Review of Literature
2. REVIEW OF LITERATURE

2.1 HISTORY

Treatment of haemophiliacs with Factor VIII concentrates (F VIII) is known to be associated with a high risk of PT-NANB hepatitis, presumably due to the inclusion of one or more infected donor plasma units in the large pools of plasma used by commercial firms to prepare F VIII concentrates (Craske et al., 1975). The early identification of three lots of F VIII concentrate implicated in the transmission of disease to two individuals in 1977 led to the recovery of proven-infectious materials, which was subsequently used for identification of virus-like particles by immune electron microscopy and possible transmission of disease to nonhuman primates. The cloning of HCV was initiated by ultracentrifugation of large volumes of chimpanzee plasma that had been shown to have an unusually high infectivity titre ($10^6$ chimp infectious dose/ml). Nucleic acid was then extracted from the centrifuged pellet. Because it was not known if the virus was DNA or RNA, a denaturation step was included before the synthesis of complementary DNA (cDNA), so that either DNA or RNA could serve as a template: conversion of RNA into cDNA involved reverse transcription using random primers. The resultant cDNA was inserted into a cloning vector λgt11, and expressed in Escherichia coli (Choo et al., 1989). After lysis of the bacteria, expressed proteins that had adhered to an overlying filter were immunoscreened with serum from a patient who had NANB hepatitis and then with radio-labelled antiglobulin. A single clone
(5.1-1) among millions tested was reactive by autoradiography. A larger clone, c-100-3, was assembled from several overlapping clones and expressed in yeast as a fusion protein using human superoxide dismutase (SOD) to facilitate expression. The c-100-3 antigen has become the basis of licensed solid-phase immunoassays that detect the complementary antibody in HCV carriers (Kuo et al, 1989; Bradley et al, 1991).

2.2 THE VIRUS

The characteristic feature of HCV is that it is a virus that has been cloned but never visualised (Fig.2.1). The genomic data suggests that HCV is a single-stranded, positive-sense RNA virus, distantly related to the arthropod-borne flaviviruses -flaviviridae group (Miller and Purcell, 1990). The RNA is bound to a nucleocapsid and the envelope is a glycoprotein. The HCV genome comprises around 9379-9481 nucleotides in length containing a single large open reading frame (orf) which is translated to yield a polyprotein from which the viral proteins are derived (Fig.2.2). There is a short untranslated region (UTR) at the 5' end of 341 nucleotides and a 3' UTR of 48 nucleotides. The structural proteins are located towards the 5' termini and the non-structural (NS) proteins towards the 3' end (Okamoto et al, 1990). The first product of the polyprotein is the non-glycosylated nucleocapsid protein, C, which complexes with the genomic RNA to form the nucleocapsid. Adjacent are two domains, E1 and E2/NS1 apparently encoding two glycosylated envelope proteins, gp33 and gp72. The E1 and E2/NS1 regions encode proteins that may be present on the surface of the infected cell and may be important antigenic
Figure 2.1. Schematic representation of the HCV
Fig. 2.2 Schematic organisation of HCV genome and encoded proteins.

- aa = amino acid number, nt = nucleotide, e = core, e = envelope, ns = non-structural; MBF = membrane binding function.
determinants. The non-structural proteins encoded by the NS2, NS3, NS4 and NS5 regions may include a helicase, protease and polymerase (Hijikata et al, 1991).

The replication mechanism of HCV is poorly understood. Like in flavi- and pestiviruses, it appears that HCV-RNA is replicated by a direct RNA-to-RNA mechanism. It has been impossible to detect DNA intermediates in serum or liver of infected individuals. The NS5 region encodes a protein with demonstrated RNA-dependent RNA polymerase activity (Chung et al, 1992). Antigenomic (minus) RNA strands have been detected in the liver (Takehara et al, 1992) and plasma (Fong et al, 1991; Shindo et al, 1992) of patients. Due to technical difficulty, detection of minus-strand RNA should be interpreted with care (Willems et al, 1993). As antigenomic strand synthesis should start at the 3' terminus, some small repeated 6-8 base pair (bp) sequences, present in both the 5' and 3' UTRs may be involved in secondary structure formation or cyclisation of the RNA genome (Inchauspe et al, 1991). The secondary structures of the 5' UTRs of HCV, bovine viral diarrhoea virus (BVDV) and hog cholera virus (HoChV) have been determined by thermodynamic modelling and enzymatic cleavage of RNA by specific ribonucleases (Brown et al, 1992). These studies revealed the presence of a large conserved stem-loop structure in the proximal part of the 5' UTR, which serves as a putative internal ribosome entry site (IRES). Studies using mono- and di-cistronic RNA constructs suggested the presence of IRES in the HCV 5' UTR (Wang et al, 1993), although other reports are contraindicatory (Yoo et al, 1992). The function of 3' UTR is less well known but is probably important for the initiation of RNA replication.
2.3 GENOTYPES OF HCV

During replication of the viral genome by the NS5-encoded RNA-dependent RNA polymerase, which is an error-prone enzyme lacking proofreading activity, each replication cycle generates mutants. Some mutations are tolerated whereas others may abolish or hamper proper functioning of the resulting viral RNA. By this method of "error and trial", variant RNA genomes evolve. These mutants are then selected on the basis of fitness within the host (quasispecies) and diverge over time within the human population (genotypes) (Oshima et al., 1991; Murakawa et al., 1992; Tanaka et al., 1992).

