CHAPTER III

LITERATURE REVIEW

HEPATITIS B VIRUS

History

The term "hepatitis A" and "hepatitis B" were introduced by MacCallum in 1947 to categorize infectious (epidemic) and serum hepatitis (homologous serum jaundice). These terms have been adopted by the World Health Organization (WHO) committee on Viral Hepatitis to replace the myriad descriptions previously applied to these disorders. The hepatitis B virus (HBV) antigen was discovered in 1963 by Baruch Blumberg and co-workers. The antigen was first found in the serum of an Australian aborigine (Au antigen) that reacted specifically with an antibody in the serum of American hemophilia patient. By 1968, Prince, Okochi and Murakami(22,23), had established that the Au antigen was found specifically in the serum of hepatitis B patients. It was first renamed hepatitis-association antigen (HAA) and then given its current name, hepatitis B surface antigen.

In 1970, Dane et al (24) detected the complete hepatitis B virion, a 42-nm double shelled particle that consisted of an outer envelope composed of HBsAg and inner core or nucleocapsid containing its own distinct antigen, the hepatitis B core antigen (HBcAg), in addition to a small, circular, partially double stranded DNA molecular and an endogenous DNA polymerase enzyme (25-26). Hepatitis B e antigen (HBeAg), was described subsequently by Magnius and Espmark (27). This antigen is a reliable marker for the presence of intact virions and thus for infectivity. The unique characteristics of these viruses led to their classification within a new family, named *Hepadnaviridae* to reflect the association with hepatitis and the DNA genome. The very small genome replicates via a unique mechanism.

Hepatitis B virus is one of the most common infectious diseases in the world, estimated by the World Health Organization to cause 1 million deaths annually. HBV infection can cause a spectrum of acute and chronic liver disease, ranging from mild, asymtomatic infection to severe, progressive liver disease. Chronic hepatitis B is a major cause of cirrhosis and has been linked to the development of primary hepatocellular carcinoma.

Biology

Hepatitis B virus is the prototype virus for a family of DNA viruses called the *Hepadnaviridae*. Ultrastructural examination of sera from hepatitis B patients shows three distinct morphological forms. (Fig.1) The most numerous, by factor of 10^2 to 10^4, are small pleomorphic, spherical , noninfectious particles containing HBsAg that measure 17 to 25 nm in diameter. Particle counts of 10^{13} per ml or higher have been detected in some sera. Tubular or filamentous forms of various lengths, but with diameters similar to those of the small particles, also are observed. Like the spherical particles, they also contain HBsAg polypeptides. The third morphological form, the 42-nm hepatitis B virion, is a complex, double-shelled particle that consists of a 7-nm outer shell or lipid-containing envelope, replete with all S gene polypeptides, and a 27-nm electron-dense inner core or capsid. The sera of infected patients may contain as many as 10^{10} infectious virions per milliliter. The complete virion has a buoyant density of about 1.22 g/cm^3 in CsCl. Its nucleocapsid contains core proteins with HBcAg and HBeAg specificitics, a small 3,200–base pair, circular, partially double–stranded DNA molecule with molecular weight of 1.6×10^6 and an endogenous DNA polymerase enzyme. (25,26) Table 1 summarizes the terms used in hepatitis B, their proper abbreviations, and a concise description.

The stability of HBV does not always coincide with that of HBsAg. Immunogenicity and antigenicity are retained after exposure to ether, acid (pH 2.4 for at least 6 hr), and heat (98°C for 1 minute; 60°C for 10 hr); however, inactivation may be incomplete under these conditions if the concentration of virus is excessively high. Exposure of HBsAg to 0.25% sodium hypochlorite for 3 minutes destroys antigenicity (and probably infectivity). Infectivity in serum is lost after direct boiling for 2 minutes, autoclaving at 121°C for 20 minutes, or dry heat at 160°C for 1 hr. HBV has been shown to retain infectivity when stored at 30 to 32°C for at least 6 months and when frozen at -20°C for 15 years.

