Review of Literature
INTRODUCTION

DNA virus that belongs to the while searching for

Pathogenesis

HBV Mutants

Precore gene mutants
Core promoter mutations
Mutations in the Envelope Gene
Mutations in the X gene
Polymerase Mutations: antiviral drug resistance

Diagnosis

Serologic Diagnosis
Molecular Diagnosis

Genotypes, Serotypes, Subtypes and Determinants

Global Distribution of HBV Genotypes and Subtypes and its association

HBV genotypes and liver disease
HBV Genotyping Methodologies
Molecular Genotyping
Serological Genotyping
HBV genotypes and therapy management

Prevention

Hepatitis B Vaccines
Active Immunization
Passive Immunization

Treatment
INTRODUCTION

Hepatitis B virus (HBV) is a small DNA virus that belongs to the family of hepadnavirus. Blumberg et al., (1967) while searching for polymorphic serum proteins discovered a previously unknown protein in the blood from an Australian aborigine (tribe). He termed this protein Australian Antigen (AuAg). Later it became apparent that this antigen was related to type B hepatitis. Dane et al., (1970) found the virus like particles in the serum of type B hepatitis infected patients. These particles were considered to be the Hepatitis B virus (HBV). The viral nature of these particles was confirmed by detection of an endogenous DNA dependent DNA polymerase within its core (Kaplan et al., 1973) and the discovery of this polymerase allowed Robinson and Greenman, (1974) to detect and characterize the HBV genome. The cloning of HBV DNA and complete nucleotide sequencing of the cloned DNA in 1979 opened new approaches to understanding the biology of HBV.

The viral genome consists of partially double-stranded DNA molecule of about 3,200 nucleotides (Lee, 1997). Nearly 400 million people are infected with hepatitis B virus (HBV), which constitutes to one-third of the world’s population. Among these, 25% will develop serious liver disease, including chronic hepatitis, cirrhosis and hepatocellular carcinoma (Zoulim and Seeger, 1994). About 80% of the cases of hepatocellular carcinoma can be attributed to hepatitis B. Five to 20% of the population of Asia and Africa are chronic carriers better termed as chronic HBV infection. Clearly, hepatitis B is an infection of major public health importance. The prevalence of HBV infection showed marked geographic differences. Regions of high endemicity (> 8 per cent) are China, South-East Asia, Sub-Sahara and Amazon basin. Regions of intermediate endemicity (2-7 percent) are Central, Eastern and Southern Europe, the Middle East, Japan and South Asia. Regions of low endemicity (< 2 per cent) are North America, Western Europe, Australia,
South America and in the United States 1.25 million are infected (Malik and Lee, 2000). In Latin America, the highest seroprevalence of antibody to HBV core antigen was found in the Dominican Republic (21.4%) followed by Brazil (7.9%), Venezuela (3.2%) and Argentina (2.1%). The lowest seroprevalence was found in Mexico (1.4%) and Chile (0.6%) (Tanaka, 2000). The average estimated carrier rate of hepatitis B virus is 4% placing India in the Intermediate range of Hepatitis B endemicity (Irshad and Acharya, 1992; Thapa et al., 1995).

In regions of high prevalence HBV infection is predominantly transmitted perinatally or in early childhood. In rare cases only, HBV are transmitted parentally. The mode of transmission in childhood is unclear as yet. In regions of low endemicity, transmission occurs mostly in adults by parenteral or sexual transmission (Sanchez-Tapias, 2000; Lok, 2000). High-risk groups are intravenous drug users as well as homosexuals. HBsAg and HBeAg positive individuals are highly infectious to others. The natural course of HBV infection depends in part on the age at the time of infection. In adults, chronic HBV-infection develops in 1-10 per cent and is characterized by the persistence of HBsAg in serum. By contrast, the development of chronic infection is much more frequent (>90 per cent) if infection occurs perinatally or in early childhood. Overall, chronic hepatitis B progresses to liver cirrhosis in 20-30 % of patients and is associated with significant morbidity and mortality from complications of liver cirrhosis as well as HCC (Wands and Blum, 1991).

**Structure and Genome Organization**

Hepatitis B virus is a hepadna virus, a small DNA virus having a genome of about 3.2Kb in length, which exhibits a partially double stranded circular configuration. There are four ORF’s within the HBV genome; pre
S/S, pre C/C, P and X ORFs. The minus strand of the DNA is almost a complete circle and contains overlapping genes that encode both structural proteins (pre-S, surface and core) and replicative proteins (polymerase and X protein). The plus strand is shorter and variable in length.

The pre S/S encode major S (i.e.) HBsAg alone, middle S [i.e. pre S2/HBsAg] and large S (pre S1/preS2/HBsAg). The middle S and large S polypeptides are carboxy-co-terminal extensions of the major S protein. The major S peptide consists of p24/gp27 polypeptides, which make up about 80% of HBV envelope protein. The middle S contains gp33/gp36 polypeptides and represents about 15% of the envelope protein. The large S consists of p39/gp42 and constitutes only 5% of the envelope protein. The pre C/C ORF encodes the viral nucleocapsid polypeptide [i.e. HBcAg] and a related, secreted polypeptide referred to as Hepatitis B 'e' antigen (HBeAg). Hepatitis B core antigen (HBcAg) is a p21 polypeptide that not only assembles to form the viral core, but also is involved in packaging of viral nucleic acid during virus core assembly. HBcAg is formed by initiation of transcription at the second initiation site of the pre C/C ORF. HBeAg is a p18 polypeptide produced from transcription initiated at the first initiation site of the pre C/C ORF. This results in a peptide with a signal sequence for secretion that is further modified post-translationally by loss of the amino terminal signal sequences and part of the carboxy terminal sequences. The P ORF encodes the HBV DNA polymerase and encompasses approximately 67% of the genome, overlapping the three other ORF's. The P ORF has three distinct domains; a terminal protein, RNase H and Reverse transcriptase. The X ORF encodes an X protein (pX) of 154 amino acids, which appear to function as a modulator of HBV transcription. In addition pX is a potent transactivating factor for several genes of the host including class I and II human leukocyte antigen (HLA) and intra cellular adhesion molecule I. (Table-1) (Fig- 1&2)
Table 1

Immunological and Virological Characteristics of hepatitis B virus (HBV)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family</td>
<td>Hepadnaviridae</td>
</tr>
<tr>
<td>Virion size and shape</td>
<td>42 nm, spherical, with 27nm capsids</td>
</tr>
<tr>
<td>Envelope</td>
<td>Large, middle, small surface proteins lipids</td>
</tr>
<tr>
<td>Nucleocapsid</td>
<td></td>
</tr>
<tr>
<td>Genome</td>
<td>Circular partially ds DNA, 3.2 kb</td>
</tr>
<tr>
<td>Proteins</td>
<td>Core protein, polymerase, ? host proteins</td>
</tr>
<tr>
<td>Replication</td>
<td>Reverse transcription of positive-strand RNA</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
</tr>
<tr>
<td>Antigens</td>
<td>HBsAg, HBeAg, HBcAg</td>
</tr>
<tr>
<td>Antibodies</td>
<td>Anti- (HBs, HBe, HBc, HBc IgM)</td>
</tr>
</tbody>
</table>
**Figure 1** STRUCTURE OF HEPATITIS B VIRUS

- Small surface protein (S)
- Medium surface protein (S + PreS2)
- Large Surface Protein (S + PreS2 + Pre S1)
- Polymerase (P)
- DNA
- Icosahedral core (R)

**Figure 2** ORGANIZATION OF HBV GENOME

Diagram showing the organization of the HBV genome with labels for pre-S1, pre-S2, DR1, DR2, 5', + strand, pre-C, X, 0.7 kb, 3.5 kb, 2.4 kb, 2.1 kb.
Clinical Spectrum
Acute Hepatitis

The clinical course of HBV runs similarly to that of Hepatitis A virus (HAV) but tends to be more severe and may be associated with serum-sickness-like syndrome. The mildest attacks are asymptomatic and are detectable only by an increase in serum transaminase levels. Alternatively, the patient may be anicteric but suffer from gastrointestinal and influenza-like symptoms. These patients are likely to remain undiagnosed unless a clear history of exposure is available. The severity of infection may vary from the symptomatic and icteric (from which recovery is typical) through to fulminant, fatal viral hepatitis. Icteric attacks in adults are marked by a prodromal period (typically 3-4 days up to 2-3 weeks) during which a patient feels sick, suffering from digestive symptoms such as anorexia and nausea and may in the later stages have mild pyrexia. Other common symptoms are mild pyrexia, rigors, malaise and occasionally severe headaches. The prodromal period is followed by the darkening of urine and lightening of faeces followed by the development of jaundice.

The consequences of acute HBV infection are highly variable. The incubation period range from 6 weeks to 6 months and the development of clinical manifestations is highly age dependent. New born generally do not develop clinical signs or symptoms and infection produces typical illness in only 5 to 15% of children 1 to 5 years of age (McMahon et al., 1985). In older children and adults, only 33% to 50% are symptomatic. When the clinical course is self-limited, viremia quickly becomes undetectable; HBeAg disappears within few weeks and is replaced by the corresponding antibody (anti HBe). HBsAg positivity is highly variable and usually becomes negative in 2-4 months. The appearance of antibodies to HBsAg (anti HBs) is the best serological indicator of recovery from the infection and may take several
months after HBsAg seroclearance. A persistence of high HBV-DNA levels and HBeAg positivity predict evolution of the infection. It is conventionally diagnosed when HBsAg persists for more than 6 months from its first detection. It is important to know that in cases of fulminant acute hepatitis HBsAg and HBV DNA are often negative due to massive hepatocellular necrosis. In such cases anti HBc IgM is the most reliable assay for the diagnosis. Fulminant hepatitis occurs in about 1 to 2% of persons with acute disease and has a case fatality ration of 63 to 93%. HBV causes acute viral hepatitis in 34-35% cases in India (Tandon et al., 1996); about 31% cases in Pakistan (Haider et al., 1994) and 35% cases of acute viral hepatitis were reported from Bangladesh (Khan and Ahmad, 1996).

**Chronic HBV Infection**

Chronic HBV infection is defined as the presence of HBsAg in serum for six months or longer after initial detection. The risk of developing chronic infection varies inversely with age and is highest (up to 90%) for infants infected in the perinatal period (Hyams, 1995). 25 to 50% of the children who are infected between 1 to 5 years of age develop chronic infection, compared to 6 to 10% of acutely older children and adults (Mahoney, 1999).

Adult acquired chronic HBV infection begins with a phase of immune clearance (Hoofnagle et al., 1981). Symptoms may or may not be present and serum liver transaminases are usually elevated. HBV DNA is present in low concentrations in serum. Patients are HBeAg positive and liver histology reveals chronic hepatitis of varying activity often with lobular inflammation. HBcAg can be detected in the nuclei and cytoplasm, and HBsAg is commonly detected in the cytoplasm. During this phase, HBV replication declines, and spontaneous HBeAg to anti HBe seroconversion can be observed at the annual rates of 2.7% (Luis Viola et al., 1981) to 25% (Hoofnagle et al., 1981).
Often a brief episode of significant elevation of serum ALT levels precedes seroconversion (Liaw et al., 1983). Transition from replicative to non-replicative infection may be smooth, rapid and clinically silent or prolonged, fluctuating and marked by recurrent exacerbations (Lok, 1992).

Once HBV infected cells are destroyed by the immune system, patients enter the third phase when the active replication ceases, and HBeAg protein disappears. Liver transaminases tend to normalize and patients have no or minimal symptoms. HBcAg can be detected in the liver tissue and typical ground glass appearance of the hepatocytes is observed. The HBV DNA gets integrated in the host’s genome and the presence of HBV DNA in the serum is only detected by PCR. The intensity and the duration of the second phase will have determined the degree of long-term carriage. At this phase of non-replicative infection, the patient is seen as a “healthy” carrier (Hoofnagle et al., 1991) although residual liver damage may be established.

Chronic Hepatitis B virus infection is a leading cause of chronic liver disease worldwide. It is estimated that 15% to 25% of people with chronic Hepatitis B virus (HBV) infection will die prematurely from cirrhosis or hepatocellular carcinoma. Chronic HBV infection induces substantial direct and indirect costs, significant mortality and morbidity. In India, 49-68% of chronic liver diseases are caused by HBV (Sundaram et al., 1990). Sarin et al., (1988) and Acharya et al., (1993) have found HBV in more than 60% of chronic hepatitis. Nayak et al., (1977) reported HBV in 80% cases of cirrhosis of the liver. Guptan et al., (1996) studied 120 patients with histologically proven HBV-related chronic liver disease, of these 15.5% patients had precore, 10.8% had surface gene mutations. The remaining 74.2% patients were infected with wild type HBV. In Pakistan, 60% cases of chronic liver disease were caused by HBV (Zuberi, 1996). In Nepal, hepatitis B virus causes 60% cases of chronic hepatitis and 40% cases of cirrhosis of the liver
(Shrestha, 1994). In Bangladesh, 40.5% cases were reported in a series of 116 cases of chronic hepatitis and cirrhosis of liver due to hepatitis B viral infection (Khan et al., 1994).

**Association with Hepatocellular Carcinoma**

Several factors are thought to contribute to the carcinogenic process including the random integration of viral DNA into chromosomal DNA, the generation of viral inserts defective in the Pre-C region and/or the S region, which are potentially transactivating, the expression of the transactivating X protein and the intracellular accumulation of viral proteins. In addition, the immune response to viral infection resulting in cell injury, inflammation and liver regeneration may also contribute.

Epidemiological studies have shown a high incidence of primary liver cancer in HBV carriers and clinical studies have shown that many primary liver cancer patients are positive for Hepatitis B surface antigen (HBsAg) and have chronic hepatitis (Slagle et al., 1992). Chronic hepatitis type B is responsible for at least 0.8 million deaths annually worldwide, linked mainly to complications of cirrhosis and HCC (Fattovich, 1998).