The first full-length HCV sequence was derived from a chimpanzee infected with a contaminated American F VIII concentrate. This sequence became known as the HCV prototype, HCV-US or HCV-1, to which each new sequence is compared (Houghton et al., 1990; Choo et al., 1991). Later, two highly similar full-length sequences from two Japanese HCV isolates were shown to be significantly different from the prototype (Kato et al., 1990; Takamizawa, 1991). The different strains were provisionally designated as "American" and "Japanese" strains. Since then, several full-length (Okamoto et al., 1991; Tanaka et al., 1992; Chen et al., 1992) and numerous partial nucleotide sequences (Kubo et al., 1989; Enomoto et al., 1990; Mori et al., 1992) from isolates collected worldwide were reported. Comparison of various HCV sequences (Kubo et al., 1989; Simmonds et al., 1990; Li et al., 1991; Tsukiyama et al., 1991) revealed the presence of similar viral variants in different areas of
the world, and initiated attempts to classify the HCV isolates into distinct groups. Initial classifications were based on various genomic regions, and the associated nomenclatures using individual isolate names were confusing.

Based on all sequence data, a phylogenetic tree containing four branches was proposed, comprising types I-IV (Okamoto et al, 1992). This tree was extended with new branches representing types V and VI by HCV isolates from Thailand (Mori et al, 1992) and similar European isolates (Chan et al, 1992). This provisional classification system was based on history and did not properly reflect the relationship between sequences.

Recently, a new classification system (Fig.2.3) was proposed based on sequence alignments of a large number of HCV sequences derived from several parts of the genome (Chan et al, 1992; Stuyver et al, 1993; Simmonds et al, 1993; Simmonds et al, 1994). Construction of phylogenetic trees revealed that comparison of 5' UTR, core, NS3 or NS5 resulted in similar classifications of HCV isolates into types and subtypes (Chan et al, 1992). Isolates belonging to the same subtype show an average nucleotide homology of > 90 %, those belonging to different subtypes approximately 80 %, and isolates of different types less than 70 % (Weiner et al, 1991). The proposed nomenclature is more functional as it is associated with the degree of homology between sequences, and new (sub)types can easily be fitted into the system.
Figure 2.3. Genotypes of HCV.
Unrooted maximum likelihood tree inferred from nucleotide sequences of the complete core region. All branch lengths are drawn to scale.
The geographical distribution of HCV genotypes is the subject of current investigations. Genotypes 1a, 1b, 2a, 2b and 3a are the most common genotypes found in the United States, Scotland, Finland, The Netherlands and Australia (van Doorn, 1994, Lau et al, 1995). Types 2 and 3 are not found in the Eastern European countries (e.g. Hungary). Types 1b, 2a and 2b are the most commonly found HCV genotypes in Japan and Taiwan. Type 3 is prevalent in Bangladesh and other parts of Asia. Type 4 is found mostly in South Africa and type 6 is found mostly in Hong Kong and Macau (Dusheiko et al, 1994; McOmish et al, 1994; van Doorn, 1994; Martin et al, 1995; Simmonds et al, 1995) In India, two major HCV genotypes have been reported-type 1 and type 3. Type 1 is the predominant genotype in Southern India whereas type 3 predominates in the northern parts of the country (Valliammai et al, 1995, Panigrahi et al, 1996) Currently genotyping can be performed by type-specific PCR primers (Okamoto et al, 1992), direct sequencing (Kleter et al, 1994), restriction fragment length polymorphism (RFLP) (Nakao et al, 1991), Southern blot hybridisation with type-specific probes (Enomoto et al, 1990; Takada et al, 1993), or reverse hybridisation to (sub)type specific probes (Stuyver et al, 1993). 11 genotypes and more than 70 subtypes have been reported so far (Fig.2.4), using the techniques mentioned above (Bernier et al, 1996).

2.4 SEROTYPES OF HCV

Sequence variation may influence the antigenic properties of specific epitopes. It had been shown that type 1-derived NS3 and NS4 antigens are not
Figure 2.4. Nomenclatures for HCV genotyping.

<table>
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<th>Representative sequence</th>
<th>Sturver</th>
<th>Bukh Purcell</th>
<th>Chan/Simmonds</th>
<th>Chu Ludes</th>
<th>Enomoto Date</th>
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adequately recognised by antibodies from patients who are infected with non-type 1 HCV genotypes (Chan et al, 1991; McOmish et al, 1993; McOmish et al, 1994; van Doorn et al, 1994). Similarly, anti-E1 envelope antibodies to recombinant type 1b E1 protein were more prevalent in type 1a and 1b HCV sera than in type 2, 3 and 4 HCV sera (Maertens et al, 1994). These findings initiated the exploration of possible serotyping assays and on the basis of branched-type-specific oligopeptides, serotyping assays have been developed. At first, these assays allowed detection of only types 1 to 3, but it has recently been extended to types 4, 5 and 6 (Bhattacheree et al, 1995; van Doorn et al, 1996).