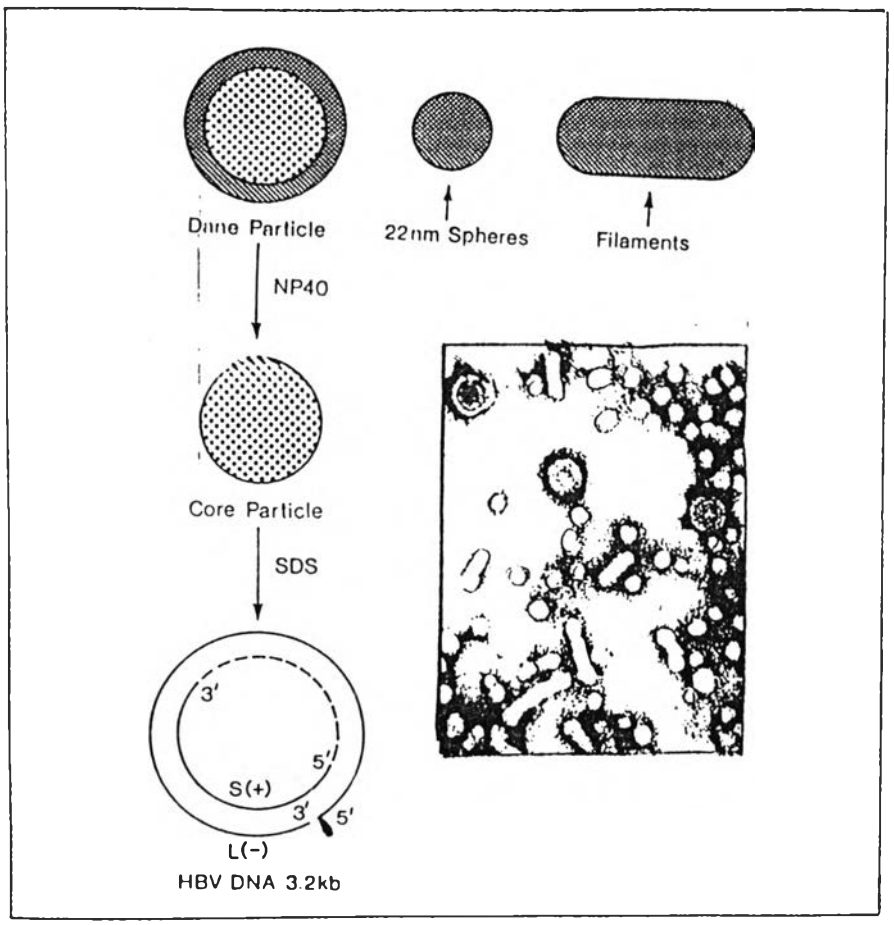


Figure 1. Schematic representation of the architecture of the HBV virion and genome. Insert: electron micrograph of concentrated serum of an HBV-infected human individual.

(From Thomas H.C. and Jones E.A. 1986. In: Recent Advances in Hepatology. No.2 Churchill Livingstone. London. 56.)

Table 1. Nomenclatures for viral hepatitis B

Term	Abbreviation	Description
Hepatitis B virus	HBV	The 42 - nm, double-shelled particle, that consists of a 7 – nm outer shell and a 27- nm inner core contains a small circular, partially double - stranded DNA molecule and an endogenous DNA polymerase. This is the prototype agent for the family *Hepadnaviridae.*
Hepatitis B surface antigen	HBsAg	The complex of antigen antigenic determinants found on the surface of HBV and on the 22 - nm particles and tubular forms. It was formerly designated Australia (Au) antigen or hepatitis associated antigen (HAA)
Hepatitis B core antigen	HBcAg	The antigenic specificity associated with the 27 - nm core of HBV.
Hepatitis B e antigen	HBeAg	The antigenic determinant that is closely associated with the nucleocapsid of HBV. It also circulates as soluble protein in serum.
Antibody to HBsAg, HBcAg, and HBeAg	Anti-HBs, anti-HBc, and anti-HBe	Specific antibodies that are produced in response to their respective antigenic determinant.

The HBV genome consists of a 3.2 kb molecule of circular dsDNA of most unusual structure. The plus strand is incomplete, leaving 15-50% of the molecule single - stranded; the minus strand is complete but contains a discontinuity ("nick") at a unigue site. The 5′ termini of the plus and minus strands overlap by about 240 nucleotides and include short direct repeats, DR 1 and DR 2, producing "cohesive" ends that base - pair to maintain the chromosome in a relaxed circular configuration. A " terminal protein" is covalently attached to the 5′ end of the minus strand, whereas a 5′ - capped oligoribonucleotide primer is attached to the 5′ end of the plus strand.

The minus strand contains four open reading frames: pre -S/S, pre -C/C, P (or Pol), and X (Fig.2). The P gene, which compromises 80 % of the genome and overlaps all the other genes, encodes a polymerase with three distinct enzymatic functions (DNA polymerase, reverse transcriptase, and RNase H) and also encodes the terminal protein primer. Gene X, spanning the cohesive ends of the genome, encodes a transactivating protein that upregulates transcription from all the viral and some cellular promoters. The C gene has two initiation sites that divide it into a pre - C and a C region, producing two distinct proteins, HBeAg and HBcAg, respectively. The pre-S/S gene encodes the envelope protein, S, which occurs in three form: a large (L) protein, translated from the first of the three in -phase initiation codons, is a single polypeptide encoded by the pre-S1, pre-S2, plus S regions of the genome and occurs in the envelope of infections virions ; a middle - sized (M) protein comprises the product of pre -S2 plus S; and finally, the most abundant product is the S protein , the basic constituent of noninfectious HBsAg particles, comprising only the product of the S ORF . All three forms are glycosylated, and the pre - S1 product is myristylated.

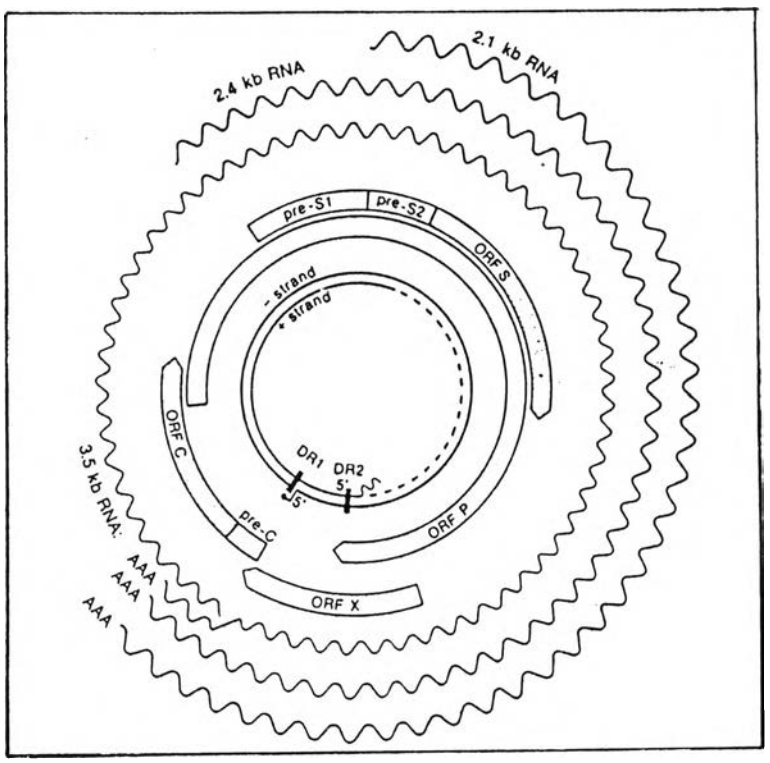


Figure 2. HBV genome Wavy lines denote viral transcripts; boxes, viral open reading frames (ORFs); arrows, direction of transcription and translation; innermost circles, structure of virion DNA; DR1, DR2, positions of direct repeat sequences involved in the priming step in viral DNA synthesis [From D. Ganem and H.E. Varmus, 1987. Annu . Rev . Biochem; **56**, 651]

