha2b

Key:
1 = Normal + nim.
2 = Normal + IFN alpha2b.
3 = Abnormal + Nim.
4 = Abnormal + IFN alpha 2b.
5 = Normal only.
6 = Abnormal (HCV) only.
Figure 6: SDS-PAGE analysis of 30 kda protein sample against nim extract and interferon alpha 2b.

Key:  2 = 30 Kda Protein and nim extract  
      3 = 30 Kda Protein and IFN alpha 2b.  
      5 = 30 Kda Protein.  
      6 = IFN alpha 2b protein.
Figure 7: SDS-PAGE analysis of 14 kda protein sample against nim extract and interferon alpha 2b.

**Key:**
1 = 14 Kda Protein and nim extract  
2 = 14 Kda Protein and IFN alpha 2b.  
3 = 14 Kda Protein only.  
4 = IFN alpha 2b protein only.
Figure 8. HPLC analysis of ALD patient blood serum.
Figure 9. HPLC analysis of HCV patient blood serum.
Figure 10. HPLC analysis of normal blood serum
Figure 11. HCV nucleic acid RT-PCR from human blood.

M = DNA marker
1, 2, 3 = Experimental samples of human blood serum containing HCV RNA.
Figure 12 (A). Nucleic acid isolation from blood containing HCV
M = DNA marker
1, 2, 3 = Experimental blood samples
Figure 12 (B). Reverse transcriptase PCR analysis of HCV from whole blood.

M = DNA marker
1, 2 = Experimental blood samples containing HCV.
Figure 12 (C). Nested PCR of HCV RNA under different dilutions.

- M = DNA marker
- 1, 2, 3 = Nested PCR after 1:10 dilution of RT-PCR 296 bp product.
- 4 = Nested PCR after 1:100 dilution of RT-PCR 296 bp product.
DISCUSSION

The recent research project has significance because in developing country like Pakistan liver problem viz. chronic liver disease (CLD) due to hepatitis C virus infections and alcoholism is continuously going to increase. Most of the people donot know that when disease had started and only come to hospitals when disease is at later stages so there is need to diagnose this disease at early stages by identifying the specific marker proteins in serum of patients as well as by molecular tests so that we can estimate the HCV load in person’s serum. Confirmed as well as random suspected samples on the basis of risk analysis were collected from various localities of Lahore and subjected to biochemical analysis. In biochemical analysis the suspected individuals of HCV, alcoholism and CLD categories showed variation with reference to each other. In biochemical tests in suspected category of each group the percentage of positive cases is variable i.e 23.33 %, 20.0 % and 13.33 % for HCV, alcoholism and CLD were found to be positive for each category. This percentage of positive cases is quite high as already reported (Chander et al., 2002; NIH report 2002) but in agreement with the percentages as described by (Scott and Garland, 2008).
The samples which were found to be positive in biochemical tests were further subjected to SDS-PAGE analysis for the identification of specific marker proteins. These marker proteins have significance regarding the early diagnosis of this disease as reported by various workers in different diseased states (Fouzia et al., 2001; Bunout, 1999). In each specific protein case level of expression was raised as well as declined in other state however these three proteins were more prominently affected in different disease states and include 14 kda, 30 kda and 60-70 kda proteins.

The 60-70 kda protein was prominent in all the three categories of subjects however its level of expression in quite high in case of positive individuals of CLD and alocholism groups who found to be positive earlier in biochemical tests. In normal individuals this 60-70 kda protein also present but its level of expression is quite low as compare to diseased groups. As serum albumin and globulin fall in the same size range therefore this protein is serum albumin and its raised level of expression is an indication of abnormal liver activity as indicated by various workers (Jacoby and Cole, 2000, Fouzia et al., 2001; Bunout, 1999).

The level of expression of 30 kda protein is almost same in all the three categories however the 14 kda protein expressed in variable way in different groups of subjects. The maximum expression of 14 kda protein was
observed in subjects suffering from HCV however this protein expression was significantly declined in alcoholism and CLD groups. This protein can also act as marker protein indicating the improper functioning of liver. This protein was not observed in healthy individuals. In literature there are some reports indicating the presence of these proteins with different molecular weights in some diseased states (Dufour et al., 2000; Schroter et al., 2001).

In healthy individuals 19 kda protein was also observed along 60 kda protein. This 19 kda protein was not present in other categories of subjects and very low intensity band of this protein observed in some HCV cases. This 19 kda protein is perhaps human interferon alpha 2 because its size is equivalent to reported size of human IFN alpha2. This protein activated in persons in response to viral infections and thus protect the subjects from further damage caused by viruses including HCV and other viruses of retroviridae as described by (Watanabe, 2004; Gines et al., 2003).

In HPLC analysis of serum proteins of alcoholism and HCV categories two and three major peaks were observed respectively. In alcoholism category the first major peak corresponds to 60 kda albumins while second peak represents 30 kda protein. However in HCV category in addition to first two peaks which correspond to 60 and 30 kda proteins, a third major peak was also observed which indicates14 kda protein. The HPLC analysis was used
for serum proteins analysis because various other workers also used the same technique for serum proteins analysis (El-ghaffar and Assad, 1967; Wallach, 2000).

The 30 kda protein as well as 14 kda protein was specifically purified from SDS-PAGE gel for further characterization in in-vitro assay. The purification quality of protein was checked by doing SDS-PAGE of purified proteins again. For in-vitro bioassay neem extract as well as purified interferon alpha 2b was used against purified proteins. The purified proteins (50 µg each) were incubated along fixed amount of neem and interferon alpha 2b protein (50 µg) at 37 °C for 3 hours and there was observed some variation in 30 and 14 kda band intensities specifically in case of nim extract however there was observed no variation in case of interferon alpha 2b case. This indicates that nim extract has some enzymatic/chemical activity as a result of which 30 and 14 kda proteins were partially degraded. The nim had already reported medicinal importance in certain viral infections like HCV (Veitch et al., 2008) and this research finding to some extent in favor of this point of view but there is need to further research on this issue to completely sort out the point at molecular level.

In case of IFN alpha 2b the 14 kda and 30 kda proteins intensities remained unchanged and this is perhaps due to fact that IFN alpha 2b protein domains
have no enzymatic activity and in-vivo systems it acts as a signal protein which alarms the cells after entry of viruses in the body so that further damage of cells could be prevented. This view is also supported by (Takaoka et al., 2003).

For HCV detection in suspected as well as confirmed cases of all three categories, RT-PCR was carried out. In confirmed cases of alcoholism and CLD the percentage prevalence of HCV was found to be 23.44 % and 26.66 % while is suspected group in case of both categories decline of percentage prevalence was observed of both above mentioned categories that is 16.66 % and 20.00 %. In case of HCV suspected group category the percentage prevalence was find out 23.33 %. HCV is major cause of CLD in world world (Yano et al., 1996) therefore its high prevalence rate can be expected in our area, which is so for part of a developing country.

The recent research project has importance because it describes not only the specific serum proteins as marker proteins in different diseased states like HCV, alcoholism and CLD but also provides information regarding the involvement of HCV in suspected as well as confirmed groups of these categories. The high percentage prevalence of HCV in all the three suspected and confirmed categories of disease states is a reflection of need to control and cure the HCV infection after proper early diagnosis either
through serum proteins analysis or through molecular analysis by RT-PCR so that further liver damage could be prevented.
References


55. Lindsay K, Trepo C and Heintges T (2001). A randomised, double blind trial comparing pegylated interferon alfa-2b to interferon alfa-