2.5 PREVALENCE

Hepatitis C is an ubiquitous disease infecting over 300 million persons worldwide. Serologic testing for anti-HCV antibodies in healthy population (mostly blood donors) suggests that prevalence rates are low in Northern Europe and the USA, slightly higher in Southern Europe and Asia, and highest in Africa (Fig.2.5). In one Italian study, however, a population of factory workers had an anti-HCV positivity rate of 10 %—nearly 100 times higher than that seen in the blood donor population Caselmann and Alt, 1996).

In a recent study, conducted by Nkengasong et al (1995), anti-HCV positivity was found to be 27.5 % in Southern Cameroon. Previously, Aceti and Taliani (1992) showed 25.6 % HCV seropositivity, Louis et al (1994) had reported a HCV-seropositivity of 12.5 % and Kowo et al (1995) had shown 13 % seropositivity among blood donor population of Africa. However, a low
Figure 2.5. Worldwide relative frequency of anti-HCV antibody as determined from healthy individuals and blood donor populations.
seroprevalence of 2 % has been reported by Frommel et al (1993) in a study conducted in Ethiopia. Likewise, there has been reports from Egypt with a high HCV seroprevalence rates. Bassily et al (1995) in his study had shown a HCV seropositivity of 26.6 % among Egyptian blood donors. The high prevalence of HCV in these countries has been hypothesised to be due to ritual tattooing, use of non-disposable or inadequately sterilised medical equipment, intravenous drug abuse and probably due to an arthropod vector.

The relative anti-HCV antibody frequencies in normal individuals or blood donors vary from 0.02 % to 0.42 % in Northern Europe. The corresponding figures being 0.02 % for Finland, 0.12 % for Scotland, 0.08 % for Netherlands, 0.06 % for the United Kingdom and 0.42 % for Germany (Krauledaat, 1989; Alter, 1991; van der Poel, 1993; Esfahani et al, 1995). The seroprevalence is found to be low, between 0.3 % - 0.5 %, in the United States of America as well (Aoki et al, 1993), although higher seroprevalence rates have been reported previously by Dawson et al, 1991 and Smalligan et al, 1995. In the study conducted by Dawson et al in US blood donors, it was seen that anti-HCV was positive in 0.8 % of voluntary blood donors and 10.49 % of paid blood donors. Smalligan et al had reported a 0.6 % HCV prevalence among American missionaries.

The seroprevalence of HCV in Southern Europe and Asia is in the medium range. Countries like Spain and Italy have reported a HCV seroprevalence of 0.5-0.85 % and 0.87 % respectively (van der Poel, 1993). The HCV seroprevalence was found to be 0.78 % among Australian blood donors as well (Allain et al, 1991).
Among the Asian countries India has reported a higher HCV-seroprevalence than other countries. The seroprevalence of HCV is seen to a tune of 0.95 % in Taiwan, 2.3 % in Indonesia, 0.8 % in Thailand and 1.3 % in Japan (van der Poel, 1993; Luengrojanakul et al, 1994; Tamura et al, 1992). The seroprevalence of HCV among blood donors in Northern India was reported to be 2.7 % by Panda et al, 1996. However, the HCV seroprevalence was found to be 0.48 % in the southern parts of the country (Abraham and John, 1995).

2.6 TRANSMISSION AND RISK GROUPS

Hepatitis C is spread by parenteral transmission of body fluid, mainly blood or blood components. Consequently intravenous injections or transfusions are the main cause of HCV transmission. In the developed countries, haemophiliacs, hypogammaglobulinaemic patients, IVDUs and haemodialysis patients are at highest risk for hepatitis C infection (Alter, 1990; Alter, 1991; Main, 1995).

2.6.1 Parenteral Transmission

Prior to current inactivation procedures, clotting factor concentrates were the highest risk blood product and it is now apparent that 60-90 % of haemophiliacs are anti-HCV positive (Alter, 1990; Brettler, 1990; Alter, 1991a). Haemophiliacs treated with only virus-inactivated coagulation components or with single donor cryoprecipitate were generally anti-HCV negative in a study by Epstein and Fricke, 1990. Studies conducted in Spain, Germany, Egypt and
USA have shown that the prevalence of anti-HCV was very high in patients who received transfusions, with rates of 60 % - 90 % in patients with chronic-transfusion-associated non-A, non-B hepatitis (Esteban et al., 1989; Roggendorf et al., 1989; Alter et al., 1991b; El-Zayadi et al., 1992). The introduction of screening of blood products has reduced the incidence of post-transfusion hepatitis but cases still occur and there has been recent concern following an outbreak of acute HCV infection following administration of immunoglobulin (Bader, 1994). HCV infection was also found to be high among haemodialysis patients, with the positivity rates ranging from 18 % to 74.2 % among various countries (Sakamoto et al., 1993; Viola et al., 1993; Okuda and Hayashi, 1996) and exceeding 90 % in some Eastern European countries (Alter, 1995). It has also been shown that anti-HCV positivity was directly proportional to the volume of blood transmitted and the duration of dialysis (Okuda and Hayashi, 1996).