There are a number of subtypes of hepatitis B virus, defined by various combinations of antigenic determinants present on the HBsAg. All have the same group - specific determinant, *a*, but there are four major subtype - specific determinants, certain pair of which (*d* and y; *r* and *w*) tend to behave as alleles, that is, as mutually exclusive alternatives. The *w* determinant displays considerable heterogeneity. Hence, the subtypes are designated: *ayw, ayw2, ayw3, ayw4, ayr, adw4, adr*, and so on. There are also some more unusual combinations, and additional determinants such as *q* and *x* or *g* have been described, as well as some variants with mutation in any of the determinants. Different subtypes tend to show characteristic geographical distributions, though they often overlap.

Replication of HBV

In the absence of a conventional cell culture system for HBV much of our current understanding of the replication of hepadnaviruses comes from studies of woodchuck, ground squirrel, or duck hepatitis viruses, or from the growth of HBV in human hepatocytes either *in vivo* or in primary culture, or following transfection of hepatoma cell lines by various HBV DNA constructs .

The HBV genome is remarkably compact and makes use of overlapping reading frames to produce seven primary translation products from only four ORFs ("genes"): *S, C, P*, and *X*. Transcription and translation are tightly regulated via the four separate promoters and at least two enhancers plus a glucocorticoid - responsive element. Transcription occurs in the nucleus, whereas replication of the genome take place in the cytoplasm, inside protein cores that represent intermidiates in the morphogenesis of the virion. Replication of the dsDNA genome occurs via a unique mechanism involving the reverse transcription of DNA from an RNA intermediate. Thus hepadnaviruses are sometimes categorized as "retroid" viruses because of the similarity in replication strategy to the retroviruses, although in a sense the two strategies are mirror images of one another. The key difference is that, in the case of the retroviruses, the plus sense ssRNA is packaged as the genome of the virion, whereas in the case of the hepadnaviruses, the ssRNA is the intracellular intermediate in the replication of the dsDNA genome (Fig. 3)

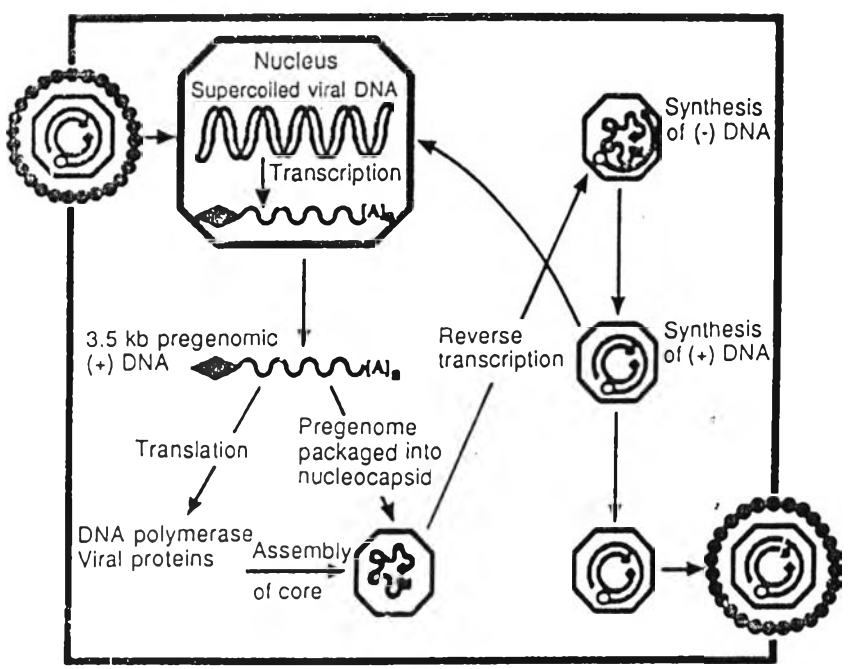


Figure 3. Replication cycle of hepatitis B virus [Modified from Civitico, G., Wang, Y.Y., Luscombe, C., Bishop, C.N., et al. 1990. J. Med. Virol: **31**: 90]

The virion attaches to the hepatocyte via a sequence in the pre - S1 protein and enters by receptor-mediated endocytosis. Following removal of the envelope, the nucleocapsid is translocated to the nucleus and the viral genome released. The short (plus) strand of viral DNA is then completed to produce a full - length *relaxed circular* (RC) dsDNA molecules . This in turn is converted to a *covalently closed circular* (*CCC)* form by removal of the protein primer from the minus strand and of the oligoribonucleotide primer from the plus strand, elimination of the terminal redundancy from the minus strand, and ligation of the two ends of the DNA . This closed circular form becomes twisted to yield what is known as *supercoiled* (*SC*) DNA, which is the template for transcription by cellular RNA polymerase II. The "minus"strand only is transcribed to give mRNAs of 2.1 and 2.4 kb, plus a 3.4 kb RNA transcript known as the pregenome that is actually longer than the genome itself because it contains terminally redundant sequences. Following transport to the cytoplasm, the 3.4 kb species is translated to yield the C (core) antigens and the polymerase, while the 2.1 and 2.4 kb transcripts are translated from three different initiation codons to yield the three forms of S (surface) antigens.