Several lines of evidence associate chronic HBV infection with the development of HCC. Worldwide, there is certain parallelism between the HBsAg carrier rate and the incidence rate of HCC; the carrier rate is high in areas of high HCC incidence and low in areas of low HCC incidence, but HCC comes far behind other types of cancer in regions where HBV infection is uncommon. Serological evidence of HBV infection is detected in about 70% of HCC patients in Africa and more than 90% in Mainland China, as compared with 10-20% of the population residing in the same areas (Tabor, 1991). A marked increased risk of HCC has been shown among HBsAg
carriers compared with non-carriers. This is evidenced by a prospective study carried out by Beasley et al., (1981) on 22,707 Chinese men in Taiwan, where it was found that the incidence of the tumor in the HBsAg-positive population was 473/100,000 persons per year, compared to only 4.6 in the hepatitis B surface antigen (HBsAg)-negative individuals (relative risk = 102) (Beasley et al., 1981). In the Far East in persons who are positive for HBsAg, the annual incidence of HCC is 0.5% for asymptomatic HBsAg carriers (Di Bisceglie et al., 1988) and 0.8% for patients with chronic hepatitis B (Liaw et al., 1985). Globally about 57% of all liver cancer deaths can be attributed to the hepatitis B virus. Hepatitis B surface antigen (HBsAg) positivity in Indian HCC patients varies from 36% to 74% with an average of 47% (Kumar et al., 1995; Ramathilagam et al., 1995; Sarin, 1996; Mujeeb et al., 1997). It is estimated that nearly 42.5 million people in India are HBsAg positive (4.7% of 900 million) (Thyagarajan et al., 1996). (Fig-3)

Pathogenesis

The replication cycle of HBV begins with the attachment of the virion to the hepatocyte. The steps, which are involved in viral entry, uncoating and nuclear translocation, are unclear. Synthesis of the plus strand HBV DNA is completed inside the hepatocyte nucleus, and the viral genome is converted into a covalently closed circular DNA (cccDNA). The cccDNA is the template for transcription of pregenomic RNA and messenger RNA. The 3.5 kb pregenomic RNA serves both as a template for reverse transcription of the first (-) strand HBV DNA as well as a messenger RNA for the production of nucleoside and polymerase proteins. Four mRNA transcripts of known function have been identified as being involved in HBV transcription. The longest (3.5 Kb) is the template for genome replication and expression of precore/core and polymerase proteins. A 2.4 Kb transcript encodes pre-S1, pre-S2 and HBsAg while a 2.1 Kb encodes only pre-S2 and HBsAg. The smallest transcript (0.7Kb) encodes the X protein.
GLOBAL PREVALENCE OF HBsAg, 2005, (CDC)
HBcAg and HBeAg are translated from a common gene. When transcribed, HBcAg is targeted to the endoplasmic reticulum, here it is cleaved, and HBeAg (the precore fragment) is secreted. HBcAg is essential for viral packaging and is an integral part of the nucleocapsid. It is not detectable in serum by conventional techniques; however, it can be detected in liver tissue in patients with acute or chronic HBV infection. HBV replication begins with binding of the virus to the cell surface and penetration (Gerlich et al., 1993; Kann and Gerlich, 1997). The virus is transported without processing to the nucleus, where the relaxed circular DNA is converted to a covalently closed circular DNA (cccDNA), which in turn acts as a template for viral RNA synthesis. HBV DNA does not integrate into the host genome during the normal course of replication.

The cellular and humoral immune responses to HBV infection are complex. Most studies suggest that HBV is not directly cytopathic to infected hepatocytes and that the cellular response to several viral proteins correlates with the severity of clinical disease and viral clearance (Chisari and Ferrari, 1995). Antibody response to the viral envelope antigens contributes to the clearance of the virus; the cytotoxic T cells mediate viral clearance by killing of the infected cells. It has been postulated that chronic HBV infection is related to a weak T-cell response to the viral antigens. Neonatal immune tolerance to viral antigens appears to play an important role in viral persistence among persons infected at birth, the basis of a poor T-cell response in adults is not well understood.

**HBV Mutants**

The host-virus relationship is a dynamic and complex interaction with several aspects, including pathogenesis and viral mutagenesis. Generation and/or selection of mutants are a reflection of attempts of the virus to resist
immune attacks of the host and to survive. The number and distribution of some HBV mutations in serum from chronic active hepatitis carriers change as function of time. Initial point mutations offer a strong advantage if they allow the virus to escape the immune responses of the host. The continued evolution of mutations, however, suggests that a second generation of mutants is selected as a result of the induction of lethal immune reactions against the first generation. The result is that the antigenic complexity of the virus increases over time. Therefore, it becomes more and more difficult for the host to target the virus and to eliminate it by immunological means. In this sense, increasing number of mutations during a phase of exacerbation of progressive liver disease might be seen as evidence of adaptive mutation in chronic HBV infection (Chuang et al., 1993).

Mutations of HBV have frequently been described. HBV shows greater mutability than previously expected, because its replication requires reverse transcription of the RNA pre-genome and this process is known to be susceptible to errors caused by the lack of proof-reading capacity (Yamamoto et al., 1994). This error replication leads to $2 \times 10^{-4}$ base substitutions per site per year i.e. four orders of magnitude more than in other DNA viruses, but less than in RNA viruses (Mimms, 1995). Mutants may also arise from recombination between co-infecting strains. HBV mutations are not limited to specific ORFs and occur in all viral genes and regulatory elements. Mutants have been identified in patients with acute or fulminant as well as chronic HBV infection; the repression of the synthesis of wild-type HBV is because of defective variants with mutation in the core region and can be catalogued as pre C/C gene mutants. Among these variants, the most common are in the Mediterranean area, mutants containing a strategic mutation at nucleotide 1896 of the pre-core region that prevents the secretion of HBeAg ("pre-core mutant") (Carman et al., 1989; Bonino et al., 1991). HBeAg negative, anti-HBe positive chronic hepatitis B accounts for 7-30% of patients with
chronic hepatitis worldwide. Prevalence rates range from 40-80% in the Mediterranean area, Hong Kong, Korea, Taiwan and Japan to 13-15% in India and China to lower rates in northern Europe and United States. Most of the variants seem to occur in the long-term natural history of wild type HBV but the exact prevalence of direct infection is unknown. Several studies have documented progression from wild type to mutation 1896 (Akara et al., 1994). The other variants include the presurface /surface (pre S/S) gene mutants, and X gene mutants. Patients in whom mutants coexist with wild type viruses are not exceptional. In fact, many infections wherein mutants are involved may be mixed infections of this type, containing different DNA templates that complement each other by trans-activation or by DNA recombination (Tu et al., 1997).

The existence of HBV mutants was first suggested when hepatitis patients were observed with detectable amounts of HBV-DNA or RNA but without serological indications of active or past infection, as demonstrated by the absence of HBsAg or anti-HBs respectively. Because HBV infections may persist for years or even decades, mutations may accumulate and become clinically significant. The time factor, the high viral replication activity (with total release into the peripheral blood of up to $10^{11}$ virus particles per day), and both immune and therapeutic pressures, are decisive determinants in this respect. Besides association with a specific course of the infection, certain mutations may have serious implications at different levels and allow a classification depending on the involved mechanism. Reduced viral clearance by immune mechanisms or antiviral therapy is called “immune escape” or “therapeutic escape”, respectively. Mutations in the envelope genes can result in a decreased or absent detectability by assay based upon antibodies to the wild-type virus (diagnosis escape) or in non-recognition by neutralizing antibodies induced by vaccination (“Vaccine escape”) (Norwak et al., 1996; Blum et al., 1997).
**Precore and core gene mutants**

Two major groups of mutations that result in reduced or blocked HBcAg expression have been identified and can be classified into a translational or transcriptional mechanism of control. The most frequently observed precore mutation is a G to A transversion at nucleotide 1896. This substitution introduces a translation stop codon (TAG) in the distal precore gene and prevents expression of the pre C/C fusion protein that functions as a precursor of HBe Ag (Carman *et al.*, 1989). Less common mutations resulting in HBeAg negativity include initiation codon mutations (at positions 1814 or 1815), a nonsense mutation at 1874, a missense mutation at 1862, and frameshift mutation (Kramvis *et al.*, 1997). The serum of patients with these mutations does not contain HBeAg and hepatocytes harboring this mutant virus, also do not present HBeAg on their surface. Because HBeAg is an important immunological target, these cells escape killing mediated by the host’s immune system. In the early stages of chronic HBV infection, when HBeAg is detectable, the wild virus survives. In the later stages, however, the virus population is reduced in number and, probably as a result of the prolonged interaction with the host’s immune mechanisms, mutations affecting HBeAg expression occur. The HBeAg-negative mutants survive, becoming the dominant virus type, and prolong the infection. In HBV genotypes B, D, E and G and in some strains of genotype C, the 1858 nt is a thymidine (T). Thus the stop codon mutation created by G1896 A (T-A) stabilizes the ε structure. In contrast the precore stop-codon mutation is rarely detected in HBV genotypes A or F or in certain strains of HBV genotype C, because the nt at position 1858 is a cystidine (C), maintaining the preferred Watson-crick (G-C) base pairing.

The 1896 stop codon mutant is often present in patients with chronic active or inactive hepatitis and in asymptomatic carriers in Mediterranean and
Oriental countries (Lee et al., 1996). In contrast, these mutants are rare in North America, Western Europe, and southern Africa (Mangia et al., 1996; Bowyer et al., 1997). This geographical pattern is determined by the distribution of HBV genotypes. In those regions where genotype A dominates, the 1896 mutant is rare. In the proximal stem of the RNA encapsidation signal or epsilon (ε), the G residue at 1896 is normally paired with a T (U) at 1858 in non-A genotypes but with a C in the A genotype (Kramvis et al., 1998). G to A switch at 1986 would result in an unstable base-pairing (A-C) at that position, destabilizing the stem-loop structure of ε and reducing the efficiency of HBV replication (Kramvis et al., 1997). In contrast in non-A genotypes this mutation creates a Watson-Crick T (U)-A base pair, stabilizing the secondary structure of ε and enhancing viral replication. A mutation at 1899, which may occur in association with the 1896 mutation or other mutations that are associated with HBeAg negativity, is another mutation that improves the stability of by providing an additional A-T (U) base pair (Kramvis et al., 1997).

A mutation at position 1862, which occurs in the bulge of ε, has been detected in asymptomatic HBV carriers, and in patients with chronic hepatitis, cirrhosis, hepatocellular carcinoma or fulminant hepatitis (Kramvis et al., 1997; Kramvis et al., 1998). This mutation could affect HBeAg expression at two levels. It occurs very close to the signal peptide cleavage site, where the signal peptide is cleaved from the precursor of HBeAg in the endoplasmic reticulum, and might therefore abrogate HBeAg synthesis. Alternatively, this mutation might interfere with reverse transcription of pregenomic RNA. Polymerase (reverse transcriptase) acts as a primer of RNA directed DNA synthesis by binding to the bulge of ε. Although binding of the template to position 1862 is not as crucial as it’s binding to 1864 and 1865, the 1862 mutation may possibly decrease the efficiency of reverse
transcription and hence viral replication. Encapsidation and replication of HBV may also be impaired by mutations in the upper stem and loop of ε (Kramvis et al., 1998).

The core gene contains both humoral and cytotoxic T cell epitopes. Mutation within immunodominant cytotoxic T cell epitopes may be exploited by viruses to evade protective immune responses critical for viral clearance. Deletion of the core gene has been reported in immunocompromised and Oriental Patients with chronic hepatitis B or hepatocellular carcinoma (Yuan et al., 1998). These deletions almost always involve loss of B and T cell epitopes and may confer a selective advantage on the virus by evading immune surveillance. Multiple point mutations in the core gene also occur in Oriental and Mediterranean patients with chronic hepatitis B (Carman, 1995). Although these mutations may be concentrated at sites with major B and T cell epitopes, it is uncertain how often the function of cytotoxic T cell epitopes is adversely affected by these mutations. Point mutations of the core gene are rarely seen in American or British Caucasian patients with chronic hepatitis B (Gray et al., 1997) suggesting that, in general, the induction of chronic liver disease is not associated with these mutations. Nevertheless, two patients chronically infected with HBV were shown to display mutant epitopes that acted as natural antagonists for T cell antigen receptor recognition and had the capacity to inhibit the cytotoxic T lymphocyte response to the wile-type epitopes (Blum et al., 1997). In this way they may have contributed to viral persistence.

In the year 1989, a precore defective HBV mutant with a G to A substitution at nucleotide 1896 resulting in a TAG stop, codon, and failure to produce hepatitis B e antigen (HBeAg) was found in hepatitis B surface antigen (HBsAg) carriers with antibody against HBeAg (anti-HBe) and severe chronic liver disease (Carman et al., 1989).
Over 350 million people worldwide have chronic hepatitis and the majority is in South East Asia and Sub-Saharan Africa. 7-30% are infected with the mutant forms (Huang et al., 2003), with an estimated hepatitis B surface antigen (HBsAg) carrier rate of 4.7% and almost 80% of CLD patients being infected with HBV in India (Thyagarajan et al., 1996). The patients with HBeAg negative chronic hepatitis B have a high risk to develop progressive chronic liver disease. High incidence of precore mutants have been reported in patients with sporadic HBsAg positive fulminant hepatitis, from the Far East (Kosaka and Jakase, 1991) and South Mediterranean Countries (Carman et al., 1991).

Furthermore precore mutations have been also detected in fulminant hepatitis type B in Israel (Liang et al., 1991) and in Japan after nosocomial, interspouse or intrafamilial transmission (Carman et al., 1991). Genotypes in Northern Europeans were 60% A, 31% D, in Southern European and Middle East’s 96% D, in Africans 53% A, 27% D, 20% E, and in East Asians 14% A 43% B, 43% G (Magnus and Ann-Sofi, 1997). The low prevalence of TAG mutation in carriers of Northern European origin has been explained by the presence of cytosine in hepatitis B virus of genotype A, which is the most common genotype in this region (Tong et al., 1992).

Precore mutants are rarely found in carriers of Northern European origin, who usually carry genotype A. Studies in Sweden show C-1858 strains in 61% of Northern European carriers, 12% of East Asian carriers (Genotype A & C), 3% of Middle Eastern carriers, 50% of African carriers (Genotype A). Thus the emergence of the precore TAG mutation is prevented by C-1858 not only in Northern Europeans but also in a significant proportion of South American, African and South East Asian carriers (Magnus and Ann-sofi, 1997).
Majority of anti HBe-seropositive HBV carriers in Bulgaria are infected with precore mutant HBV. The precore variant with a stop codon at nucleotide position 1896 is the dominant virus strain in anti–HBe chronic carriers with severe liver disease. Precore variant virus are associated significantly more often with high viral DNA titres in the serum than infection with the mixed type. Anti-HBe-positive with predominant precore variant infection and high viremia had more severe disease than patients with low viremia and mixed infection with wild type and precore mutant virus (Nikolai et al., 1992).

Precore sequence alters profoundly the properties of the viral core protein. The generation of the p22c expressing clone from the p25c expressing clone by deleting the precore sequence excludes the possibility that the differences between p25c and p22c were due to differences in the gene ‘C’ part (Angela and Velkar, 1986). HBV mutants with precore region defects incapable of directing the synthesis and secretion of HBeAg increasingly prevail in persistent carriers and finally replace the predecessor, wild type HBV after these carriers seroconvert to anti-HBe (Okamoto et al., 1990).