The prevalence of anti-HCV was also high, 40-90 %, in patients with percutaneous exposure through intravenous drug abuse, mainly by shared hypodermic needles and syringes (Alter, 1991a; Alter MJ, 1991; Alter, 1995). Health care professionals exposed to accidental inoculation with infected blood represent another risk group (Jochen, 1992; Herbert et al., 1992). In a Japanese study, 5 % of subjects with a history of needle stick injuries involving HCV-infected patients showed evidence of HCV infection (Kiyosawa et al., 1991).
Transmission of HCV has also been reported due to tattooing needle by Abildgaard et al, 1991. Anti-HCV antibodies and HCV-RNA have been detected in Urine (Morris et al, 1992), oral fluid (Thieme et al, 1992) and tears (Feucht et al, 1995). Transmission of HCV infection by a human bite has been documented (Dusheiko et al, 1990). It is not yet clear whether HCV infection can be transmitted by kissing.

2.6.2 Sexual Transmission

Sexual transmission of HCV is thought to occur but at a much lower rate than hepatitis B or HIV (Krauledaat, 1989; Esteban et al, 1989; Main, 1995). Before anti-HCV testing, it was thought likely that heterosexual transmission played an important role in the spread of NANB hepatitis. Later studies involving tests for HCV suggest that this is unlikely. Although sexual transmission of HCV is known to occur, it seems relatively uncommon in both heterosexuals and homosexuals, and one study failed to find the virus in semen samples (Morris et al, 1992). However, there does appear to be a tendency for patients with many sexual partners to have an increased risk for HCV infection and sexual transmission may occur when the index case has a high viral burden (Riestra and Carcaba, 1991).

2.6.3 Perinatal Transmission

Perinatal transmission of HCV may represent an important means by which high HCV carrier rates are maintained in some areas, and has been clearly demonstrated using PCR to compare viral genotypes (Alter, 1991c;
Inoue et al., 1991; Nishiguchi et al., 1992a). Nucleotide sequencing of the NS5 region of HCV isolates from Japanese mothers and their children has shown 100% similarity, while variation in this region of the HCV genome in other Japanese patients has been reported as around 10% (Nishiguchi et al., 1992a).

### 2.6.4 Other modes of Transmission

It has been seen that 20-40% of anti-HCV cases do not have any of the above mentioned risk factors (Alter, 1990; Alter, 1991). A possible role for insect vector transmission, in endemic areas such as Egypt, has not been excluded.

Intrafamilial transmission has been reported, although the degree of risk is yet to be confirmed (Nishiguchi et al., 1992b). One group of workers have suggested that intrafamilial transmission occurs when index patients have a high viral load (Riestra and Carcaba, 1991).

Transmission of HCV among multi-transfused thalassaemics, pregnant women and patients undergoing haemodialysis has been reported in studies from India by Arankalle et al. (1995) and Agarwal et al. (1993). Arankalle et al. have reported a 24.5% seropositivity for HCV among haemodialysis patients and 5.7% HCV positivity in multiply-transfused patients. They had also screened 430 pregnant women and 86 children below the age of 5 years and found that none of them were anti-HCV reactive. In the other Indian study of multi-transfused thalassaemics by Agarwal et al., it was seen that 16.7% of the 72 patients screened were found to be anti-HCV positive.
2.7 NATURAL HISTORY OF HCV INFECTION

2.7.1 Acute Hepatitis C

Only 5 to 10 % of acute hepatitis C is associated with symptoms or signs of acute hepatitis (Fig.2.6). Acute hepatitis C has an incubation period ranging from six to twelve weeks, although it may be as short as two to 24 days in patients acquiring infection from blood products, such as factor VIII; the average incubation period is seven to eight weeks (Alter, 1990; Alberti and Realdi, 1991; Main, 1995). While the symptoms of acute hepatitis A and B are similar, acute hepatitis C infection is far more likely to be mild or asymptomatic, with less-severe disease. In one study of transfusion-related acute NANB hepatitis, only 25 % of cases were icteric and less than 10 % were seriously ill. In hepatitis C, serum ALT levels may rise rapidly, upto 10 times the upper limit of normal, and then fall abruptly; may rise and stay at plateau; or (typically) may fluctuate over weeks or months (Alter MJ et al, 1992).