Replication of the viral genome occurs via a mechanism absolutely distinct from that of any other DNA virus. The RNA pregenome associates with the polymerase and core protein to form an immature core particle in the cytoplasm. Within this structure the reverse transcriptase, primed by the virus coded terminal protein, transcribes a complementary (minus) strand of DNA. Meanwhile, the RNase H progressively degrades the RNA template from its $3'$ end, leaving only a short $5'$ oligoribonucleotide which, following transposition to base - pair with a complementary site on the newly synthesized minus strand, serves as the primer for the DNA polymerase to transcribe a DNA plus strand. Some of the core particles, containing newly synthesized viral DNA, are recycled back into the nucleus to amplify the pool of HBV genomes available for transcription. The remainder are assembled into virious before the plus strand of the genome has been completed. The cores bud through those areas of endoplasmic reticulum into which the L, M, and S antigens have been inserted, thereby acquiring an HBsAg-containing lipid envelope. Vesicles transport the virions to the exterior without cell lysis (28).

HBV Variants

Viral variants sometimes emerge during HBV infection. This occurs secondary to spontaneous genetic mutation of the virus coupled with internal and/or external selection pressures (29). Overtime, increasing numbers of viral variants and quasi-species can accumulate in HBV-infected patients. Often, these variants have no clinical impact, but certain types of HBV variants may affect therapeutic response and disease progression. The most important of these are described as follows.

Pre- core mutant HBV

Mutations in the pre-core region of the HBV genome can result in loss of expression of HBeAg, a viral protein that may influence the anti-HBV immune response (29). The worldwide distribution of pre-core mutant hepatitis B has not been well documented; however, this variant is known to be particularly prevalent in the Mediterranean region and possibly in Asia (29-30). The pathogenicity of pre-core mutant HBV relative to the wild-type virus is uncertain, but pre-core mutant HBV has been reported to cause significant disease (31). Of particular importance is the association of pre-core mutant HBV with impaired responsiveness to interferon-alpha (IFN-α) therapy (32). Management of pre-core mutant hepatitis B is therefore problematic. Although the majority of Northern European and U. S. patients with chronic HBV infection and active viral replication are HBeAg positive. Many Southern Eropean and Asian patients have severe liver disease and active viremia in the absence of HBeAg. Whether pre-core mutants are ethiologically important in these syndromes or whether they merely emerge in association with severe disease remains controversial. Moreover, if they are etiologically associated, the mechanism by which the virus causes these syndromes is unknown. Several hypotheses have been proposed, including 1), heightened immune response against core peptide expressed on hepatocytes in the absence of "immune deflection" by HBeAg in serum; 2), direct cytopathicity of truncated HBeAg fragment, which is synthesized as a result of early termination of translation of pre-core/ core mRNA ; and 3), enhanced replication of mutant virus over wild- type virus because of increased stability of hairpin stem loop of HBV which encoded the encapsidation signal for viral packaging.

Mutations in the core promoter region have recently been proposed to result in failure of HBeAg of production. These mutant viruses, which involve nuclotide substitution positions 1762 and 1764 may decrease transcription of the gene segment encoding HBeAg. Although there has been a great deal of interest generated the discovery of HBV variants, the clinical significance of these mutant viruses, particularly those described in association with severe liver disease remains controversial.

Surface antigen HBV mutants

Mutation within the HBV S- gene, leading to changes in HBsAg, can result in failure of hepatitis B vaccination to prevent transmission (33). Such variants are therefore becoming a concern for public health programmes designed to control the spread of hepatitis B. At present, however, HBsAg variants have not been associated with diminished responsiveness to therapeutic intervention. Immune escape to neutralizing antibodies occurs in a highly conserved region of HBV know as the "a" determinant (34-35). This hydrophilic region from amino acids 124 to 147 is believed to be important in eliciting protection against infection. A guanine to adenine nucleotide substitution at the highly conserved amino acid 145 appears to result in major antigenic changes in the virus.

HBV polymerase variants

The HBV polymerase is essential for viral replication and is highly conserved among different, naturally occurring strains of HBV. Few spontaneous mutations of the polymerase gene have been reported. However, the HBV polymerase is the target of nucleoside analogues, including lamivudine, which are used to treat hepatitis B, and HBV variants with polymerase gene mutations have emerged in conjunction with nucleoside analogue therapy. Substantial information has been assembled concerning HBV polymerase variants, particularly those associated with lamivudine therapy. Together, these data suggest that these variants have impaired replication competence, and improvements in liver disease often persist despite the emergence of HBV variants during therapy (36).

Low- level HBV infection

The existence of HBsAg-negative HBV infection has been long debated. Two hypotheses have been proposed to explain atypical serologic profiles in patients with detectable HBV DNA. The first suggests that mutations in the viral genome result in impaired antigen production by the virus. Numerous mutation in HBV have been reported that may facilitate viral persistence. The second suggests that the host immune system keeps the virus in a quiescent or latent state until immunosuppressive therapy results in viral reactivation.

Pathogenesis of Hepatitis B virus

HBV usually causes of damage indirectly by stimulating the host's immune system to attack infected hepatocytes (37). HBV-infected cells express viral proteins on there surface; these become targets for cytotoxic T lymphocytes (CTLs). Paradoxically, chronic HBV infection appears to result from an inadequate primary immune response, but progressive liver disease is caused by persistent immunologic assault on chronically infected hepatocytes.

The immune response to infected hepatocytes cause inflammation and necrosis within the liver. This is the active, and potentially most reversible, component of liver disease. Inflammatory parameters tend to fluctuate naturally; necrosis result from inflammation.

If necro- inflammatory activity persists, it can eventually lead to replacement of damaged cells with fibrous tissue rather than with new, health cells. This process of fibrosis can eventually from the noudular growths that define cirrhosis. Such damage may be irreversible, and can result impair liver function and potentially fatal outcome.