HBV variants bearing mutations in the precore region, which abrogate the expression of HBe protein, have been readily isolated from chronic hepatitis B patients with circulating anti-HBe antibodies (Tong et al., 1992). The occurrence of hepatitis B virus precore mutants, which arise during spontaneous or interferon induced seroconversion from HBeAg to anti-HBe, are thought to be selected by immune pressure. A higher response rate to interferon therapy was observed in patients with wild type (75%) than in patients with precore mutant (40%). The data support the hypothesis that precore defective HBV represents viral mutants with an increased capacity to resist exogenous alpha interferon (Zhang et al., 1996).
Severe hepatocellular injury continues to occur during infection with an HBV variant intrinsically incapable of producing HBeAg strongly suggesting that continuous synthesis of HBeAg is not required for the progression of HBV induced CLD (Paul P. Ulrich et al., 1990). Prevalence studies reported from Japan and Israel have shown that the precore TAG mutant was found in 80 – 100% of the patients with fulminant hepatitis B but in none of the patients with acute self-limiting hepatitis B. 7.3% positivity for surface mutants and 9.7% positivity for precore mutants was reported in CLD cases of Eastern India 15.5% positivity of precore mutants and 10.8% positivity of surface mutants was reported in Asian-Indian patients with CLD (Guptan et al., 1996). Patients with precore mutations were always asymptomatic, often presenting with ascites (67%) and jaundice (55%).

The selection of hepatocytes replicating this HBeAg negative virus is probably due to the lysis of the HBeAg positive hepatocytes by anti-HBe. Because the cells infected with the HBe–negative virus express HBC peptides like hepatocytes infected with HBe-positive virus, emergence of the mutant virus implies absence of cytolytic T cells reactive with HBC protein. Deleted mutants of HBV-DNA in the core region were found to exist in more than half of the patients with chronic hepatitis. Heterogenicity was found in the nucleotide sequences of the precore and core gene including defective mutants in precore with chronic HBV carriers. Products of these variant genes may change the immunological properties of HBcAg (or) HBeAg and subsequently, the clinical course of chronic HBV carriers (Takaji Wakita et al., 1991).

HBeAg negative HBV mutants do not play a predominant etiologic role among North American patients with fulminant hepatitis B. Severe liver damage in chronic hepatitis B infection is related to the clustering missense mutations in codon (48-60) and (84-101) of core gene. The emergence of
precore stop codon mutation and missense mutations around the carboxy-terminal processing site of precore / core protein (codons 147–155) may be the adaptive mechanism of hepatitis B virus to decrease production and secretion of viral protein and retain the viral persistence.

Analysis of DNA sequences of HBV genomes isolated from Japanese patients with fulminant hepatitis B, showed that precore mutations are commonly associated with the fulminant hepatitis (Kiyoshi Hasegawa et al., 1991). The precore mutation at the 28th codon, the 29th codon or both was a significant factor in severe forms of hepatitis B in spouses of anti –HBeAg positive carriers of HBV. The HBV population with the 29th codon mutation expanded in the spouse along with a significant rise in the concentration of circulating HBV (Shigeru Yotsumoto and Mineokojima, 1992).

The analysis of core promoter and precore mutations in HBsAg positive children, revealed a striking presence of both BCP and precore mutants in anti-HBe positive infants with fulminant and severe hepatitis B. The combination of core promoter mutants 1762 –T / 1764-A and a precore stop codon in HBV genotype D virus strains could be one factor in the pathogenesis of fulminant hepatitis B in infants. There was a high prevalence of precore mutants in HBV sequences with genotype D and a low prevalence in sequences with genotype A, which suggests that HBe Ag positive cases with genotype A have little chance of selecting precore mutants after anti-HBe seroconversion. The distribution of precore mutants between the different genotypes can be explained by the different stabilization energy of each genotype as a result of the introduction of mutation (Rodriguez et al., 1995).

In patients undergoing liver transplantation for HBV related liver disease, infection with precore mutant virus was associated with severe
recurrent disease following transplantation (Angus et al., 1995). Reduced immune recognition would allow greater HBV replication within hepatocytes post transplantation. Patients with precore minus HBV infection, HDV replication and spreading might be less efficient because of a hampered helper function provided by the HBV variant (Brunetto et al., 1990).

Cumulative epidemiological and biological observations suggest that the severity of liver damage and outcome of chronic hepatitis B are influenced not solely by the intensity and competence of the host’s antiviral immune response (Ferruccio Bonino et al., 1991). Accumulation of multiple mutations within the precore and core gene region in chronic HBV patients with liver injury and the relative absence of precore and core region mutations in chronic HBV patients without liver injury (Hosono et al., 1995). HBeAg-Tg model illustrates that the secretion of a viral protein (ie. HBeAg) which may preferentially deplete inflammatory Th1 cell may represent a viral strategy to promote persistence. High prevalence of precore mutant viruses in anti-HBe carriers with chronic liver disease suggest that monitoring of virus sequence type and DNA level may be of prognostic value for liver disease sequelae. Allele specific PCR is an efficient method for the detection of these viral variants (Lo et al., 1992). Co-existence of serum HBeAg and precore mutants was possibly the result of a release of intracellular core particles from massive necrosis of liver cells and cross reactivity between HBcAg and HBeAg (Hong-Yuan Hsu and Mei-Hwei, 1995).

Studies in Hong Kong, showed that severe disease occurs in patients with serine 15, who do not have large numbers of core amino mutations, indicating that the core variation is not the cause of severs disease but is a result of immune pressure, which appears less active (or) less focused in those with the serine 15 variant. Precore (or) BCP mutants were not associated with severe liver disease in Western India. Precore mutants appeared to favor an
asymptomatic carrier state. HBV-DNA levels and Pre-C mutants were identified as independent variables influencing outcome of chronic HBV infection. (Arankalle and Gandhe, 2003).

**Core promoter mutations**

The core promoter plays a central role in HBV replication and morphogenesis, directing the transcription of both pregenomic RNA and precore mRNA. It overlays the 3’ end of the X gene and the 5’ end of the pre C/C gene. Sequence variation in the core promoter is limited because of its pivotal role in viral replication. The double mutation, A to T transversion at 1762 and G to A transition at 1764, is often present in patients with chronic hepatitis, hepatocellular carcinoma, fulminant hepatitis, and less often in asymptomatic carriers, immunosuppressed patients, and in carriers without HBV markers (Kramvis et al., 1999). The mutations are accompanied by a reduced level of HBeAg expression resulting from decreased binding of liver enriched factor to this region and the conversion of a nuclear receptor binding site to a hepatocyte nuclear factor I binding site. The effect of the double mutation on viral replication is uncertain. A mutation at 1653 in the core upstream regulatory sequence (CURS) is frequently found in patients with fulminant hepatitis (Gunther et al., 1998) and nt 1753-1757 in patients with fulminant hepatitis or hepatocellular carcinoma (Kidd-Ljunggren et al., 1997), both in association with the double mutant. A number of deletions of the core promoter have been described, usually in patients with chronic hepatitis, hepatocellular carcinoma, asymptomatic carriers, and in HBV infection without serological markers. Most deletions result in a frame shift and truncation of the X protein at its C-terminal end, which is essential for its trans-activating function (Kramvis et al., 1999).
Mutations in the Envelope Gene

The Pre-S sequences exhibit the highest heterogeneity of the HBV genome (Gunther et al., 1999). Point mutations, deletions and genetic recombination's within the Pre-S genes have been identified in HBV DNA sequences obtained from the sera of inactive carriers. HBV genomes that cannot synthesize Pre-S2 proteins occur frequently and are the dominant virus populations in these persons (Gunther et al., 1999). The Pre S2 region overlaps the spacer region of the Pol protein, which is not essential for enzyme activity. Most hepatitis B vaccines contain the major HBsAg protein and induce an immune response to the major hydrophilic region, located from residue 99-170 which includes the 'a' determinant or neutralization domain of HBV. The anti-HBS response produced to this region is associated with protective immunity. Mutations within this epitope have been selected during vaccination (Carman et al., 1990) and following treatment of liver allograft recipients with HBIG (Carman et al., 1996). Most isolates contain a mutation from glycine to arginine at residue 145 of HBsAg (sG145R) or from aspartate to alanine at residue 144(sD144A) and this mutation has been associated with vaccine failure (Carman et al., 1990).

Mutations in the X gene

The X gene protein exhibits numerous activities affecting intracellular signal transmission, gene transcription, cell proliferation, DNA repair and apoptosis. HBX up-regulates a number of viral genes and a wide variety of cellular genes. Its transactivating functions are mediated by activation of transcription factors, modulation of cell signaling pathways. HBX may play a role in the development of hepatocellular carcinoma (Arbuthnot et al., 2000).
Mutations in the X region can involve the regulatory elements that control replication, such as the basal core promoter and Enhancer II. Because the basal core promoter encompasses nt 1742-1802 and overlaps with the X gene in the concomitant reading frame, the A1762T plus G1764A core promoter mutations also cause in the X gene at xK130M and xV131I. In addition, nearly all deletions or insertions in the basal core promoter result in shift of the X gene frame and lead to the production of truncated X proteins. These shortened X proteins typically lack the domain in the C terminus (amino-acids 130-140) that is required for the transactivation activity of HBxAg (Gunther et al., 1999).

Polymerase Mutations: antiviral drug resistance

The advent of treatment with nucleoside and nucleotide analogs has resulted in the selection of quasispecies containing mutations in the HBV Pol gene. Antiviral resistance to lamivudine has been mapped to the YMDD locus in the catalytic or C domain of HBV Pol, (Stuyver et al., 2001) and specific mutations selected are designated as rtM2041/V/S (domain C) rtL180M (domain B). Resistance to adefovir dipivoxil is associated with mutations in the B and D domain of the enzyme (Angus et al., 2003) and the major mutations associated with adefovir-resistant HBV are designated as rtN236T (domain D) and rtA181T (Angus et al., 2003). A mutation of the polymerase gene that might be linked to viral persistence was reported. Polymerase dysfunction, in the form of an inability to package pregenomic RNA into core particles, appeared to result from a single missense mutation in the 5' region of the gene. Trans-complementation in-vitro by the full-length wild type polymerase gene resulted in restoration of the replication competence of the mutant.
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Diagnosis

Serologic Diagnosis

Infection with HBV is complex and normally results in the sequential production of HBV-specific antigens and antibodies in the serum of an infected patient. The most practical and reliable way of making a laboratory diagnosis of HBV infection involves the detection of these serologic markers. A number of commercial solid-phase antibody capture and competitive inhibition RIAs and EIAs are available and are well established for the identification of Hepatitis B surface antigen (HBsAg), total antibody to hepatitis B core antigen (anti-HBcAg), hepatitis B e antigen (HBeAg), antibody to HBeAg (anti-HBe) and antibody to HBsAg (anti-HBs). These assays are routinely used to differentiate acute from chronic HBV hepatitis, to evaluate the infectivity or immune status of a patient and to screen blood products and organ donors. Serologic testing is the cost effective and definitive means of diagnosis in chronic HBV infection as the clinical symptoms of HBV, is difficult to distinguish from other forms of hepatitis. Acute HBV infection is detected by diagnosis of anti HBc IgM. HBeAg is also detected during acute infection. During convalescence, HBsAg and HBeAg are cleared, anti HBs, anti HBc and anti HBe develop. Anti HBs is a protective antibody and it neutralizes the virus. The presence of anti HBs following acute infection indicates recovery and immunity from reinfection. Anti HBs is also seen in persons who have received hepatitis B vaccine. Anti HBC (Total) indicates current or past exposure and viral replication. Anti HBc IgG appears shortly after HBsAg among persons with acute HBV and persists throughout life. Therefore anti HBC (Total) is not a good marker for persons with acute infection, only anti HBc (IgM) must be tested. In persons with chronic HBV infection, HBsAg remains persistent throughout life and anti HBc IgM become undetectable after six months of acute infection.
Molecular Diagnosis

Detection of HBV DNA in serum or tissue of infected patients has been shown to be a more direct and sensitive means of measuring viremia and viral infectivity than conventional serological tests. Conventional Southern blot, dot blot and slot blot, nucleic acid hybridization techniques and PCR have been developed for detection of HBV DNA (Janssen et al., 1993). The liquid hybridization method has a lower limit of detection of 1.6 pg/mL and is considered to be more sensitive than slot blot hybridization assays, which have sensitivity of 10 to 500 pg/mL. HBV DNA by PCR is considered to be the most sensitive method and may be positive when other HBV DNA tests are negative and is more sensitive than slot or dot blot hybridization assays and detects HBV DNA levels of approximately 100 to 1000 genomes, however PCR are prone to false-positive results. Detection of HBV DNA by hybridization indicates significant viral replication and a high probability of active liver disease (similar to that of HBeAg). Monitoring of the levels of HBV DNA by quantitative assays is useful marker of active replication, disease progression and response of treatment in chronic HBV infection.

Genotypes, Serotypes, Subtypes and Determinants

A genotype is generally defined as the genetic constitution of an organism or cell. In an evolutionary context, the term is sometimes more specifically applied to the stable forms into which, for example, viruses develop after undergoing a number of changes over a prolonged period, provided they have been characterized on the basis of their genomic sequences. If they have been characterized serologically, they are often labeled as subtypes or serotypes. It is sometimes presumed that the nature of genotypes or subtypes does not affect a hepatitis B infection and its clinical course (Grop, 1998). This subject, however, remains controversial.
To avoid confusion about the terminology, it is recommended that 'serotype' and 'serological subtype' be used as synonyms and that the less specific expression 'subtype' on its own be avoided. For similar reasons, the classical definition of the concept 'genotype' is advisable, particularly because the term has been applied to HBV strains (A-F) with unknown stability (Magnius and Norder, 1995).

In case of HBV, a number of serotypes have been described. The existence of the common 'a' determinant of HBsAg and the occurrence of at least two mutually subdominants, d or y and w or r, resulted in four serotypes adw, ayw, adr and ayr. They have a differential geographical distribution and have proven their value as epidemiological markers. The conventionally classified serotypes are sometimes erroneously called genotypes. Genetic analysis has, however, revealed that the four mentioned serotypes do not unequivocally correspond with single genotypes (Grop, 1998). At present, six HBV types (A-F) and nine serotypes have been defined. The 'a' determinant is of the greatest importance, since it is the dominant epitope cluster of HBV, conferring protection and binding most of the anti-HBs present in hyperimmune serum. Besides, it is highly conserved among the HBV serotypes, and for all of these reasons, HBsAg is a crucial component of current vaccines (Halder and Margolis, 1998).

The 'a' determinant is part of the major hydrophilic region (MHR) of HBsAg and its tertiary structure has been shown to be important for its antigenicity. In an originally proposed model it consists of 23 amino acids, situated between positions 124 and 147, and forms two loops protruding from the outer surface of the virus (Waters et al., 1992). Most specific antibodies obtained from vaccines bind to a region composed of nine amino acids, between positions 139 and 147. On the other hand, the 'a' determinant has to be considered as a conformational cluster of epitopes extending at least to

29
position 120, because antibody binding around amino acid 122 is affected by variation in the 139-147 region, whereas, antibody binding to amino acids 139-147 is in turn, influenced by variation between positions 120 and 124. Diagnostic assays designed to detect HBsAg make use of specific antibodies raised against the extended ‘a’ determinant (Carman et al., 1997).