The timing of seroconversion to anti-HCV is very variable, hence most patients remain anti-HCV negative during the early phase of illness, although anti-HCV tests may be transiently positive immediately after the transfusion due to passive transfer of antibodies from the donor. In a few patients, anti-HCV antibodies appear as early as two to four weeks after the onset of hepatitis, while in others they do not appear until months after serum aminotransferase levels become elevated. Diagnosis of acute hepatitis C is still based largely on patient history, epidemiology and exclusion of other causes or PCR testing for HCV-RNA (Alberti and Realdi, 1991; Main. 1995).
Figure 2.6. Natural history and markers in chronic hepatitis C.
In India, the percentage positivity of HCV among transfusion-related AVH has been reported to be 12.5 % by Irshad and Acharya (1994); 25 % by Khuroo et al (1993) and 27 % by Mehta et al (1992). None of the patients with sporadic or epidemic NANB hepatitis were positive for anti-HCV.

2.7.2 Fulminant Hepatitis

The role of HCV in fulminant hepatic failure (FHF) has remained controversial. A study from Japan reported the presence of HCV-RNA or anti-HCV antibody among most patients with FHF (Yanagi et al, 1991), whereas studies from France (Feray et al, 1993), the United States (Wright, 1993) and the United Kingdom (Sallie et al, 1991) have not been able to implicate HCV as an important cause of FHF. In an Indian study by Acharya et al (1996), it was seen that HCV-RNA alone was present in 7 cases and HCV-RNA along with HEV-RNA was seen in 11 cases among 50 NANB-FHF cases tested. This indicated that either HCV infection caused the disease or a HCV carrier state made these patients more susceptible to another hepatotropic viral infection. In other reports from India by Irshad and Acharya (1994) and Arankalle et al (1995) it was seen that HCV was positive in 45 % and 7.9 % of FHF cases respectively.

2.7.3 Chronic hepatitis C

The characteristic feature of HCV infection is the large number of patients, an estimated 50-80 %, who develop the chronic form of the disease, defined as raised liver enzymes and anti-HCV positivity for more than 6
months and/or histological findings consistent with chronic liver inflammation (Esteban et al., 1991; Muller et al., 1996). There is little tendency for spontaneous recovery and, although the majority of patients are asymptomatic during the first 10 years after infection, the risk of developing cirrhosis and HCC is relatively high. The initial stage of the chronic disease is classified histologically as chronic persistent hepatitis (CPH). This stage may persist for as long as 20 years before conversion to chronic active hepatitis (CAH) and ensuing late sequelae such as cirrhosis and eventually HCC (Alter, 1990; Main, 1995; Muller et al., 1996).

Chronic hepatitis C rarely causes obvious distress to the patient and the appearance of jaundice is characteristic of advanced disease. Serum ALT level tend to fluctuate over months or years in chronic hepatitis C, generally ranging between two and eight times the upper limit of normal. Although anti-HCV positive patients with persistently normal serum ALT levels are usually considered "healthy" carriers of the virus, recent evidence suggests that this may not be the case (Esteban et al., 1991; Muller, 1996). In fact, HCV-RNA levels may be better predictors of disease severity. An Italian study using PCR for detection of HCV-RNA had shown that HCV-RNA-positive subjects invariably have liver disease independently of serum transaminase levels (Fischer et al., 1996).

Chronic hepatitis C has a distinct liver pathogenesis and can be differentiated from type B hepatitis and autoimmune hepatitis by liver histology (Bach et al., 1991). Portal lymphoid aggregates and lymph follicles
that consist mainly of B lymphocytes are far more frequent in hepatitis C than in hepatitis B or autoimmune hepatitis. Parenchymal steatosis which is thought to be a cytopathic effect of the HCV, is present in more than 50% of liver biopsies with hepatitis C, far more frequently than in hepatitis B and autoimmune hepatitis. Hepatitis-associated bile duct lesions are found far more often in hepatitis C than in hepatitis B (Bach et al, 1991; Lefkowitch et al, 1993).

The prevalence of hepatitis C virus among Chronic liver diseases (CLD) in India has been reported to be between 12.5% to 48.5% (Amarapurkar et al, 1992; Mehta et al, 1992; Khuroo et al, 1993; Irshad and Acharya et al, 1994; Panigrahi et al, 1994; Issar et al, 1995)

2.7.4 Hepatocellular carcinoma

Epidemiologic studies have shown a strong link between chronic NANB hepatitis and HCC, and this link has been confirmed by the high prevalence of anti-HCV antibodies among patients with liver cancer. In Europe, the USA and Japan, HCV may be more important than HBV in this context (Epstein and Fricke, 1990; Main, 1995; Caselmann and Alt, 1996).

The difference in HBV-induced HCC and HCV-induced HCC is that although the HBV lacks an oncogene it is capable of integrating its DNA into human cellular DNA, including chromosomal rearrangements or insertional mutagenesis, whereas the HCV is a non-integrating agent which predisposes to HCC through cirrhosis. In a study comparing the gross pathology of
HBV-seronegative and seropositive HCCs (Okuda et al, 1984), the expanding type was found in 82% of the former and only 36.8% of the latter, while the other multi-nodular/spreading types were more frequent in the latter (58% vs. 18%). Majority of juvenile patients with HCC are positive for HBsAg, presumably acquired from the mother. HCV infection is very uncommon before 15 years of age. HBsAg-positive HCC patients are generally younger than HBsAg-negative patients, and the majority of the latter are now known to have HCV infection (Okuda et al, 1984).