Clinical Features

Acute Infection

The incubation period from acute exposure to clinical symtoms ranges from 60 to 180 days. Clinical presentation ranges broadly from asymtomatic infection to cholestatic hepatitis

with jaundice and rarely to liver failure. In acute infection (fig. 4A), HBsAg and markers of active viral replication (HBeAg and HBV DNA by hybridization assays) become detectable approximately 6 weeks after inoculation, prior to the onset of clinical symtoms or biochemical abnormalities. These tests remain positive throughout the prodromal phase and during the early clinical phase of the illness. Biochemical abnormalities usually coincide with the prodromal phase of the acute illness and may persist for several months. With the onset of symptoms, the anti-HB core IgM becomes detectable. Anti-core IgM may persist for many months, and anti-HB core IgG may persist for many years if not for a lifetime. Anti-HBs is the last serologic test to become positive and is a marker of resolving infection (as HBsAg titers fall). Much has been made of the serologic "window" period for infection when neither HBsAg nor anti-HBs is detectable, and anti-HB core IgM is the only marker of acute infection. Although this may occur, with the currently available serologic assays, it is rare.

The biochemical diagnosis of acute hepatitis largely depends on measurements of serum bilirubin and aminotransferases. Serum alanine aminotransferases (ALT) is typically higher than serum asparatate aminotransferases (AST), and elevations of both aminotransferases are usually 500 IU/L or greater. Bilirubin elevations are usually modest (5-10 mg/dl) although levels may be higher in the setting of hemolysis or renal failure.

The most profound complication of acute HBV infection is fuminant liver failure, defined as the onset of hepatic encephalopathy within 8 weeks of the onset of symtoms. Although this is an infection complication (occuring in fewer than 1% of cases), the prognosis is poor once encephalopathy has developed. When patients present with acute hepatitis, it is essential to obtain tests of hepatic synthetic function (prothrombin time, serum albumin). Evidence of a prolonged prothrombin time (international normalized ratio of 1.5 or greater or prothrombin time of 17 seconds or longer) should raise concern regarding the potential development of fulminant hepatic failure. If clinical symtoms of hepatic failure develop, patients should be refered for consideration of liver transplantation.

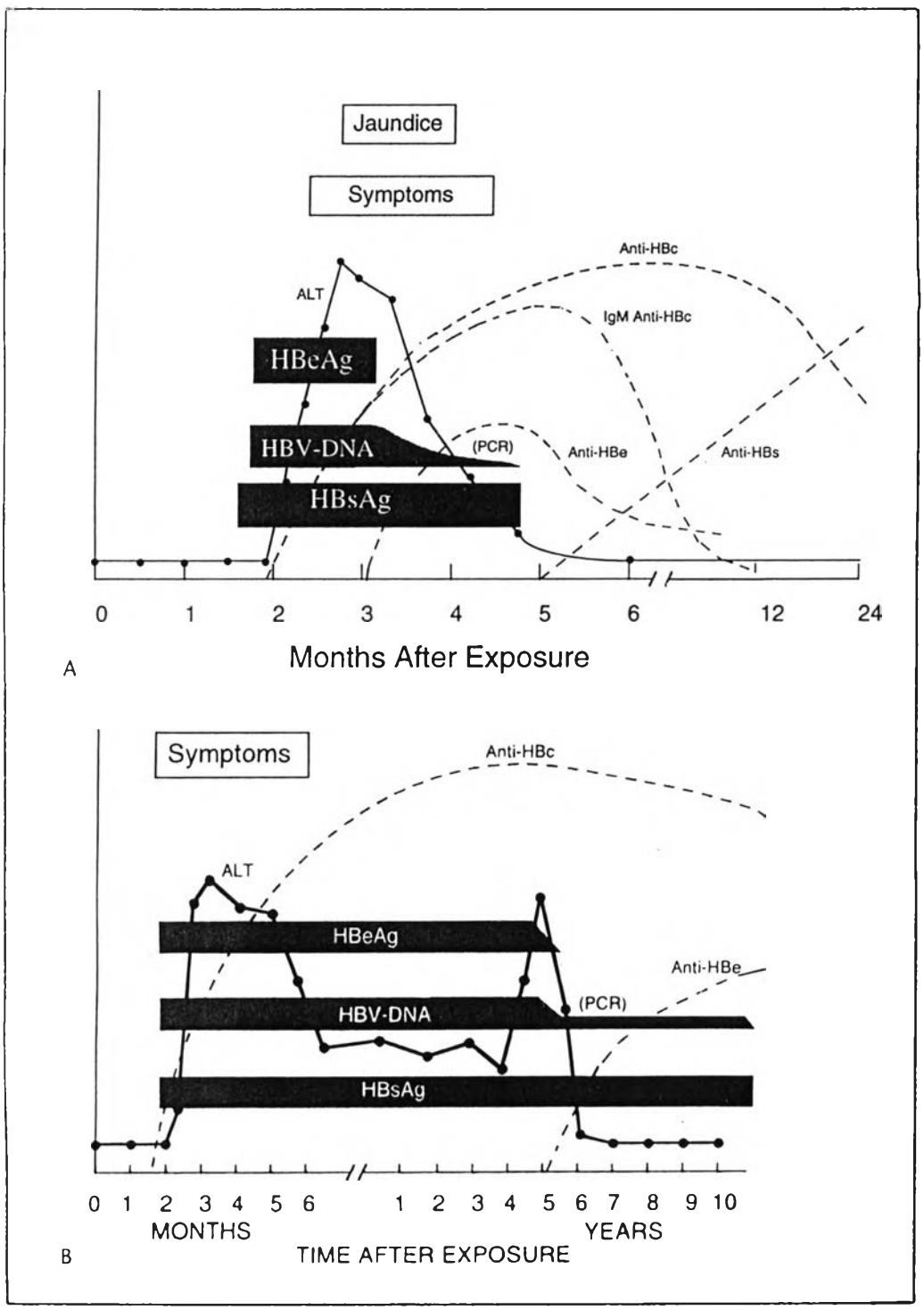


Figure 4. Sequence of events after acute HBV infection. (A) Acute HBV infection with resolution (B) acute HBV infection progressing to chronicity (From Hoofnagle and Di Bisceglie.1991. Semin Liver Dis; **11**:73)