Global Distribution of HBV Genotypes and Subtypes and its association

HBV is a member of the Hepadnaviridae family. The partially double-stranded circular DNA within its nucleocapsid consists of only about 3200 base pairs (Ganem and Varmus, 1987). For such small DNA virus, the HBV genome is not only characterized by considerable complexity, but exceptional degree of molecular variation. Over time, this spontaneous tendency for mutations has led to the emergence of at least eight genotypes (designated A to H), defined by the divergence of 8% or more with reference to the complete nucleotide sequence. Genotypes and serotypes are useful tools in understanding the epidemiology of HBV infection.

It is apparent that these genotypes show a distinct geographic distribution: in general, Genotype A is found in north-west Europe, United States and Sub Saharan Africa; Genotype B and C predominate in east and southern Asia (Theamboonlers et al., 1999), with latter prevailing in Oceania; Genotype D is associated with southern and central Europe (Borchani-Chabchoub et al., 2000), the Middle East, many parts of Central Asia, South America and India (Arankalle et al., 2003); Genotype E occurs uniquely in West and Southern Africa (Norder et al., 1994); and Genotype F found in Central and South America (Blitz et al., 1998) as well as in Polynesia. Genotype G has recently been localized in the United States and France (Stuyver et al., 2000) while an eighth genotype H has also been discovered in Central America (Arauz-Ruiz et al., 2002).
HBsAg has three major antigenic determinants two of which are allelic, “d, y” and “w, r” as well as “a” which is common to all strains that have so far been identified (Robinson, 1983). There are ten reported subtypes of HBV with the predominate types being adw, ayw and adr, in terms of geographical distribution (Bancroft et al., 1976). The subtype ayr is not found commonly but is observed in isolated pockets in Oceania. In Far East, some less common and unusual subtypes have been identified including awr, adwr, adyr, adyw and adywr (Robinson, 1983). These subtypes represent antigenic make-up on individual particles indicating some form of genetic recombination or other mechanism that has resulted in phenotypic mixing. In India ayw and adw are predominant, at least since 1979 (Thyagarajan et al., 1979). In China serotypes adr and adw2 were leading serotypes, adr was encoded completely by genotype C, while the majority of serotype adw2 was encoded by genotype B (Xia et al., 2001). A Study carried by Arankalle and Gandhe, (2003) on tribes of Andaman revealed all genotype D isolates belonged to ayw serotype, whereas serotype adw was encoded by genotype A. It has also been reported that ayw is not a simple subtype but is split into four different categories ayw1, ayw2, ayw3 and ayw4 and that this additional subtyping could differentiate more precisely the geographical distribution of HBV; ayw1 has been found only in Vietnam, ayw2 is the predominant subtype in the Mediterranean, ayw3 is prevalent in Greece and Yugoslavia along with ayw2 and ayw4 is mainly found in West Africa and also Central Africa along with ayw2 (Courouce-Pauty et al., 1983). (Table-2)

The most frequent combination of genotypes and subtypes are genotype A (subtype adw2, ayw1) prevalent in Western Europe, United States, Central and South America. Genotype D (subtype ayw2, ayw3 and ayw4) in the Mediterranean area, Central Asia and India. Genotype B (subtype adw2, ayw1) in East Asia and South East Asia. Genotype C (subtype adr, adrq-, ayr and adw2) in pacific Islands and South East Asia, Genotype E
Table 2

HBV Genotypes, Corresponding Serotypes, and Global Distribution

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Serotype</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>adw2, ayw1</td>
<td>North-west Europe, United States, sub-Saharan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Africa, Central and South America</td>
</tr>
<tr>
<td>B</td>
<td>adw2, ayw1</td>
<td>East Asia, South – East Asia</td>
</tr>
<tr>
<td>C</td>
<td>adw2, adrq+, adrq-, ayr, adr</td>
<td>East Asia, South-east Asia, Pacific Islands</td>
</tr>
<tr>
<td>D</td>
<td>ayw2,ayw3,ayw4</td>
<td>Mediterranean and Central Europe, Middle East,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Central, Asia, India, South America</td>
</tr>
<tr>
<td>E</td>
<td>ayw4</td>
<td>West Africa, Southern Africa</td>
</tr>
<tr>
<td>F</td>
<td>adw4q-, adw2, adw4</td>
<td>Central America, South America, Polynesia</td>
</tr>
<tr>
<td>G</td>
<td>adw2</td>
<td>United States, France (and still to be determined)</td>
</tr>
<tr>
<td>H</td>
<td>adw4</td>
<td>Central America</td>
</tr>
</tbody>
</table>

Erwin Sablon and Fred Shapiro, 2004
(subtype ayw 4, adw 2) predominant in West Africa, Genotype F (subtype adw 4q-, adw2, adw4) in Central America and Polynesia and Genotype G (subtype adw2) United States and France. Genotype H (subtype adw4) prevalent in Central America.

HBV genotypes and liver disease

Of the two billion persons worldwide who come into contact with HBV, approximately 6% of the global population (more than 400 million) fail to resolve acute viral infection and become chronic carriers, eventually placing between 15-25% of such individuals at risk of life-threatening sequelae of end-stage liver disease (Lai et al., 2003). It has become clear with time that clinical management of chronic HBV disease cannot be easily stereotyped since the epidemiology, natural history and response to the treatment of chronic HBV disease are far from uniform. There has been considerable variability with respect to the course of disease, complications during the course of treatment and outcome. Such heterogeneity can depend on factors such as the age at which infection is acquired, geographical, and ethnic, competence of the immune system and predisposing genetic factors such as preexistent mutations and genotype. Treatment approaches and possible responses depend to a large degree on patient’s HBeAg-positive or-negative status, ALT levels, possible co-infection with other viruses and severity of hepatic disease at time of treatment, degree of viral suppression, pre-existence of molecular variants, emergence of drug resistance and genotype.
HBV Genotyping Methodologies
Molecular Genotyping

HBV genotyping methodologies, including commercially available tests (Ex. Direct sequencing, Reverse Hybridization, PCR/genotype-specific primers) and home brew assays (Ex. RFLP and Multiplex PCR) have been reviewed recently (Bartholomeusz and Schaefer, 2004). In general, these methods include (i) Sequence Analysis; (ii) RFLP; (iii) Genotype-specific primers for single or multiplex amplification; (iv) Hybridization assay and (v) serological assays.

Sequence analysis-preceded by PCR amplification and followed by phylogenetic analysis-remains the corner stone of HBV genotyping and determination of new sequences. A commercially available genotyping test is the TRUGENE HBV Genotyping test (Bayer Diagnostics, Tarrytown, NY). Sequencing is limited, however, for detection of samples containing mixed genotypes. Standard DNA sequencing technology provides accurate and complete DNA sequence information and is applicable to any part of the 3-2 Kb HBV genome (Clarke and Bloor, 2002). A serious handicap of sequencing is its inability to detect viral resistance even the mutated virus still makes up a relatively large fraction (up to 30%) of the entire HBV population (i.e., mixtures of wild type and mutant species). This limits its use for detecting upcoming resistance at an early stage (Aberle et al., 2001). Furthermore, it tends to be time consuming and labor intensive, not readily adaptable to high throughput screening and is amenable to analysis only by well-trained personnel. For their part, home-brew RFLP based tests are widely used for research purposes, including HBV subtyping (Chen et al., 2004) and must necessarily is adapted when single nucleotide polymorphisms are present. RFLP methodology is as accurate as direct DNS sequencing (Jardi et al., 1999) but unlike sequencing, can detect samples mixed
virus populations containing mutant virus making up 5% to 10% of the virus population (Niesters et al., 2002). Nevertheless, the procedure is generally long and tedious (multiple PCRs, multiple enzyme digestions) and requires skilled personnel since a specific endonuclease reaction has to be developed for each separate mutation to be analyzed.

Genotype type primers for PCR have been developed (Naito et al., 2001) and commercialized (Genome Science Laboratories, Fukushima, Japan) (Kato et al., 2003) as well as for multiplex-PCR amplification (Kirschberg et al., 2004). This system allows the identification of types A through F. This assay system may be useful for rapid and sensitive genotyping of the HBV genome when their epidemiological, pathological or transmission studies of this agent are carried out in large scale.

A widely used commercial assay based on reverse hybridization is the INNO-LiPA HBV Genotyping test (Innogenetics, Gent, Belgium), in which labeled PCR-derived amplicons are hybridized to genotype-specific oligonucleotides immobilized on nylon strips (Hussain et al., 2003). Such tests are particularly sensitive and useful for detection of mixed genotype infections. Importantly, the test can detect variations early during the emergence of viral resistance even when the variant represents only a minor fraction of the total viral population (Lok, 2002). This is especially relevant for patients at high risk for disease progression. Finally, a novel approach to genotyping is the recently developed test based on real-time PCR and melting curve analysis, although this test has not yet been extended for use beyond genotypes B and C (Payungporn et al., 2004).
Serological Genotyping

Various serological assays have also been developed (Usuda et al., 2000) including a commercially available ELISA kit (HBV Genotype EIA, Institute of Immunology, Tokyo, Japan) amenable for larger studies. HBV genotypes were determined by an ELISA with mAb. The 4 ORFs for transcription are controlled by 4 promoter elements (preS1, preS2, core and X) and 2 enhancer elements (Enh I and Enh II). The mAbs were raised against genotype specific epitopes in pre S2-region product of HBV and labeled with HRP using a commercial kit. From the amino acid sequence found by reaction with mAbs genotypes A-F determined. Genotype G was determined by the combination of preS2-based ELISA genotype kits for genotype D and HBsAg subtype ‘adw’ (Kato et al., 2001). Sensitivity may, however be compromised in the presence of genotype mixtures or low levels of HBsAg in the sample.

HBV genotypes and therapy management

The study of possible clinical ramifications of HBV genotypes (Kao, 2003) is the subject of intense clinical research, with evidence accumulating that HBV genotype influences HBeAg seroconversion rates, the natural history and severity of liver disease, development of anti-viral drug resistance and precore /core promoter mutational patterns, as well as response to treatment. With respect to HBeAg seroconversion, substantial evidence has accumulated that the rate of seroconversion differs according to genotype, especially with respect to the widely studied differences between Far East Asian genotypes B and C (Kao et al., 2004). In particular, patients chronically infected with genotype B seroconvert earlier and more rapidly to anti-HBe; genotype B patients also carry the precore stop codon mutation more frequently.
Disease progression is also clearly influenced by the genotype that is present. Numerous Far East Asian studies indicate that genotype C is linked with more active liver disease than genotype B (Chan et al., 2003), while an Indian investigation indicated more severe disease consequences when carrying genotype D than A (Thakur et al., 2002). Furthermore, genotype also plays a role in the development of mutations. Although some studies showed that the rates of acquired drug resistance mutations to lamivudine were similar for genotypes B and C (Yuen et al., 2003), a recent European investigation determined that selection of mutations differed between genotypes A and genotype D. Genotype C is associated with more core promoter mutants than genotype B especially the double core promoter mutation T1762/A1764 (Miyakawa and Mizokami, 2003). There have been several trials exploring the impact of genotype on response to treatment with interferon-alfa (Seo et al., 2004) and Lamivudine (Chien et al., 2003). The general pattern that is tentatively emerging points to better outcomes with genotype B compared to genotype C. Recent studies showed that HBeAg-negative patients infected largely with genotype D, specific viral sequence patterns present before therapy appeared to be predictive of long term response to lamivudine treatment (Ciancio et al., 2004). However, all the studies carried that examined the influence of genotyping were of an exploratory nature and limited in size. Larger and well-controlled trials are needed to determine its definitive role in therapy management.

Prevention
Hepatitis B Vaccines
Active Immunization

The first commercially available vaccine, which was licensed by WHO in the early 1980s was plasma, derived HBsAg subunit vaccine (Szmuness et al., 1982). Although this vaccine provided excellent immunogenecity and
protective efficacy, it was not widely accepted because of unnecessary concerns of other blood borne infectious agents. The most common vaccine available in the market today is derived from a recombinant yeast source. The small hepatitis B surface protein (SHBs) is generated by the yeast cells. Expression of this protein by yeast results in SHBs particle formation. However, particles are not secreted by the yeast. Disruption of yeast cells is performed in order to liberate the produced spheres into solution. These particles are then purified through Ultrafiltration, Chromatography and Ultra centrifugation. The purified particles are then absorbed onto aluminium hydroxide to which thiomersal is added to preserve the solution. The two yeast-derived vaccines licensed in most countries are Engerix-B (SmithKline Beecham, Philadelphia, PA) and Recombivax HB (Merck & Co., West Point, PA). Both products are structurally and chemically similar with less than 2% yeast protein remaining in solution. Recombivax HB, however, is treated with formaldehyde before its adsorption onto alum. As both are yeast-derived, the S-protein is not glycosylated (as yeast does not possess the correct post-translational machinery to do so). Both, thankfully, appear to be quite effective as vaccines, allowing for immunization against the various forms of HBV.

The vaccines, however, should not be frozen, as this appears to be deleterious to its immununogenicity. Studies have shown that freezing of these vaccines results in lower immune responses. There are also other forms of immunization and vaccines. However, the ones mentioned above generally appear to be the most effective and the most widely used. Typical vaccination schedules are 0, 1 and 6 months or 0,1,2 and 10 months. The 0,1 and 6 month vaccination schedule is preferred for routine pre-exposure prophylaxis. The four-dose schedule may be preferred for immuno-compromised patients or for post exposure prophylaxis. It has been recommended that a booster dose be given for every five to 7 years after the initial vaccination. Infants may also be
vaccinated this way. However, there have been some rare reports of adverse reactions to yeast derived vaccines. Some possible adverse reactions include skin, rheumatic, vasculitis, ophthalmologic, hematological and neurological reactions (Grotto et al., 1998). The immunogenicity and protective efficacy of hepatitis B vaccination is directly related to the levels of anti HBs titers of greater than 10mIU/ml after a primary vaccination series. The vaccines produced by each manufacturer have to be evaluated in clinical trials to determine the age specific dose that achieves the maximum seroprotection rate.

**Passive Immunization**

HBIG is prepared by the Cohn Oncly fractionation procedure from the serum containing high titers of anti HBs and is standardized to 100,000 IU of anti HBs/ml. HBIG is effective often in combination with Hepatitis B vaccine, as post exposure prophylaxis following a) Perinatal exposure for an infant born to an HBsAg positive mother. b) Percutaneous or mucous membrane exposure to an HBsAg positive blood c) Sexual exposure to an HBsAg positive person (Beasley et al., 1983).