2.7.5 HCV and autoimmune hepatitis

A number of studies have reported anti-HCV positivity in patients with autoimmune hepatitis. There seems a strong case for an association between HCV infection and expression of anti-liver-kidney-microsomal -1 (anti-LKM-1) antibody, considered characteristic of autoimmune type 2 hepatitis (Epstein and Fricke, 1990; Miyakawa and Kako, 1992; Main, 1995). Serologic evidence of HCV infection was found in over 80% of Italian patients with anti-LKM (Lenzi et al, 1990). Japanese authors (Miyakawa et al, 1994) have identified a peptide (GOR) which is coded by a human gene; they have also sequenced the corresponding epitope. Interestingly, as GOR shares an amino acid sequence with a peptide (cp 10) coded by the core region of HCV, autoantibodies against GOR are formed in the course of HCV infections. A German study has suggested that anti-GOR positivity may reflect HCV-specific autoimmunity, and that HCV can induce both anti-GOR and anti-LKM-1 antibodies (Michel et al, 1992). In this study, patients with autoimmune hepatitis who were
anti-HCV positive/anti-GOR-positive were significantly older and less likely to be female than anti-HCV-negative/anti-GOR-negative patients. They also had lower disease activity and significantly lower anti-LKM-1 titres, and responded well to immunosuppression.

2.8 DIAGNOSIS

The diagnosis of HCV infection is done by either the exclusion tests and surrogate markers testing or the much reliable HCV antibody and RNA testing.

2.8.1 Surrogate markers

Initial reports have stressed the importance of ALT testing and anti-HBc IgM testing for the diagnosis of HCV infection, as elevated ALT levels and anti-HBc IgM positivity correlated well with anti-HCV positivity (Alter et al., 1981). Later studies have shown the insignificance of these surrogate marker testing and have shown anti-HCV to be a reliable marker (Barrera et al., 1991).

2.8.2 Antigen detection

Visualisation of complete HCV virions has been extremely difficult primarily because of the low titre of the virus in clinical samples and this has a direct effect on antigen detection as even the antigenaemia is equally low. Currently, HCV antigens are detected only in the liver biopsies by using immunohistochemical techniques (Infantolino et al., 1990) or by immunoperoxidase electron microscopy (Shimizu et al., 1995). These tests are
done at a research level and commercially there are no antigen-detection systems available for HCV.

2.8.3 Antibody detection

Anti-HCV is detected by ELISA and an array of supplemental or confirmatory tests (Fig.2.7) (Younossi and McHutchison, 1996).

2.8.3.1 ELISA

The enzyme-linked immunosorbent assay has been the mainstay screening test for the detection of antibodies to HCV. Not only is the technique fairly simple, it is easily reproducible, simple, inexpensive and can be automated and performed on large number of specimens without difficulty. Obviously, this type of system has advantages particularly in screening large populations, such as blood donors. The first generation (Gen.) ELISA for anti-HCV utilised the c-100-3 antigen to capture the antibody. While serum anti-c100 was found to be good marker for chronic HCV infection in transfusion-related and sporadic NANB hepatitis, there was a high false-positive rate among low-risk populations, namely blood donors. Stored samples and patients with hypergammaglobulinaemia also gave spurious results.

Subsequent refinement of the I Gen.ELISA led to the introduction of a II Gen.ELISA which differed from the I Gen. assay in its ability to detect antibodies to proteins derived from three distinct regions of the HCV genome.
namely the c22-3, c33c and c200 (a composite antigen). Anti-c33 and anti-c22 antibodies not only occur more frequently in serum than anti-c100, but also appear earlier in the course of the disease and thus may be useful in identifying acute as well as chronic infection in some patients (Vernelen et al, 1994; Buffet et al, 1994).

More recently, a third generation test incorporating the recombinant antigens from the NS5 region to detect the corresponding antibody, in addition to being able to detect antibodies against antigens from the core, NS3 and NS4 regions of the HCV genome has been made available commercially. In this III Gen.ELISA some of the antigens have also been reconfigured to enhance their sensitivity (Buffet et al, 1994).

Attempts have been made to develop tests to detect anti-HCV antibodies in the early phase of acute infection. An interest in IgM antibodies to HCV structural and non-structural proteins has also more recently been evaluated. IgM class antibodies to the c100 antigen have been evaluated in the setting of acute infection with somewhat conflicting results. The presence or disappearance of IgM core antibody has also been suggested as a possible marker to predict response to interferon therapy for individuals with chronic HCV infection.

2.8.3.2. Supplemental Assays

Along with the introduction of the initial ELISA-based systems and the need to diagnose HCV infection accurately, confirmatory or supplemental
assays were soon introduced. Out of the commercially available supplemental assays (Fig. 2.8), the most commonly used assay is the recombinant immunoblot assay (RIBA) which is a nitrocellulose-strip based assay with HCV antigens coated onto it.