Chronic infection

When infection progresses to chronicity (fig. 4B) the early clinical and biochemical events are very similar to those in acute infection. However, in chronic infection, HBsAg, HBeAg and HBV DNA remain positive for 6 months or longer. Following the acute phase of infection, ALT levels often remain persistently abnormal (from 50 to 200 IU/L). Anti-HB core IgM titer typical fall to undetectable levels after 6 months, but may become detectable again during reactivation of infection. Anti-HB core IgG persists indefinitely. HBV DNA is detectable by hybridization assays during the acute and chronic phase of disease. With time, there may be a spontaneous loss of HBV DNA and HBeAg, frequently in association with a "flare" of ALT and seroconversion to anti-HBe positivity. Spontaneous loss of HBsAg is rare. Anti-HBs may be detected simultaneously with HBsAg in serum, but this occurs is fewer than 10 % of cases. In chronic infection, active viral replication (HBV DNA positivity) can occur in the absence of HBeAg.

Extrahepatic Manifestations

Extrahepatic findings are common with acute HBV infection. Arthralgias and rashes occur in 25% of cases. A more severe serum -sickness-like syndrome with immune complex deposition occurs more rarely and can result in angioneurotic edema. Polyarteritis nodosa with a systemic vasculitis can occur with either acute or chronic HBV infection.This syndrome typically presents with abdominal pain due to arteritis of the medium-sized arteries , which results in ischemia to the intestine or gallbladder. Other manifestations of HBV-associates vasculitis include neuropathy (mononeuritis), renal disease, cutaneous vasculitis , arthritis and Raynaud 's phenomenon .

Viral Persistence and Hepatic Carcinogenesis

The association of persistent HBV infection with the development of primary hepatocellular carcinoma (PHC) is one of the most intriguing aspects of this complex and inscrutable pathogen. As noted previously, this association was first suggested by epidemiologic investigations with the documented striking geographic correlation between area of HBV

endemicity and regions of high PHC prevalence. Subsequent studies have shown that even in low-prevalence areas for HBV, patients with hepatoma are 5-70 times more likely to be HBsAg carriers than are matched controls. PHC appears to follow primary HBV infection by a long interval-usually 25-35 years and generally is accompanied by significant chronic hepatitis or cirrhosis; patients who acquire infection early in life are at the greatest risk for PHC development. The magnitude of this risk has recently been estimated by Beasley and co-workers in prospective studies of long-term carriers in Taiwan: the relative risk of PHC development in such carriers is 250-fold that of non-carriers matched for age, race and sex. This makes persistent HBV infection one of the strongest risk factors yet identified for any human neoplasm.

HBV window period

The HBV window period can likewise be studied by using panels of samples from seroconverting plasma donors or archived samples from subjects in posttransfusion hepatitis studies. Data from posttransfusion cases indicate that HBsAg is first detected about 50 to 60 days after transfusion. As with HIV, virus can be detected at low levels in plasma for several weeks before HBsAg detection, as determined by testing serial samples collected before HBsAg positivity from plasma seroconversion panels. Analyses of the HBV DNA concentrations in serial plasma donations yielded an average 4-day doubling time for HBV DNA during this preseroconversion phase. Thus, whereas HCV doubles every few hours and HIV doubles every day, HBV levels in plasma increase relatively slowly. For HBV, the levels of DNA in plasma have been correlated with infectivity in chimpanzees. Replicate dilutions of plasma that had previously been pedigreed with regard to chimpanzee infectivity were tested by PCR assays to determine the dilution at which the assays became negative. On the basis of these data, it has been estimated that 10 to 20 HBV particles or DNA equivalents are equivalent to 1 chimpanzee-infectious dose. These results address the issue of the sensitivity of Nucleic acid amplification testing (NAT) that is needed to interdict infectivity. On the basis of the level of HBV DNA at HBsAg seroconversion, the doubling time, and the chimpanzee infectivity data, it can be estimated that infectivity may exist as much as 40 days before detectable HBsAg (38). However, because of the relatively high sensitivity of HBsAg assays and because the concentration of HBV DNA is relatively low (average, <1,000 copies/mL; range, 1-2,400 copies/mL), during this prolonged phase of HBV infectivity before the appearance of HBsAg, NAT pooling strategies

will allow the detection of only a small portion of donations made in the pre-HBsAg-infectious window period.

Laboratory diagnosis of HBV infection

Serology

Serological markers for clinical diagnosis of HBV infection have been well characterised, and are now complemented by newer molecular biological techniques. Current serologic assays for the diagnosis of acute and chronic HBV infection (HBsAg and HBeAg) are both sensitive and specific HBsAg, HBeAg, anti-HB core, and anti-HBe are detected by standard enzyme-linked immunoassays (EIAs) or RIA (radioimmunoassay). These assays rely on the sandwich principle or competitive binding immunoassays. The detection of HBsAg indicates active HBV infection, and the detection of HBeAg indicates active viral replication and increased infictivity. HBeAg positive carriers have more than 10^{5} genomes per ml serum. Immunity after infection with HBV is characterised by the presence of anti-HBs and anti-HBc antibodies in serum. Immunity after vaccination is characterised by the presence of anti-HBs. The HBsAg RIA assay is capable of detecting HBsAg at a level approximately 0.25 to 0.5ng/ml. The EIA technique is at least as sensitive as RIA. Improvement in the test has reduced the number of repeatably false-positive reactions and specificity is almost comparable to that of RIA.