**Treatment**

Advances in the treatment and prevention of hepatitis B virus (HBV) infection represent among the most dramatic achievements of modern medicine and have resulted from fully exploiting discoveries in molecular immunology and virology as well as rational drug design. Many antiviral agents have been investigated as candidates for chronic Hepatitis B virus infection. In 1976, two studies one with leukocyte interferon and one with β-interferon have suggested that it can affect the serologic profile of chronic HBV infection (Greenberg et al., 1976). The most promising anti-viral agent
is α-2b interferon, which has been licensed by the Food and Drug Administration. The goals for treatment are three folds. They are to eliminate infectivity and transmission of HBV to others, to arrest the progression of liver disease, improve the clinical prognosis and to prevent the development of hepatocellular carcinoma.

But currently, interferon treatment is the method of choice for treatment of chronic Hepatitis B. The recommended regimen is either 5 million units daily or 10 million units three times a week; given subcutaneously for 4 months (Mahoney, 1999). However, patients who are positive for HBsAg and negative for HBeAg and DNA, are not likely to benefit from interferon treatment.

Nucleoside Analogue

First generation nucleoside analogues like Vidarabine, Acyclovir, Ganciclovir, Zidovudine, Ribavirin, Didanosine and Zalcitabine did not effectively suppress viral replication and had serious side effects. The second-generation nucleoside analogue like Fialuridine, Lamivudine, Famciclovir, Lobucavir, Adefovir, BMS 200475 and Emtricitabine were more effective in treatment. Fialuridine, a fluoro-iodo-arabinofuranosyl-uracil nucleoside, markedly suppressed HBV DNA (Fried et al., 1992) but caused multisystem toxicity due to mitochondrial dysfunction in patients treated for more than two months (McKenzie et al., 1995).

Lamivudine

Lamivudine therapy is being studied as a possible method of treatment for chronically infected hepatitis B virus carriers. So far, evidences show a tolerated and sustained suppression of HBV replication during treatment.
There also appears to be a decrease in HBe antigen and HBs production in patients treated (Nevens et al., 1997). In-patients receiving long-term therapy with Lamivudine, resistance has been noted in the form of mutations at the YMDD locus (Atkins et al., 1998). The best mutation described so far is the substitution of alanine or isoleucine for methionine at residue 552.

**Lobucavir**

Lobucavir is a guanosine nucleoside analogue with activity against many viruses. In a study by Heathcote et al., (1998) in 81 patients treated with Lobucavir for 12 weeks at doses of 200 to 800 mg, there was suppression of HB DNA than in those who received placebo (P<0.001). Extended course of treatment with Lobucavir was found safe and efficacious (Bloomer et al., 1998). The major side effects included mild anorexia, dizziness and abdominal pain. The manufacturer recently halted the clinical testing of Lobucavir because of concerns about a possible association between long-term administration and neoplasia in mice and rats.

**Famciclovir**

Famciclovir, an acyclic guanine derivative, which also inhibits HBV DNA polymerase (Bartholomeusz et al., 1997). In a preliminary trial, Famciclovir, given 500mg three times daily, suppressed HBV DNA in all patients and resulted in HBeAg seroconversion in a small minority of patients. Famciclovir resistance (Bartholomeusz et al., 1997) is associated with mutation at residue 528 (domain B) of the HBV DNA polymerase. This is the reason for Famciclovir to overcome Lamivudine resistance and for this reason; it is less attractive than other nucleoside analogues.
Adefovir

Adefovir is an adenine nucleotide analogue that is administered as the prodrug Adefovir dipivoxil. Clinical trials (Bloomer et al., 1997; Gilson et al., 1998), have suggested that Adefovir may be effective as first line monotherapy for the treatment of chronic HBV infection. In studies where 12 weeks of Adefovir treatment at daily doses of 30mg or greater resulted in reduction of 4 log copies in levels of HBV DNA (P<0.001) compared with controls. Levels of HBV DNA returned to baseline after treatment in patients who did not seroconvert. Although Adefovir is well tolerated, the development of renal injury in patients treated with doses of 30mg and higher suggests that a cautious approach towards adverse side effects is needed.

Immunomodulator Therapy

Immunomodulators are non-HBV specific and HBV specific. Non-HBV specific immunomodulators include Thymosin, Interleukin-2, Interleukin-12 and Levamisole. HBV specific immunomodulators include pre S or S peptide vaccination, cytotoxic lymphocyte epitope vaccination and DNA vaccination (Malik, 2000). Treatment with thymosin for 6-12 months has been associated with a greater HBV DNA and HBeAg response (Andreone et al., 1996; Chien et al., 1998) mutations have also been reported after thymosin treatment (Tang et al., 1998). Large scale randomized controlled studies in humans are to be conducted.

Combination Therapy

Combination therapy as in the case of HIV treatment may yield a better response. Combination therapy should include an antiviral, which will reduce the viral load, an immunomodulator, which might eliminate residual
intracellular virus and therapeutic immunization, which will induce the loss of the carrier state. As on date combination therapy (Perillo et al., 1990) like the one conducted in United States and Canada with Lamivudine and Interferon did not show improvement in patients who did not respond to treatment with Interferon alone. Although the initial results are not very promising, such combination therapies could hold an answer for effective treatment of chronic HBV infection.

Although interferon therapy may be used in a selected group of patients, it is a short-term therapy and does not rule out the use of Lamivudine if seroconversion doesn’t occur. Although Lamivudine can be used for all patients with HBV infection who have active viral replication, the drawback to nucleoside analogue monotherapy is the development of mutated HBV strains that are resistant to these drugs.
HEPATITIS C VIRUS (HCV)

Introduction

HCV Virion

Structure and Genome Organization

Clinical Spectrum

- Acute Hepatitis C
- Chronic Hepatitis C
- Hepatocellular carcinoma

Diagnosis

- Contribution of HCV in causing Liver Diseases
- Acute hepatitis and fulminant hepatic failure
- Chronic Liver Diseases
- Progression of Liver Disease

Treatment

Genetic Diversity

HCV Genotypes and Geographical Distribution

Genotypes and HCV Genetic Heterogeneity as Epidemiological Markers

Methods for HCV Genotyping

- Molecular Genotyping
- Serological Genotyping
- Response to Interferon Therapy
INTRODUCTION

Hepatitis C virus (HCV) is a member of the genus Hepacivirus in the Flaviviridae family. It is responsible for most parenteral non-A, non-B hepatitis (NANBH) virus infections. In 70% of cases HCV infection is characterized by virus persistence leading to chronic infection with manifestations like chronic hepatitis, cirrhosis and hepatocellular carcinoma (Saito et al., 1990). It is estimated that 170 million individuals are infected with HCV worldwide (Pileri et al., 1998). An astonishingly high proportion of individuals acutely infected with HCV become chronically infected (approx. 70%) and more than 40% of these individuals will actually develop liver cirrhosis (EASL, 1999). Interestingly, a strong association has been found between HCV infection and development of HCC (Bukh et al., 1995). This virus is globally distributed with anti-HCV rates among donors throughout the world ranging from 0.3-1.5% (Alter et al., 1997). The prevalence of anti-HCV antibodies in the Indian population varies from 0.3-1.8% (Deshpande and Khodaji, 1998; Shah et al., 1998). In India the HCV carrier rate is approximately 1.2% (Nishioka, 1994; Tandon et al., 1994) of which 70% are RNA positive. About 15-25% of patients with acute hepatitis recover and remain HCV RNA negative on multiple determinations over the years of follow up evaluation (Alter et al., 1997). The virus is transmitted parenterally as well as through perinatal route. In parenteral transmission the routes of infection include transfusion of blood and blood products, abuse of intravenous drugs, tattoos, sexual or household transmission (Watson et al., 1996). Perinatal transmission from mother to child (Ohto et al., 1994) and transmission by breast milk (Kumar and Sahul, 1998) have been reported. Thus the hepatotropic virus has emerged as an important pathogen posing a challenge to the medical community worldwide.
In 1989, Choo et al., first isolated part of the HCV genome by immuno screening a cDNA library derived from plasma of a chimpanzee chronically infected with HCV. The HCV genome was considered to be a positive stranded RNA molecule of about 10 kilo bases (Choo et al., 1989). An anti-HCV enzyme linked immunosorbent assay (ELISA) using recombinant HCV antigen, which was expressed in yeast, was also simultaneously developed by Chiron (Kuo et al., 1989). Thus HCV was established as a major causative agent of parenteral non-A, non-B hepatitis worldwide.

**HCV Virion**

Analysis of the structure of HCV particles has been hampered by the low titer of virus infectious sera and also due to the difficulty of replicating the virus in culture systems. Particles with diameter of 45 to 65 nm have been observed by electron microscopy in human plasma, chimpanzee liver and experimentally infected (Shimuzu et al., 1996) or transfected cell lines (Mizuno et al., 1995). Some studies have suggested the possibility that HCV particles are present in the circulation as immune complexes (Hijikata et al., 1993) or in association with serum lipoproteins (Prince et al., 1996).

**Structure and Genome Organization**

The genome of HCV comprises a positive stranded RNA molecule of about 10 kilo bases (Choo et al., 1989). A large Open Reading Frame (ORF) extends throughout the HCV RNA genomic sequence and encodes a single polypeptide of about 3010 amino acids (Chen et al., 1992). The genome contains two untranslated regions at the 5’ and 3’ ends of the genome. The 5’ UTR is ~340 nucleotides long, whereas the 3’ UTR has a tripartite structure. Interestingly, computer assisted analysis of the hepatitis C virus genome has revealed the presence of an additional out-of frame ORF
overlapping the core gene in the +1 frame (Ina et al., 1994; Smith and Simmonds, 1997). This novel ORF codes for 124-160 codons in most of the hepatitis C virus strains. Thus a putative polypeptide of ~14-17 kDa could be potentially synthesized by an alternate translation mechanism (Xu et al., 2001 and Varaklioti et al., 2002). Another recent study showed that ribosomal frame shifting could actually occur in three frames: zero, +1 and +2 leading to the formation of different translation products (Choi et al., 2003). The large polypeptide precursor encoded is processed co-and post translationally by host and viral proteases to yield a variety of structural proteins and non-structural proteins. Structural proteins are processed from the N-terminal region of HCV polyprotein precursor, beginning with an RNA-binding nucleocapsid of basic charge (C; 21-22kDa) followed by two glycoproteins (E1;32-35 kDa and E2;68-72 kDa). Additional proteins derived from core (16 and 19 kDa) and p7 (7 kDa) that lies between E2 and NS2 have been found. The non-structural proteins (NS2, NS3, NS4A, NS5A and NS5B) are found in the remaining portion of the polyprotein (Grakoui et al., 1993). (Table-3) (Fig- 4 & 5)

Clinical Spectrum
Acute Hepatitis C

HCV accounts for approximately 20% of cases of acute hepatitis, 70% of chronic hepatitis and 30% of end stage liver disease (Hoofnagle, 1997). The acute infection has an incubation period of 7 weeks and is symptomatic only in one third of patients (Barrera et al., 1995). Serum aminotransferase (ALT) levels generally increase 1.5 to 10-fold the upper limit of normal and decrease to the normal range as signs and symptoms resolve (Conry-Cantilena et al., 1996). In one study of transfusion-related acute NANB hepatitis, only 25% of cases were icteric and less than 10% were seriously ill. Although antibodies to HCV are usually, though not always, present at the time of onset
<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>GENOMIC REGION</th>
<th>AMINO ACID POSITION</th>
<th>MAJOR FUNCTIONS</th>
</tr>
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<tbody>
<tr>
<td>p21</td>
<td>Core</td>
<td>1-191</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>gp31</td>
<td>E1</td>
<td>192-383</td>
<td>Envelope glycoprotein</td>
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<td>gp68-72</td>
<td>E2/NSI</td>
<td>384-809</td>
<td>Envelope glycoprotein</td>
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<td>E2-A</td>
<td>E2/NSI</td>
<td>747-809</td>
<td>No known</td>
</tr>
<tr>
<td>p7</td>
<td>E2/NSI</td>
<td>801-1026</td>
<td>NS2/NS3 metallo-protease Component</td>
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<td>p23</td>
<td>NS2</td>
<td>801-1026</td>
<td>NS2-3 protease Component</td>
</tr>
<tr>
<td>p70</td>
<td>NS3</td>
<td>1027-1657</td>
<td>Serine-protease: helicase; NS2/NS3 metallo-proteased Component</td>
</tr>
<tr>
<td>p8</td>
<td>NS4A</td>
<td>1658-1711</td>
<td>NS3/4 protease –cofactor</td>
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<tr>
<td>p27</td>
<td>NS4B</td>
<td>1712-1972</td>
<td>Membrane-associated replicase Component?</td>
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<tr>
<td>p56-58</td>
<td>NS5A</td>
<td>1973-2420</td>
<td>Not known</td>
</tr>
<tr>
<td>p68</td>
<td>NS5B</td>
<td>2421-3011</td>
<td>RNA-dependent RNA polymerase</td>
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Figure 4  STRUCTURE OF HEPATITIS C VIRUS

Figure 5  ORGANIZATION OF HCV GENOME

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of symptoms, HCV RNA is detectable in serum within one to two weeks of exposure and increase to levels of 10\(^6\) to 10\(^8\) genomes/ml at the time of symptoms, and then disappears during resolving disease. Only 20% of patients have symptoms that are usually intermittent, vague and non-specific (Hoofnagle, 1997). They include fatigue, lethargy, malaise, lack of energy, nausea, poor appetite, arthralgia, fever, weakness and weight loss. Symptoms are rarely incapacitating but they can cause decrease in the quality of life. This clinical syndrome occurs within 2-26 weeks of exposure and lasts for 2-12 weeks.

**Chronic Hepatitis C**

In patients who develop chronicity, HCV RNA persists and ALT levels remain elevated and anti-HCV remains to be positive for more than 6 months and histological findings consistent with chronic liver inflammation (Muller, 1996). The course of chronic HCV infection is variable. The factors that lead to chronic hepatitis C are not well defined. 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 (Main, 1995; Muller, 1996). The quasispecies nature of HCV and the tendency of the envelope gene of the virus to mutate rapidly may be key factors responsible for the process. Since only 15% of patients clear HCV infection, while majority do not, it may be thought that cellular immunity plays an important role in HCV infection.
Patients with chronic HCV often have no symptoms but may complain on-specific symptoms such as fatigue, muscle aches, anorexia, right upper rant pain and nausea. Cirrhosis occurs in less than 20% of the patients hepatitis C, usually becomes detectable in the second or third decade after infection. An undermined proportion of chronic infections are asymptomatic with normal liver enzymes and relatively normal liver histology.

Factors that influence the rate of progression of chronic hepatitis C to cirrhosis and hepatocellular carcinoma include alcohol abuse, age at time of infection, liver histology at initial biopsy and viral titre. The role of other factors, such as viral genotype, co-infection with HBV or HIV, gender and immunogenetic factors are less well understood. The prevalence of hepatitis C virus among chronic liver disease (CLD) in India has been reported to be between 12.5% to 48% (Amarapurkar et al., 1992; Irshad and Acharya, 1994; Panigrahi et al., 1994; Issar et al., 1995).