The I Gen.RIBA incorporated the recombinant HCV antigens (the original isolated clones) 5-1-1 and c-100-3 but was soon replaced by the II Gen. RIBA (RIBA 2.0), even before it could be licensed.

The RIBA 2.0 incorporated the HCV recombinant antigens 5-1-1, c100-3, c33c and c22-3, coated onto the nitrocellulose strip. Reactivity to two or more bands was considered as anti-HCV reactive. Samples reacting with only one of the 4 bands was considered indeterminate.

Recently a III Gen. RIBA (RIBA 3.0) has been introduced which incorporated an additional NS5 recombinant antigen to its existing c100-3, c33 and c22 antigens. 5-1-1 has been excluded in this assay. This assay has been introduced and used throughout Europe and Asia but is not as yet licensed in the USA. The significance of this assay is that it resolves the RIBA 2.0 "indeterminate" into clear-cut positives or negatives. The increased sensitivity of this assay is not due to the addition of NS5 but due to increased reactivity to the reconfigured c33 antigen (Vernelen et al, 1994). Both the c33 and c22 are good indicators of active viral replication, as evinced by positive HCV-RNA findings (Buffet et al, 1994) and it has been suggested that the main factor responsible for indeterminate RIBA 3.0 pattern could be immunosuppression (Pawlotsky et al, 1996). The RIBA 3.0 pattern could also indicate the possible
Table 2.7. HCV proteins/genomic regions used in anti-HCV antibody tests.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Protein</th>
<th>Coding Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening Tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Generation ELISA</td>
<td>c100-3</td>
<td>NS4</td>
</tr>
<tr>
<td>II Generation ELISA</td>
<td>c200, c22</td>
<td>C, NS3, NS4</td>
</tr>
<tr>
<td>III Generation ELISA</td>
<td>c22, c33, c100, NS5</td>
<td>C, NS3, NS4, NS5</td>
</tr>
<tr>
<td>Confirmatory assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Generation RIBA</td>
<td>c100-3, 5-1-1</td>
<td>NS3, NS4</td>
</tr>
<tr>
<td>II Generation RIBA</td>
<td>c100-3, 5-1-1, c33, c22</td>
<td>C, NS3, NS4</td>
</tr>
<tr>
<td>III Generation RIBA</td>
<td>c100-3, c33, c22, NS5</td>
<td>C, NS3, NS4, NS5</td>
</tr>
</tbody>
</table>

Figure 2.8. HCV antigens incorporated into supplementary assay testing systems.

<table>
<thead>
<tr>
<th>Assay</th>
<th>HCV antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIBA 30</td>
<td>Core, NS3, NS4, NS5</td>
</tr>
<tr>
<td>Matrix</td>
<td>Core, NS3, NS4</td>
</tr>
<tr>
<td>Latex III</td>
<td>Core, E2/NS1, NS3, NS4, NS5</td>
</tr>
<tr>
<td>DB-2 blot</td>
<td>Core, NS3, NS4</td>
</tr>
<tr>
<td>Murex blot</td>
<td>Core, NS3, NS4, NS5</td>
</tr>
</tbody>
</table>
genotype as seen in a study conducted in Scotland (Chan et al, 1991). It was found that infection with genotype 2 and 3 strains of HCV tended to occur without c-100-3 antibody and patients with type 3 infection tended not to have anti-c33 antibody. It was also seen that 90% of type 1 infections, but only 22% of type 2 and 3 infections, were associated with c100-3 antibodies.

2.8.4 HCV-RNA detection

Direct tests for HCV antigens in serum are not available, so HCV-RNA is currently the best marker of viraemia and infectivity (Gretch et al, 1992). Viral RNA is detected much earlier than other markers and may be found within days of infection (Fig. 2.9). The nucleic acid is extracted using different procedures like the Proteinase K method (Gretch et al, 1992), the guanidinium isothiocyanate method (Panigrahi et al, 1995), rapid heat method (Antonello et al, 1992) or by the various commercially available extraction kits, which are based on one of the above extraction principles. The genome, being an RNA, is converted to a cDNA using a reverse transcriptase (RT) and then this is amplified by the PCR. At present the reverse transcription and PCR is performed in a single tube. The detection of HCV-RNA by RT-PCR is greatly dependent on the source of primers used (Bukh et al, 1992). Primers derived from the 5' UTR for the double nested-PCR, enhance sensitivity and specificity (Cha et al, 1991). Of late, even the 3' UTR is being used as a site for the detection of HCV-RNA with a single round PCR (Umlauft et al, 1996). Though serum is used for the detection of HCV-RNA on a regular basis, there have been increasing reports on RT-PCR performed on liver sections/biopsies (Sallie
Figure 2.9. Pattern of HCV markers during HCV infection
et al, 1992). Furthermore, detection of minus strand of HCV-RNA by the RT-PCR may also be a means of determining sites of active viral replication (Sherker et al, 1993).