HBV DNA Detection

Methods of HBV DNA detection can be broadly clasified into those that are dependent on hybridization of labeled probe to the DNA with quantitation achieved by comparing the result with known standards [Genostic assay (Abbott Laborotoies, Abbott Park, Chicago, IL) and branched DNA assays (Chiron Corporation ,Emeryville , CA)] and polymerase chain reaction (PCR) based assays.

Dot or Southern Blot Hybridization techniques

The dot blot hybridization test for serum HBV-DNA correlates with infectivity and is an important means of determining both the presence of viral genomes and the response to antiviral treatment. Human serum and tissue can be analyzed for HBV DNA sequences by dot hybidization techniques. In this technique, HBV DNA in the sample is immobilized on a nitrocellcellulose or nylon membrane and hybridized to radioactive probe. This method is capable of visual detecting 0.1 to 1.0 pg (28,000 to 280,000 genomic equivalents) of HBV DNA sequences within 24 h , with a 2 to 10 fold increase after 5 days of autoradiography At least 10^3- 10^5 virions have to be present in a specimen to be detected by dot blot assay. Liquid-phase molecular hybridization assays for detection and quantitation of HBV-DNA have also been developed, in this assay the extracted HBV-DNA is mixed with ^{125}I – labelled nucleic acid probe and allowed to hybridize, then free and hybridized probe are separated by column chromatography. Chemoluminescent assays are under investigation.

Southern blot assay is the most useful in the study of liver DNA samples, allowing detection of integrated or free episomal HBV-DNA.

Branched – DNA Anplication Assay

The Chiron Amplex-HBV assay is a sandwich nucleic acid hybridization method for the quantitative detection of HBV DNA in human serum. It is available for research use only (not for use in diagnostic procedures). In this assay, synthetic oligodeoxyribonucleotides containing unique primary segments designed to hybridize to the minus-sense strand of HBV DNA are covalently attached to a secondary fragment through branch points. These target probes hybridize to HBV DNA and to complementary synthetic fragments bound to a solid phase. On another level, the secondary fragments of the target probe direct the binding of multiple copies of synthetic branched-DNA amplifier molecules that contain repeated nucleotide sequences that hybridize with enzymemodified alkaline phosphatase-labeled probes. The detection scheme relies on alkaline phosphatase-catalyzed chemilumminescence emissions from a dioxetane substrate. The results are recorded as luminescence counts on a plate luminometer light emission which are proportional to the amount of HBV DNA present in each specimen, and HBV DNA is quantitated

by comparing light emission values in the test specimen against those obtained on standards. As few as 70,000 HBV DNA equivalents per ml can be detected with this assay.

Polymerase Chain Reaction Assay (PCR Assay)

Recently, the PCR assay, which makes possible detection of as few as 10 HBV DNA molecules per ml, has been used for amplification of HBV DNA in serum. This assay is 100 to 1,000 times more sensitive than the dot hybridization technique for detecting HBV DNA. Synthetic oligonucleotide primers that are complementary to a conserved sequence on the genome that will be amplified are prepared. The procedure allows for denaturing the DNA to separate the strands of the DNA duplex that contains the target region, annealing the primers to their complementary regions, and DNA synthesis by extending the primers. The discovery of a thermostable enzyme isolated from *Thermus aquaticus*, called *Taq* DNA polymerase, has increased the specificity of the reaction by allowing extension to take place at an elevated temperature. Denaturation, annealing, and extension constitute a cycle and are accomplished by varying the temperature of the reaction. Products of the previous cycle act as templates for succeeding cycles, resulting in an exponential doubling of the DNA. Thus, for every 20 cycles, the target region is amplified about a million fold, and a second round of 20 cycles with a different pair of primers results in $>10^{12}$ amplification. Alternatively, the second round of amplification may be replaced by a sensitive detection method such as hybridization to a labeled probe.

A number of PCR procedures for detecting HBV DNA in serum have been described. Viral DNA can be purified from serum by proteinase K digestion, phenol and chloroform extraction, and precipitation with ethanol, following which the DNA is redissolved in water. The amplification is carried out in a DNA thermal cycle with primers that anneal to highly conserved sequences of the HBV genome.

Epidimology of HBV infection

Hepatitis B virus is transmitted by percutaneous exposure to blood, blood products, and blood-contaminated instruments. Intimate contact, especially sexual contact, and perinatal spread from mother to newborn are the two other common modes of HBV transmission. The various epidemiologic pattern found in hepatitis B requires that the source of infectious virus be known. This usually the blood, but virus also has been detected in a variety of other body secretions and excretion, including semen, saliva or nasopharyngeal fluids and menstrual fluid. There is no convincing evidence that airborn infections occur. Feces have not been incriminated as a source of infection, presumably because the virus is inactivated by enzymes present in the intestinal mucosa or derived from the bacterial flora. Virus is sometime present in urine, but its presence is probably of limited epidemiologic importance. Exceptions may occur when person are exposed to chronically infected, diapered infants or to the urine of HBsAg–positive uremic patients undergoing hemodialysis. Gastric juice is not a source of HBV, but HBsAg has been detected in both bile and pancreatic juice even though these secretions are inhibitory to HBsAg reactivity. Pleural fluid, ascitic fluid, cerebrospinal fluid, tears, and breast milk also have been positive for HBsAg. It should be assumed that all biologic fluids from HBV-infected patients may be infectious and capable of transmitting HBV, especially in a situation in which percutaneous or mucous membrane exposure has occurred because this is the most efficient route for introducing the virus into the susceptible host.