**Hepatocellular carcinoma**

Most patients with HCV-related HCC appear to have developed the tumour as a consequence of long-standing infection accompanied by chronic and progressive liver damage. Progression from acute-transfusion hepatitis C to HCC through the intermediate steps of chronic persistent hepatitis (CPH), chronic active hepatitis (CAH), and cirrhosis has been described in individual cases and may take as long as 18 years (Kiyosawa et al., 1990). There is persistently elevated ALT levels observed in patients with HCV associated HCC, which confirms the activity of liver disease, which is characterized by inflammation, necrosis and regeneration.
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, USA and Japan, HCV may be more important than HBV in this context (Main, 1995; Caselmann and Alt, 1996). The first study from north India reported 15.1% anti-HCV positivity among 53 HCC patients investigated (Ramesh et al., 1992). A 10% anti-HCV positivity was noted among 20 HCC patients examined from different part of north India (Kapoor et al., 1999). Based on the study of 78 HCC patients from south India, Hussain et al, (1999) concluded that HCC incidence due to HCV infection was low. Another study from south estimated 14.7% anti-HCV positivity among 170 HCC cases investigated (Murugavel et al., 1999). In India HCV is the cause in only 4% of HCC, while dual infection with HBV and HCV is the cause in 8% cases. HBV alone constituted as the cause in 71% Patients (Sarin et al., 2001).

The difference in HBV-induced HCC and HCV-induced HCC is that 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-seropositive and seronegative 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).
Diagnosis

Before the identification of HCV and the development of specific viral markers, diagnosis of the infection, then called as NANBH (non-A, non-B hepatitis), was made (a) if the individual had no serological markers for hepatitis A and B viruses (b) if there was no obvious potential cause for hepatitis such as infection with EBV, CMV and alcohol and drug abuse (c) if there was no evidence of any metabolic disorder such as Wilson’s disease or hemochromatosis. With the advent of specific tests for HCV infection, it became evident that the majority of NANBH cases in Western Europe, United States and Japan were due to HCV.

Serodiagnosis is accomplished by monitoring the presence of circulating antibodies to HCV using commercial ELISA kits. The first generation ELISA kit comprised of the c100-3 epitopes from the NS4 region, in the second generation ELISA kit the c22-3 and c33-c proteins were incorporated in addition to c100-3. The third generation kits comprised of c100-3, c22-c and NS5 recombinant antigen. In the fourth generation ELISA, synthetic peptides to core, NS3, NS4 and NS5 were incorporated instead of recombinant antigens. With the use of successive new generation ELISA kits the detection of anti-HCV antibodies has been shortened from 16 weeks to 6-8 weeks after infection (Gretch, 1997; Schiff et al., 1999)

Positive ELISA reactions are confirmed using a supplementary test RIBA (Recombinant Immunoblot Assay) (Ortho Diagnostic Systems, NJ) and LIATEK (Line Immunoassay) (Organon Technical), which comprises of individual antigens separated on a paper strip. Although in high risk groups like patients with clinical liver disease and /or elevated alanine
aminotransferase (ALT) levels, the confirmation rate is high. In low risk
groups like voluntary blood donors, many positive ELISA results are
unconfirmed. CDC issued fresh guidelines for HCV result reporting (Alter
et al., 2003). Presently, two frequently used sensitive techniques to monitor
viremia are Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and
branched DNA (bDNA) assay. While the former can detect as few as
10 molecules of the viral RNA within a few days of viral infection by PCR
amplification of the reverse-transcribed HCV cDNA, the latter involves a
rapid, convenient and quantitative assay for capturing of viral RNA using
immobilized DNA oligomers. Other methods that have been developed for
detection of HCV antigens such as immunofluorescence, in situ hybridization
and immunostaining techniques although these methods have so far been used
only in experimental situations rather than in clinical diagnostics.

In view of the observations that different HCV strains vary in their
response to interferon therapy and may have different pathogenicity, it is
becoming increasingly important to be able to diagnose the particular type
and subtype of the infecting HCV. In this context nested RT-PCR, type
specific RFLPs and INNO-LiPA (Line probe Assay) (Innogenetics, Belgium)
are important. Due to the lower titre of the circulating virus, a direct assay for
the viral antigen has not been developed. The commercially available
diagnostic tests for HCV antibody and RNA include the different enzyme
immunoassays manufactured by various companies but they cannot
discriminate between ongoing and resolved infection. Since no in vitro system
for growing the virus efficiently is available and no sensitive immunoassay to
identify the HCV antigens in blood is available.
Contribution of HCV in causing Liver Diseases

Acute hepatitis and fulminant hepatic failure

HCV is responsible for < 8% of cases with sporadic acute viral hepatitis in India (Das et al., 2001). The acute phase of hepatitis C is often mild or inapparent and usually less severe than hepatitis A & B. As far as India is concerned, hepatitis E virus (Arankalle et al., 1993) and not hepatitis C virus represents the etiologic agent (Irshad and Acharya, 1994). This is in contrast to reports from the west wherein almost 90% sporadic non-A, non-B hepatitis cases could be attributed to HCV (Hollinger and Lin, 1992). Except for two unusual reports from North India (Singh et al., 1998) association of HCV with acute liver failure among Indian patients does not seem to be frequent (Mehta et al., 1992). Factors responsible for drastically different association of HCV and fulminant hepatic failure in north Indian patients from the same city, especially when proportion of sporadic hepatitis patients is uniformly low, needs to be analysed. Except for Japan, HCV seems to play a minor role in causing fulminant hepatic failure (FHF) (Bendre et al., 1999).

Chronic Liver Diseases

Almost 80% cases previously classified as non-A, non-B or cryptogenic chronic hepatitis or cirrhosis were diagnosed as hepatitis C as soon as serological assay became available (Hopf et al., 1990). It was soon apparent that 50-70% of HCV infections lead to chronicity. Dual infections with HBV and HCV were shown to result in suppression of HBV replication but acceleration of progression of liver disease, the result being more severe lesions in patients infected with both viruses (Chan et al., 1991). Different findings, with no apparent suppression of HBV replication, have been reported in cases of post-transfusion hepatitis C superimposed on chronic hepatitis B (Fattovich et al., 1991). HCV also has been documented to play an
important role in alcoholics with chronic liver disease (Koff and Dienstag, 1995). Hepatitis C is an important cause of chronic liver disease including cirrhosis among the Indian patients (Sood et al., 1999). Approximately 10-20% of such patients are anti-HCV positive (Issar et al., 1995). Among the pediatric population chronic liver disease due to HCV was seen in 7.5% of patients (Dangwal et al., 1997).

**Progression of Liver Disease**

The role of HCV genotypes in the progression of liver disease is one of the most controversial areas of research. There appears to be significant biologic variation in HCV disease expression in the host over the length of the infection (typically the life of the patient). Currently, investigators are divided into those who strongly believe in differences in pathogenicity among genotypes and those who do not. Conclusions have been derived from indirect evidence, because conducting accurate investigation to answer these questions has been difficult. The role of genotypes as an independent factor in the progression of liver disease cannot be separated from the roles of other cofactors such as viral load, alcohol intake and the length of HCV infection. Patients may not provide accurate information about drug use or the amount of alcohol intake. Therefore, the time of HCV acquisition often is not known. Because of the overall slow progression of liver disease in HCV-infected patients, prospective studies frequently are not possible.

In patients with chronic HCV, infection with genotype 1b is associated with more severe liver disease and more aggressive course than infection with other HCV genotypes. Similar to others (Zein et al., 1996) found that HCV genotype 1b was significantly more prevalent among patients with liver cirrhosis and those with decompensated liver disease requiring liver
transplantation than among with chronic active hepatitis C. There is compelling evidence that hepatocellular carcinoma occurs more frequently or emerges earlier among HCV-infected Japanese patients (Takahashi et al., 1993; Yano et al., 1993) than among HCV carriers in Western countries (Hopf et al., 1990). HCV genotype 1b was present in most of the patients with HCV-associated hepatocellular carcinoma studied by Zein et al, (1996). Similarly, Reid et al, (1994) determined the HCV genotypes in 28 patients with hepatocellular carcinoma and found that 19 (68%) were infected with HCV genotype 1b and the rest were infected with mixture of HCV genotypes that always included genotype 1b.

Some reports refute the association mentioned above (Lau et al., 1996; Naoumov et al., 1997). A possible and simple explanation may reconcile these reported discrepancies. Zein et al, (1996) found that patients infected with HCV genotype 1b were older than those infected with other genotypes. Thus HCV genotype 1b is a marker for more severe HCV associated liver disease, because it reflects a longer time of infection rather than a more aggressive form of hepatitis C. Zein et al, (1995) and Gordon et al, (1997) reported that in liver transplant recipients, HCV genotype 1b is associated with earlier recurrence and more severe hepatitis than other genotypes. Although others have reported similar findings, some authors have suggested that there is no association between genotype and HCV recurrence after transplantation (Boker et al., 1997). The difference in the duration of infection that may have been a factor in non-transplant- associated HCV patients is not likely to explain the discrepancies in the literature about post transplantation HCV.
Treatment

More than 100 million people worldwide are infected with HCV infection. Majority of them have no symptoms although they can transmit this blood-borne virus to others. People who have been infected for decades may present for treatment because of complications related to advanced liver diseases. The public health implications of HCV has come to light only recently due to epidemiological studies that pointed out the magnitude of infection and an alarming increase in morbidity and mortality due to HCV related disease. Recent studies on natural history of HCV infection indicate that the majority of people with chronic HCV infection have relatively mild disease with slow progression and many of them die probably with the disease, rather than of it. Currently, treatment is recommended for patients have persistently elevated ALT levels or fibrosis with moderate inflammation of the liver. Treatment for HCV relied initially on the antiviral effects of interferon. Treatment with standard dose of interferon alfa (3 million U, 3 times a week for 12 months) normalizes ALT levels and leads to the disappearance of HCV RNA from serum in approximately 40 % of patients with chronic hepatitis C (Hoofnagle and Di Bisceglie, 1997). However this response is transient in majority of patients. Only 10-15% of patients have a sustained response after treatment is stopped. Longer duration of treatment (18-24 months) or higher doses of interferon alfa (upto 30 million U per week) and treatment with other types of interferon (pegylated) may improve the response, many patients still relapse. Moreover all these treatment modalities are costly and poorly tolerated (Poynard et al., 1996; Farrell et al., 1998).

Response to interferon therapy differs among different HCV genotypes. Of the two predominant genotypes, HCV 1a and HCV 1b, the former predominates in America and Europe while the latter predominates throughout most Asian countries. Recently the NS5A gene of type 1b has
been sequenced and mutations within a discrete region of it has been recognized and termed as the ISDR (interferon sensitivity determining region). This has been compared with the sequence of the interferon sensitive phenotype. It was found that the strains closely matching the prototype HCV 1b NS5A sequence correlated with complete interferon resistance (Gale et al., 1997). The high frequency of IFN resistant HCV in the human population suggests that HCV, like many other animal viruses, has evolved mechanism to block the IFN mediated response to viral infection. Although the function of NS5A and its role in viral replication is unknown, it has been suggested that NS5A, by an ISDR directed mechanism, may mediate IFN resistance by interacting with and repressing one or more IFN-induced anti-viral gene products (Gale et al., 1997).

Presently a combination therapy of interferon alfa 2b (IFNα-2b) and ribavirin has been found to have impressive rates of sustained virological response leading to the absence of HCV RNA in serum 24 weeks after completion of treatment. In the last years antisense oligonucleotides have been proposed as a promising new class of therapeutic agents. The mechanisms by which oligonucleotides are known to inhibit gene expression are distinct from the action of therapeutic agents as interferons and nucleoside analogs. Antisense oligonucleotides are small single stranded DNA molecules that can be targeted to a complementary sequence of an RNA molecule. The binding of antisense oligonucleotides to RNA can block the translation by hybrid arrest of the translational machinery or by the induction of ribonuclease H activity, resulting in the cleavage of the RNA portion of the hybrid. The substitution of a non-bridging oxygen atom in the internucleotide phosphate by a sulphur atom (phosphorothionate) makes the antisense oligonucleotides resistant to the action of nuclease without influencing their biological activity. Several studies have reported the inhibition of viral gene expression in cultured cells by the use of phosphorothioate oligonucleotides (Seki and Honda, 1995).
Genetic Diversity

Genetic variability of HCV exists at several different levels. Most obvious is the substantial genetic divergence of the main genotypes of HCV, which frequently show specific geographic ranges in human population and associations with particular risk groups for infection. Below this and arising from sequence drift over a much shorter period is the variability observed between individual variants (or strains). Much of the sequence diversity observed between such strains (such as 5-8% divergence observed between variants in epidemiological unlinked genotype 1a, 1b and 3a infections) reflects processes of neutral sequence drift over time after the introduction of HCV into new risk groups in the 20th century. It is also possible that some of the sequence divergence may be phenotypically selected changes, associated with adaptation for replication in different individuals with different immune responses to infection. Finally, HCV diversifies measurably within an infected individual overtime, forming what has been described as "quasispecies". This pre-existing genetic variability, combined with an extremely large replicating population size of HCV in a chronically infected individual provides a large pool of genetic variants that can readily adapt to new selection pressures such as immunological recognition and antiviral treatment.

HCV Genotypes and Geographical Distribution

It is important to note that the distribution is not static; as frequency of infection increases and the prevalence of different genotypes and subtypes within a country or region changes. Comparison of nucleotide sequences of variants recovered from infected individuals in different risk groups for infection and from different geographical regions has revealed the existence of at least 6 major genetic groups. As an average over the complete genome,
these differ in sequence by 30-35% of nucleotide sites, with more variability concentrated in regions such as the E1 and E2 glycoproteins, while sequences of the core gene and some of the non-structural protein genes such as NS3 are more conserved. Least sequence variability between genotypes is found in the 5’ untranslated region, where specific sequences and RNA secondary structures are required for replication and translation functions. Despite the sequence diversity of HCV, all genotypes share the genome organization, each containing an ideal complement of co-linear genes similar or identical size. However, contrasting with this general observation is the marked variation in their capability to express a further protein generated by a translational frameshift at codon 11 of the core gene (Xu et al., 2001; Varaklioti et al., 2002), where both the frame shift site and potential size of this novel coding sequences are very poorly conserved between and within genotypes. This contrasts greatly with the evolutionary conserved nature of so many other aspects of HCV replication, and supports our recent proposal that this “gene” is a computational artifact arising from RNA structure-imposed constraints on 3rd codon position variability in the core gene (Tuplin et al., 2002).