Though RT-PCR has been universally accepted as a very good method for the detection of HCV-RNA, there have been efforts to develop much rapid and advanced testing methods. As a result of these efforts we have the ligation-dependent PCR (Hsuih et al, 1996), Taq-man fluorogenic detection system (Morris et al, 1996), long-PCR (Tellier et al, 1996).

The PCR has a greatest disadvantage in picking out false-positive or the problem of amplicon contamination (Epstein and Fricke, 1990; Main, 1995). Currently it is felt that no one assay is enough to correctly diagnose HCV infection as it is seen that not all antibody-positives are HCV-RNA positives and vice-versa (Reesink et al, 1993). The anti-HCV positive cases which are HCV-RNA negative may be cases of false-positivity, past infection or HCV-RNA being present in low quantities beyond the detection limit of PCR (Zaaijer et al, 1994). The HCV-RNA positivity in anti-HCV negative cases could be due to an early phase of illness where the antibody formation have not yet been elicited or due to immunosuppression (Pawlotsky et al, 1996).

2.9 TREATMENT

Despite the difficulties with diagnosing acute HCV infection a number of trials suggest that early antiviral therapy with interferon can reduce the risk of chronicity (Omata et al, 1993). Pilot studies suggested that interferon
alpha (α-IFN) was of potential benefit for patients with chronic hepatitis C infection (Davis et al., 1989).

Within a few days of starting therapy a decrease was noted in the transaminase values. Larger controlled trials confirmed these observations (DiBisceglie et al., 1989). Approximately 50% of patients respond but half of them will subsequently relapse giving a cure rate of only 20-25%. A 6 month regime of interferon alpha given at a dose of 3 MU thrice weekly for 6-12 months appears well tolerated (Davis et al., 1989; DiBisceglie et al., 1989). There is increasing evidence that more prolonged courses are associated with higher sustained response rates. In a controlled study with 108 patients, 6 months of α-IFN was compared with 12 months and better results followed the longer treatment period (Jouet et al., 1994).

It also appears that levels of viraemia and genotype may be important in determining the response to treatment. Genotype 1b is particularly associated with a poor chance of recovery (Hopf et al., 1996).

Ribavirin(1-β-D-ribofuranosyl1,2,4-triazole-3-carboxamide), a guanosine analogue, has also been used for the treatment of chronic HCV. This pilot study showed a drop in the ALT values whilst on therapy and subsequent analysis has shown a fall in HCV-RNA values (Riechard et al., 1991). The major application of ribavirin in chronic hepatitis C, however seems to be in combination with α-IFN as preliminary results suggest a much greater chance of sustained virological and biochemical response than either therapy alone (Chemello et al., 1995).
Antiviral therapy is monitored by means of quantifying the serum levels of HCV-RNA before and after the treatment. Currently serum HCV-RNA is quantified by techniques like the competitive RT-PCR (Mayerat et al, 1996) or the branched-DNA (b-DNA) assay (Lau et al, 1993).

2.10 PROPHYLAXIS

There are many problems in hepatitis C vaccine development. First is the lack of protective or neutralising antibody in hepatitis C and secondly, the heterogeneity of the HCV genome. The lack of protective antibody has been evidenced by reinfections in humans- and chimpanzees (Farci et al, 1992). Furthermore, the amino terminus of the surface glycoprotein E2 (gp 70), which may be a major antigenic domain on the virion surface, is highly variable which results in emergence of variants during peaks of viraemia and escape neutralisation by antibody.

Choo et al, 1994 expressed the surface glycoproteins of the prototype strain of HCV in cultured human cells using recombinant vaccinia virus. Of the 7 chimpanzees immunised with these viral proteins, 5 were protected against challenge with live virus; the 2 poorest responders, in terms of antibody levels, had short, resolving acute infections. It remains to be determined whether a cocktail of antigens might protect against a variety of HCV strains.
2.11 NEWER NON A-E VIRUSES

Even after all the reliable immunoassays and molecular probes for the detection of HCV and HEV infections became available, it was evident 10-20% of the NANB hepatitis are caused by agents other than HCV and HEV. The agent that was discovered by transmitting the serum of a surgeon (with initials GB) to a primate was distinct from hepatitis A, B, C, D and E virus (Deinhardt et al, 1967). The discovery of 3 novel flavilike viruses in the GB agent, namely GB virus-A (GBV-A), GB virus-B (GBV-B) and GB virus-C (GBV-C) and their similarity to HCV suggest that GBV-A, GBV-B and GBV-C are candidate agents for non-A-E hepatitis cases. The genomes of these agents are similar to those of other pesti- and flaviviruses (Fig.2.10). Across their entire orf, the GB agents exhibit 27% sequence identity to each other and approximately 28% identity to HCV-1 (Deinhardt et al, 1967; Simon et al, 1995).
Figure 2.10. Phylogenetic analysis of helicase region from various members of flaviviridae. This tree is unrooted.