Each of the major genetic groups of HCV contain a series of more closely related subtypes, typically different from each other by 20% in nucleotide sequences compared with >30% between genotypes (Simmonds et al., 1993). Some, such as genotypes 1a, 1b and 3a have become very widely distributed as result of transmission through blood transfusion and needle sharing between infecting drug users (IDUs) over the past 30-70 years, and now represent the vast majority of infections in western countries. These are the genotypes most commonly encountered clinically, and for which most information has been collected on response to interferon and other antiviral treatments.
A different pattern of sequence diversity is observed in parts of Africa and South East Asia. Here, there are close associations between genotypes and specific geographical regions. For example, infections in western Africa are predominantly of genotype 2 (Wansbrough – Jones et al., 1998; Candotti et al., 2003), while those in Central Africa, such as the Democratic Republic of Congo (DRC) and Gabon are by genotype 1 and 4 (Menendez et al., 1999; Ndjomou et al., 2003). In both regions, there is a remarkable diversity of subtypes; for example, 20 of 23 HCV-seropositive blood donors in Ghana (west Africa) were infected by genotype 2, but each corresponded to different and previously undescribed subtypes (Candotti et al., 2003). This diversity is reproduced in Guinea, Benin and Burkina Faso (West Central Africa), where 18 different subtypes of genotypes 1 and 2 were found in samples from 41 HCV-infected individuals (Jeannel et al., 1998). These field observations reflect both huge genetic diversity of genotypes 1,2 and 4, and also its likely long-term presence in human populations in these parts of Africa. Taking this geographical mapping further, genotypes 3 and 6 show similar genetic diversity in South and Eastern Asia (Mellor et al., 1995).

The model suggested by these genotype distributions is that HCV has been endemic in sub-Saharan Africa and South East Asia for a considerable time, and the occurrence of infection in Western and other non-tropical countries represents a relatively recent emergence of infection in new risk groups for infection (Ndjomou et al., 2003). In the 20th century, parenteral exposure to blood-borne viruses became frequent through the wide spread adoption of blood transfusion since the 1940s, the medical use of unsterilised needles for injections and vaccinations (a practice that continues in many developing countries) and most specifically to industrialized countries, injecting drug use and sharing of injection equipment. These new routes for transmission account for the epidemiological and genetic evidence for recent epidemic spread of HCV over the past 50 years in Europe, Egypt and elsewhere (Coehrane et al., 2002and Ndjomou et al., 2003).
Although HCV genotypes 1, 2 and 3 appear to have a worldwide
distribution, their relative prevalence varies from one geographic region to
another. HCV subtypes 1a and 1b are the most common genotypes in the
United States (Zein et al., 1996). These subtypes are also predominant in
Europe. In Japan, subtype 1b is responsible for up to 73% of cases of HCV
infection (Takada et al., 1993). HCV subtypes 2a and 2b are relatively
common in North America, Europe and Japan. subtype 2c is found commonly
in Northern Italy. HCV genotype 3a is particularly prevalent in intravenous
drug abusers in Europe and United States. HCV genotype 4 appears to be
prevalent in North America and Middle East (Chamberlain et al., 1997) and
genotypes 1b and 3 are predominantly found in India. In south India
Valliammaei et al., (1995), sequenced 24 HCV infected patients of which 21
showed HCV genotype 1 and other three to be of HCV genotype 3.

Recently a study in South India by Sukanya et al., (2003) sequenced
90 patients with chronic HCV infection for genotype determination. The
genotype profile most frequently detected was genotype 3 followed by
infection with genotype 1. Genotype 4 was seen in 5 patients and one patient
had infection with HCV genotype 2 (Sukanya et al., 2003). In north India,
Panigrahi et al., (1996) sequenced 11 HCV samples, among which 7 samples
were found to be genotype 3 and three samples were found to be genotype 1;
one sample was co-infected with 1 and 3 genotype. Genotypes 5 and 6 seem
to be confined to South Africa and Hong Kong respectively (Cha et al., 1992).

The geographic distribution and diversity of HCV genotypes may
provide clues about the historical origin of HCV (Smith and Simmonds,
1997). The presence of numerous subtypes of each HCV genotype in some
regions of the world, such as Africa and South East Asia, may suggest that
HCV has been endemic for a long time. Conversely, the limited diversity of
subtypes observed in the United States and Europe could be related to the recent introduction of these viruses from areas of endemic infection. (Table-4) (Fig-6)

**Genotypes and HCV Genetic Heterogeneity as Epidemiological Markers**

Because of geographic clustering of distinct HCV genotypes, genotyping may be a useful tool for tracing the source of an HCV outbreak in a given population. Examples include tracing the source of HCV infection in a group of Irish women to contaminated anti-D immunoglobulins (Power et al., 1995). All of these women were infected with HCV genotype 1b, a genotype identical to the isolate obtained from the implicated batch of anti-D immunoglobulin. Genotyping was used to trace the source of outbreaks in Germany (Hohne et al., 1994). More recently, genotyping and molecular characterization of HCV isolates provided evidence for a patient- to- patient transmission of HCV during colonoscopy (Bronowicki et al., 1997). The index case as well as the two other infected patients had HCV genotype 1b. Nucleotide sequencing of the NS3 region showed that the three patients had the same isolate (100% homology), strongly suggesting a common source of infection. Suspected non-conventional routes of HCV transmission could also be investigated by molecular analysis of HCV strains from different persons. These include the vertical and sexual routes. Weiner et al. (1993) showed that a single predominant HCV variant was transmitted to an infant born to a mother infected with multiple variants. A specific 12-nucleotide insertion in the E2 hypervariable region of the HCV genome was noted in the vertically transmitted sequence of an infant born to a mother infected with two different genotypes, each composed of multiple heterogenous sequences (Aizaki et al., 1996). These data may suggest a potential role of HCV heterogeneity and genotypes in mother-to-infant transmission of HCV (Zein, 1997). Reports on the sexual transmission of HCV infection are conflicting. The detection of
<table>
<thead>
<tr>
<th>HCV GENOTYPE</th>
<th>DISTRIBUTION</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Found worldwide: In the US, 1a and 1b are equally distributed and constitute ~ 70% of infections. 1a and 1b are also the most frequent genotype in Europe, although 1b is more common than 1a in Japan, 1b constitutes ~ 70% of infections. High subtype variability is found in endemic regions in Africa.</td>
</tr>
<tr>
<td>2</td>
<td>Found worldwide; 2a and 2b are the most common subtypes in the U.S., Europe and Japan. In the U.S. genotype 2 accounts for ~ 15% of infections. 2c is widely distributed in Italy. High subtype variability is found in endemic regions in Africa.</td>
</tr>
<tr>
<td>3</td>
<td>Found worldwide; 3a is associated with intravenous drug users in the US and Europe. Other genotype 3 subtypes are common in Nepal, Bangladesh, India, and Pakistan.</td>
</tr>
<tr>
<td>4</td>
<td>Found primarily in North Africa and the Middle East. 4a is responsible for the majority of HCV infections in Egypt.</td>
</tr>
<tr>
<td>5</td>
<td>5a is responsible for ≥ 50% of infection in South Africa and is unusual elsewhere.</td>
</tr>
<tr>
<td>6</td>
<td>6a is common in Hong Kong and Macau. (15-20% of infection). Laos, Cambodia, Thailand and Vietnam all harbor minor populations.</td>
</tr>
<tr>
<td>7-9</td>
<td>(Clade 6 subtypes) Found primarily in SE Asia (Vietnam, Thailand, Cambodia, Laos, Myanmar)</td>
</tr>
<tr>
<td>10-11</td>
<td>(Clade 6 subtypes) Found primarily in Indonesia.</td>
</tr>
</tbody>
</table>

Figure 6

GLOBAL PREVALENCE OF HEPATITIS C VIRUS 2004, (CDC)
anti-HCV positivity ranged from 0% in partners of transfusion-associated hepatitis patients (Everhart et al., 1990) to 8% in male homosexuals (Esteban et al., 1989) and 5% in household contacts (Ideo et al., 1990). A possible explanation is that sexual transmission occurs only in association with specific HCV genotypes or in presence of specific mutations along the HCV genome. As with vertical transmission, samples from patients with suspected sexual transmission of HCV have undergone nucleotide sequence analysis to confirm the similarity of sequences obtained from sexual partners and thus the common origin of these HCV strains (Chayama et al., 1995). Although the data are suggestive of a role of HCV heterogeneity in sexual transmission, this speculation needs to be confirmed. Although (Zein et al., 1996) found no association between HCV genotypes and the mode of HCV acquisition in their population, others have provided evidence for such an association (Pawlotsky et al., 1995). It has been suggested that genotypes 3a and 1a are closely associated with intravenous drug use and that genotype 1b is seen more often in patients who acquired HCV through blood transfusion. This information may be useful in tracing sources of HCV epidemics.

Methods for HCV Genotyping

Molecular Genotyping

Several indigenous and commercially available methods for the determination of HCV genotypes have been developed that are based on molecular or serological methodologies. The molecular genotyping assays can be divided into four categories: i) PCR with genotype specific primers (GS-PCR); ii) Restriction fragment length polymorphism (RFLP) assays; iii) Sequencing based methods and iv) Hybridization assays.

Because differences in geographical distribution, disease outcome, response to therapy among HCV genotypes
methods for determining the HCV genotype may become an important clinical test. The reference standard and most definitive method for HCV genotyping is sequencing of a specific PCR-amplified portion of the HCV genome obtained from the patient, followed by phylogenetic analysis. Investigators of HCV genotyping have used sequence analysis of HCV NS5, core, E1, and 5' UTRs. However, direct sequencing is impractical on a large scale because of the complexity of the procedure. Even with the introduction of automated sequencing methods that do not require radioactive isotopes, only a few laboratories are equipped to perform these procedures on a regular basis. Finally, sequencing of amplified DNA does not usually identify mixed infections with two different HCV genotypes.

Other methods that have been reported depend mainly on the amplification of HCV-RNA from clinical specimens, followed by either reamplification with type-specific primers or hybridization with type specific probes (Okamoto et al., 1992; Widell et al., 1994) or by digestion of PCR products with restriction endonucleases that recognize genotype-specific cleavage site. HCV genotyping by using type-specific primers was first introduced by Okamoto et al., (1992) and used primers specific for the core region. This method lacked the sensitivity and specificity (Xavier and Bukh, 1998). Without modification, this method was able to detect subtypes 1a, 1b, 1c, 2a and 3a. However, modifications have been introduced to improve the sensitivity and specificity of this method (Widell et al., 1994; Okamoto et al., 1996) but more studies are required before the efficiency of this genotyping method can be compared with that of other methods. Several DNA hybridization assays for HCV genotyping have been described. A commercial kit (INNO LiPA) for HCV genotyping has been introduced in Europe by Innogenetics (Zwijndre, Belgium) and is based on hybridization of 5'UTR amplification products with genotype specific probes (Stuyver et al., 1993). Although the initial version of INNO LiPA had lower sensitivity, the newer
version is capable of discriminating among HCV subtypes 1a, 1b, 2a to 2c, 3a to 3c, 4a to 4h, 5a and 6a. It has been shown that genotyping methods using 5’UTR, including INNO LiPA may not distinguish subtype 1a from 1b in 5 to 10% of cases and also may not distinguish between subtypes 2a and 2c (Smith et al., 1995). Others have used restriction enzymes to determine a restriction fragment length polymorphism. In this method, a PCR amplified DNA fragment is digested into fragments with different lengths by enzymes (restriction endonucleases) that recognize cleavage sites specific for each genotype (Xavier and Bukh, 1998). Investigators have used different regions of the HCV genome for restriction fragment length polymorphism, including NS5 and 5’ UTR (Nakao et al., 1991). Although all these methods are able to identify correctly the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating among subtypes (Bukh et al., 1995). Moreover, all these PCR-based methods have the shortcomings. They are expensive and time-consuming and require specialized facilities to ensure accurate results and prevent contamination. Their reliability may further be compromised if viral RNA is lost in the serum or plasma through storage or improper laboratory handling or if it is absent from the circulation during sample collection. The advantages of PCR-based methods include reliability if performed accurately and the ability to obtain information relevant to the molecular pathogenesis of HCV.

Serological Genotyping

More recently, investigators identified genotype-specific antibodies that could be used as indirect markers for the HCV genotype (serotyping or serologic genotyping) (Mondelli et al., 1994). Serologic genotyping has several advantages that make it suitable for large epidemiological studies. These advantages include the low risk for contamination and the simplicity of the assay. However, serologic typing seems to lack specificity and sensitivity,
which limits its usefulness. Two commercially available serologic genotyping assays have been introduced over the past 3 to 4 years. The RIBA was introduced by Chiron Corp., and contained five different serotype-specific peptide sequences taken from the NS4 region and two serotype-specific peptide sequences taken from the core region of the HCV genomes for genotypes 1, 2 and 3 (Dixit et al., 1995). The second serologic genotyping assay is the Murex HCV serotyping enzyme immune assay (Murex Diagnostics Ltd.), which is based on the detection of genotype-specific antibodies directed to epitopes encoded by the NS4 region of the genomes for genotypes 1 through 6. These two assays have been compared and showed a concordance rate of more than 96% for genotypes 1, 2 and 3 (Gish et al., 1997). A recent study by Beld et al., (1998) showed high reliability of HCV serotyping by the RIBA SIA (Chiron Corp., Emeryville, California) in immunocompetent individuals infected with genotype 1a. However, the assay had low sensitivity in samples containing genotype 3a or in samples from patients coinfected with HIV. These findings suggest that the use of this assay may be limited at this time, particularly in geographic regions where genotype 1a is not prevalent. Similarly, Songsivilai et al., (1998) showed that serotyping had poor sensitivity for samples from patients infected with HCV genotype 6. Unlike the two previous studies, a study conducted in the United States reported high concordance between serologic genotyping and molecular genotyping assays (Gish et al., 1997). These findings suggest variation in the reliability of these assays based on the distribution of HCV genotypes in a specific geographic area. The choice of typing method for HCV should be based on the expertise in a specific laboratory or institution and the goal of typing. To identify all subtypes and to identify novel sequences if present, PCR amplification followed by sequencing should be the method of choice. However, the goal in treatment trials is frequently to separate patients infected with genotype 1 from those infected with other genotypes a task that could be done adequately by any of the methods mentioned.
Response to Interferon Therapy

Since the discovery of HCV, considerable effort has been devoted to defining the factors that may be important in predicting the long-term response to interferon therapy (Zein et al., 1995). The interferon dose, duration of treatment, viral RNA level (Weiland et al., 1993) and liver histology (Lin et al., 1991) all seem to play a role in predicting response. Beginning with observational data, the clearest difference between genotypes is in their susceptibility to treatment with interferon (IFN) monotherapy or IFN/Ribavirin (RBV) combination therapy. Typically, only 10-20% and 40-50% individuals with chronic infection with genotype 1 on monotherapy and combination therapy respectively exhibit complete and permanent clearance of virus infection. This long-term response rate is much lower than the 50% and 70-80% observed on treatment of genotype 2 or 3 infections (Zeuzem, 2004). This difference has proved to be highly significant in patient management, and has led to the use of higher doses and longer durations of treatment for type 1 and 4 infections to achieve acceptable efficacy.

Despite this wealth of observational data, we still lack basic understanding the mechanism of these differences, mainly because the in vivo mechanism of action of exogenous IFN or RBV remains largely unknown. Insights into mechanism of treatment resistance might be obtained through investigations of the inhibitory effect of IFN or IFN/RBV on the in vitro replication of subgenomic or full-length genomic replicons of HCV (Pietschmann et al., 2002; Blight et al., 2003). Replication of the replicon can be inhibited by the addition of exogenous IFN (Frese et al., 2001; Lanford et al., 2003), at least in part through inhibition of translation (Wang et al., 2003). This model has, however provided only very limited information on treatment resistance, mainly as a result of poorly understood current limitations on the model system. The range of HCV variants that can be cultured is extremely
restricted, limited to genotypes 1a and 1b, both equivalently IFN-resistant clinically, although a full-length replicon of the more clinically sensitive genotype 2a has recently been described (Kato et al., 2003). Secondly, their in vitro replication requires or is enhanced by “adaptive” amino acid changes in NS5A and NS3 (Bartenschlager et al., 2003), even though these play no role in natural infections and actually attenuate replication in experimentally infected chimpanzees (Bukh et al., 2002). Mutations in NS5A are particularly problematic because they cluster in a region of the protein associated clinically with resistance to IFN therapy, and which interacts with PKR and other host cell defenses as part of an evasion strategy. It is therefore unclear whether IFN treatment responses can be realistically modeled in this artificial in vitro system.

In the future, the replicon model will be of great value in the development and assessment of antiviral activity of newly developed protease and RNA polymerase inhibitor for HCV therapy (De Francesco et al., 2003), and for investigating the development of antiviral resistance (Lu et al., 2004). The model is, at present, again limited by the lack of availability of replicons from other genotypes, particularly as there are concerns that antiviral agents specifically modeled on the active sites of genotype 1b protease or RNA polymerase may not be as active against corresponding sites of other subtypes or genotypes (Holland-Staley et al., 2002). Very recently, it was indeed found that non-genotype 1–infected individuals were non-responsive or only weekly responsive to short-term treatment with the BILN 2061 to genotype 2 and 3 proteases (Thibeault et al., 2004). Genotype-specific differences in response to the new generation of antiviral agents will be a major research priority in the future.
HEPATITIS D VIRUS (HDV)

Introduction
Structure
Genome
Diagnosis
HDV and HCC
Coinfections
Prevalence Patterns
Cirrhosis of Liver
INTRODUCTION

In 1977, Rizzetto et al., identified a new viral agent in the nuclei of hepatocytes in patients with chronic HBV infection. Subsequent studies revealed that this newly discovered antigen was related to a new virus, now termed hepatitis D virus (HDV) or delta agent. HDV is a defective RNA virus that requires the helper function of hepatitis B virus (HBV) for replication. HDV is associated with a severe form of acute hepatitis, and HDV superinfection in a chronic hepatitis B surface antigen carrier often results in a progressive type of chronic hepatitis leading to liver cirrhosis and hepatocellular carcinoma (Rizzetto et al., 1983).

Structure

HDV is a unique mammalian virus consisting of a 36 nm particle covered with HBsAg. It has an RNA genome that encodes for the HD antigen (HDAg). HDV is unable to establish autonomous infections but is driven to replicate in the presence of an ongoing HBV infection. Thus it behaves like a defective virus dependent for in vivo infection on complementary helper functions provided by HBV. HDV is similar to virioids and virusoids, infectious agents of plants; in analogy to virusoids it cannot form infectious particles by itself and needs the HBsAg coat of HBV to be assembled into a virion.

Genome

The genome of HDV is an incomplete, single-stranded, circular RNA molecule of negative polarity, with 1700 nucleotides long and contains a very high intramolecular base pairing similar to the genomes of plant virioids. Although many ORFs have been identified, only two have been characterized:
one that encodes for delta antigen protein (HDAg) and other that deals with viral replication. Three genotypes of HDV have been cloned and sequenced. There is 30% divergence in amino acid sequences among different genotypes.

**Diagnosis**

The diagnosis of active HDV infection is usually made by the presence of circulating antibody to HDV (anti-HDV). However, the demonstration of anti-HDV does not necessarily indicate ongoing HDV infection. More direct markers of HDV infection include the detection of hepatitis delta antigen and RNA in serum and liver (Smedile *et al*., 1986). The recent development of polymerase chain reaction (PCR) enables the detection of small amounts of HDV-RNA in serum and liver. The detection of HDV-RNA by PCR is probably the most reliable and sensitive test of ongoing HDV infection (Simpson *et al*., 1994). It has been reported that PCR can detect circulating HDV-RNA in virtually all patients with HDV-related chronic liver disease (Simpson *et al*., 1994).

**HDV and HCC**

Although the high prevalence of delta agent among the chronic carriers of HBV has been known in recent years (Raimondo *et al*., 1982), whether the new agent has any role in the oncogenic capacity of the HBV has not been explored. The strong association of chronic HBV disease and HCC has been well established. Smedile and associates have presented evidence that delta infection induces severe liver damage in previously asymptomatic carriers and aggravates the chronic active hepatitis (CAH) in symptomatic patients (Smedile *et al*., 1981). Thus, these patients are alleged to have a rapid progression to chronic liver disease. Such a chronic liver disease, leading to coarsely nodular cirrhosis, is a likely candidate for development of HCC.
A strong association between chronic hepatitis D virus (HDV) infection, independent of HBV and HCC has yet to be demonstrated. In Italian patients with cirrhosis, a similar proportion with and without HCC was found to have serologic markers of HDV infection (Kew et al., 1984). In areas in HBV-related HCC is very common (such as Southern Africa and Taiwan), very few cases of HCC associated with chronic delta hepatitis have been found (Verme et al., 1991). Patients with HBsAg and HDV super infection were found to develop active cirrhosis and HCC at an earlier age than patients with HBsAg alone (median age 56 years versus 48 years) (Kew et al., 1984).

In a woodchuck animal model, HDV infection does not seem either to prevent or to enhance the development of HCC. Like HCV, HDV is an RNA virus that does not seem to become integrated within the host genome. Hepatitis delta antigen is not often detected in HCC tissue, in patients with chronic delta hepatitis and HCC, but it may be found in the surrounding non-tumourous liver. HDV might act as an indirect promoting factor by causing severe liver disease and accelerating the development of cirrhosis (Kew et al., 1984). Ongoing necroinflammatory changes and hepatic regeneration might be expected to enhance the development of HCC in some cases.

Whatever the biologic contribution of HDV to the development of HCC, the prevalence of HDV in most HBsAg-positive populations is so low as to make any clinically significant contribution unlikely, particularly because some regions endemic for HBV infection report low seroprevalence rates of anti-HDV. Presumably, control and prevention of HBV by mass vaccination would simultaneously reduce or eliminate delta hepatitis.

There is insufficient data to answer the critical question of whether or not the delta agent inhibits the appearance of HCC or, conversely, if it stimulates rapid progression of CLD to the extent that death occurs before
HCC can develop. A major difference between the group of patients with chronic forms of HBV infection is the age at which the infection was acquired and the duration of the infection. The oriental patients apparently acquired their infection in perinatal period, the remainder in adolescence to young adulthood. Thus full assessment of the effects of chronic delta super infection on the course of chronic HBV disease must await accumulation of sufficient numbers of epidemiologically and culturally similar patients.

Coinfections

Prevalence Patterns

Worldwide several studies have been carried out with an aim to assess whether co-infection by HBV and HCV is associated with a higher risk of developing HCC than each infection alone. Because some HCC patients have serologic evidence of co-infection with HBV and HCV (Ruiz et al., 1992). Many studies have indicated that progression from chronic hepatitis to cirrhosis and HCC was accelerated by dual infection (Chuang et al., 1992; Hadziyannis et al., 1993), and that co-infection was found in 10-15% of HCC patients (Liang et al., 1993). Although a large number of HCC cases occurring in the world are likely to be caused by these infections, the relationship between them is unknown.

A positive interaction has been hypothesized, since some researchers found liver damage with dual infection than with infection by one virus only (Fong et al., 1991), while others reported higher HCC incidence among cirrhotics with dual than single infections. However, the risk of developing HCC in subjects with both infections has been estimated accurately. The major difficulty in investigating the relationship between HBV and HCV infections is the rarity of concurrent infection in subjects without clinically evident liver disease. Therefore, only large cohort studies among “healthy”
carriers or case-control studies including people unaffected by liver disease as controls are of sufficient scale to enable the interaction to be assessed properly. No single study performed to date is large enough, but there are many etiologic studies on HCC and on both infections, permitting a meta-analysis.

The meta analysis carried out by Donato et al., (1998) to assess the pattern of interaction between HBV and HCV infections in determining the risk of HCC, with aim of elucidating the pathogenic mechanisms of the two viruses in liver carcinogenesis. And it has revealed a reciprocal negative confounding between HBV and HCV infections as already observed by some researchers (Sun et al., 1996). The reciprocal negative confounding is probably due to interference between the two viruses. In fact, HCV super infection on the HBsAg carrier state can suppress HBV replication or terminate the HBsAg carrier status (Liaw, 1995), the core protein having been found to have a trans-suppressing capacity on HBV replication (Shih et al., 1993). On the other hand, HCV replication also has been found to be suppressed by active HBV replication among patients with chronic hepatitis B, as shown by the finding of HCV-RNA detected in the liver but not in serum (Pontisso et al., 1993). Some findings indicate that the viruses show alternative dominance in replication in patients with dual infection (Koike et al., 1995).

The main result of their analysis is the finding of positive synergism between the two virus infections in increasing HCC risk, as compared with each infection alone. Although the number of controls with co-infection was small (n=8), the OR for dual HBV and HCV infection seems to be greater than sum, and lower than the product, of the OR for each infection. Co-infection has also been shown to increase the risk of developing chronic hepatitis and cirrhosis as compared with each infection alone (Tsai et al., 1996).
HBV related HCC cases were younger than HCV-related cases, probably because of different ages at the onset of chronic infection, since most HCC patients acquire HBV infection in childhood, while HCV infection occurs in adulthood. Early age of acquiring infection is also possible explanation for the higher risk of HCC among people who become chronic carriers at an early age is probably higher than that of individuals infected in adulthood (Kew, 1996). An alternative explanation is that patients with HBV-related cirrhosis have high mortality from complications of liver disease other than HCC, and therefore are not likely to survive long enough to develop HCC (Blum, 1994). Reciprocally, the lower RR for HCC due to anti-HCV/HCV RNA positivity in countries where HBV is highly endemic may be due to the negative interaction between the two virus infections.

Several studies in which the risk factor was calculated with a control group suggested a synergistic or an additive effect of co-infection (Kaklamani et al., 1991). According to Chuang et al. (1992), the relative risk for HCC was 1.96 with positive HBsAg, 27.12 with anti-HCV, and was elevated to >10.05 when both were considered simultaneously.

The study carried out by Mohammed et al. (1997) to assess the clinical and histological significance of dual infection with HBV and HCV, has revealed that cirrhosis and HCC was more common in patients with dual infection than in the controls, and liver disease seems to be more severe in patients with dual infection than patients infected with single virus.

Cirrhosis of Liver

Cirrhosis is a common precursor of HCC, as reported in multiple epidemiological studies from the US and around the world and this association between cirrhosis and HCC has been known for many years. Most
of these studies have shown that, in 70-90% of cases of HCC there is associated hepatic cirrhosis. This association is based on autopsy and clinical studies showing a high prevalence of cirrhosis in non-neoplastic liver tissue of patients with HCC and a high incidence of HCC development in patients with cirrhosis (Kew and Popper, 1984; Johnson and Williams, 1987). More recently, several prospective follow-up studies with periodic ultra-sound examinations of the liver have been conducted in patients with cirrhosis. Based on data from these studies, the annual risk of developing HCC for patients with cirrhosis ranges from 3% to 10% (Ikeda et al., 1993). These studies clearly indicate that patients with cirrhosis are high-risk populations for HCC.

Several studies have addressed the issue of whether cirrhosis etiology may influence progression to HCC. Multivariante analysis of the major etiological factors indicates that alcohol abuse, HBsAg positivity and anti-HCV seropositivity are each an independent variable associated with an increased risk for HCC in the cirrhotic patient (Ikeda et al., 1993). If the factor causing cirrhosis, for example alcohol in alcoholic cirrhosis and iron in the case of haemochromatosis is removed, the risk of HCC persists, unchanged. In case of males the risk is higher and increases with age and duration of chronic liver disease. 30-40% of the patients who died of cirrhosis were found on autopsy to have HCC. In Taiwan, the risk of developing HCC was 1000-times greater in cirrhotic male carriers of HBsAg than in non-cirrhotic HBsAg negative males (Beasley, 1988). In endemic areas, an etiology common to the two pathologic disorders, namely chronic infection with HBV or HCV, accounts for the vast majority of cases. In low endemicity areas, it seems likely that cirrhosis makes the hepatocytes more susceptible to environmental carcinogenic agents such as alcohol. In some patients HCC could be the inevitable consequence of long-standing hepatic disease, whilst in others it could be an independent response to a hepatic insult common to
HCC and cirrhosis. A key factor in the pathogenesis of HCC development in patients with cirrhosis is liver cell regeneration. Cell regeneration during cirrhosis, unlike in normal livers, may be oncogenic since it is associated with abnormal hormone patterns, alteration in the liver array of parenchymal cells, altered production of growth factors and abnormal ancogene expressions (Munoz and Bosch, 1987).

HCC can complicate cirrhosis of varying etiologies and 60-90 percent of HCC occurs in cirrhotic livers (Lai et al., 1981). Macronodular cirrhosis carries the greatest risk of HCC. Macronodular cirrhosis precedes or accompanies a majority of HBV (over 80% in Asia and 40-60% in Africa) and HCV-associated HCCs in children as well as at older ages. It has been shown that South Africans with macronodular cirrhosis are at higher risk for the development of HCC compared to Caucasians with micronodular cirrhosis (Becker and Chatgidakis, 1961).

It is a general belief that HCC development following cirrhosis is the result of regenerative and proliferative processes accompanying the chronic necroinflammatory changes associated with various aetiologies. This is supported by the finding that necroinflammatory in WHV-infected woodchucks precedes the development of HCC (Popper et al., 1987). Alternatively, cirrhosis of the liver may alter the metabolism of environmental carcinogens and thus enhancing HCC development, even with low concentrations of carcinogens.

However, in patients who are HBsAg-positive, there are two evidences that cirrhosis probably plays a role in the causation of HCC by the mechanisms suggested above. Firstly, in the Taiwan study of Beasley and Hwang (1983), when the HBsAg carriers were broken down into those with and those without cirrhosis, the relative risks of development of HCC were