The HBsAg carrier rate varies from country to country, with higher rates being reported from developing countries with primitive or limited medical facilities. The prevalence of HBsAg is lowest (less than 0.5%) in countries with the highest standards of living, such as Great Britain, Canada, United States, Scandinavia, and some other European nations. Very high rates (5% to 20%) have been reported for China, Africa, Oceania and the South Pacific, the Middle East, and some parts of South America (39). In Thailand. there are about 6-10% HBsAg carriers in general population. The prevalence of hepatitis B surface antigen in chronic hepatitis was 40-60%, in cirrhosis 30%, and in hepatocellular carcinoma 50%-70%(40). Data from National blood center (1996-1999), the prevalence of HBsAg carriers in blood donor was 5%.

In endemic areas of Asia and Africa, the epidemologic patterns of infection are different from those seen in North America and Western Europe, serologic surveys indicate that the most infections in these geographic regions occur in infants and children as a result of maternal-neonatal transmission or close children contact. Percutaneous exposure with contaminated sharps is always possibility in these countries. An annual incidence of infection among children of 3% to 5% has been projected. The carrier rate and prevalence of specific antibody tend to decline with advancing age in these high-risk groups.

In low-risk areas of the world, the highest incidence of the disease and peak prevalence of anti-HBs are often seen in teenagers and young adults. Despite the low incidence of disease present in the general U.S. population, certain groups who are sexually promiscous or who have frequent contact with blood or blood products have a high rate of hepatitis B infection. Percutaneous transmission by iatrogenic invasive procedures represents the most common identifiable mode of spared, with injection drug users constituting the largest cohort of carriers. Perinatal spread is correspondingly less common.

HEPATITIS C VIRUS

History

The discovery of the viruses responsible for type A and type B hepatitis and the development of diagnostic serological tests for these virus infections during the 1970s led to the unmasked recognition of a third type of infectious hepatitis that was responsible for most cases of post-transfusion hepatitis. To distinguish the disease from better known forms of hepatitis, they refered to it by the ungainly but descriptive name of non-A, non-B hepatitis (NANB). The causative agent remained frustratingly elusive for over a decade has still not convincingly cultured *in vitro* nor visualizied by electron microscropy. Researchers from Chiron Corp. and the Centers for Disease Control and Prevention (CDC) finally identified a virus-specific antigen associated with NANB infection. This seminal discovery led to a rapid explosion in the understanding of hepatitis C virus (HCV). Including recognition of its primary role in post-

transfusion hepatitis, its proclivity to establish long-standing persistent infection, and its association with chronic hepatitis, cirrhosis, and hepatocellular carcinoma (41-42).

Properties of Hepatitis C Virus

Hepatitis C virus is an enveloped virus, approximately 40-50 nm in size while a buoyant density of 1.1 g/cm^3 and sensitivity to chloroform. The genome consis of a positive – stranded RNA molecule containing approximately 9,500 nucleotides, a single long ORF for a polyprotein of 3011 amino acids. HCV is a member of the flavivirus family but HCV is sufficiently unique to be placed in its own genus. This family comprises three genera: Flaviviruses, Pestiviruses, hepatitis C virus genus which itself comprises a heterogeneous group of RNA viruses, known collectively as hepatitis C virus. Nucleotide sequence variation has been observed in genomes amplified from serum of patients with HCV infection, and cloning of RNA amplified from patients infected with HCV has confirmed the heterogeneity of agent responsible for post – transfusion and sporadic hepatitis C (43).

The structure and assembly of HCV is being intensively examined. There is also paticular interest in the significance of strain variation and in examining the possibility that unique biological differences exist in discrete genetic variants, which result in differences in infectivity, severity or spectrum of disease and response to treatment.

The Genetic Organization of HCV

The genome of HCV was discover in 1989 and contains a positive–stranded RNA molecule of ca. 9500 nucleotides. The nucleotide sequence of the RNA genome was determined from an overlapping series of bacterial cDNA clones and was shown to contain a single large open reading frame (ORF) extending throughout most of the genomic sequence. The large ORF therefore appears to encode a large viral polyprotein precursor from which individual viral proteins are processed. The 5′ terminal region of the RNA genome is highly conserved among different viral isolates from around the world exhibiting primary sequence identities of >90%. Figure 5 shows a schematic organization of the HCV genome along with its encoded proteins and putative functions. Virion structural proteins appear to be processed from the N-terminus of the

polyprotein precursor through the action of the host signal peptides. A presumed RNA-binding nucleocapsid protein of basic charged is processed from the extreme N-terminus of the polyprotein yielding a protein of ca.20 kDa. This is followed by two gylcosylated proteins that are thought to correspond with virion envelope glycoproteins (E1, gp33, and E2, gp70: Fig. 5). By analogy with the organization of the pestiviral and flaviviral genomes, it appears that the large remainder of the HCV polyprotein is processed to produce a large variety of presumed nonstructural proteins involved in some aspects of viral replication, translation, and assembly. Polypeptide species corresponding to nonstructural (NS) proteins 2 (23kDa), NS3 (70kDa), NS4a (10kDa), NS4b (27kDa), NS5a (56kDa), and NS5b (70kDa) have all been identified. A small NS2a polypeptide of ca. 6kDa has been observed in *in vitro* translations. NS3 encodes both helicase and protease enzymes. The ATP-binding helicase is probably involved in unwinding the RNA genome during replication and/or translation, while the NS3 protease has been shown recently to be responsible for processing the downstreame NS proteins. The HCV NS3 protease belongs to the trypsin class of enzymes and has serine at its active catalytic site. Unlike the flaviviral NS3 proteases, the upstream NS2b protein species (23kDa) of HCV is not required for processing of the NS4 and NS5 protein. However, very recent data indicated that the NS2/NS3 cleavage of HCV is mediated by a different protease encoded within the HCV NS2 protein of ca. 23kDa. Fig. 5 indicates the various protease (host-and viral-encoded) involved in processing of both the structural and nonstructural proteins of HCV (44).

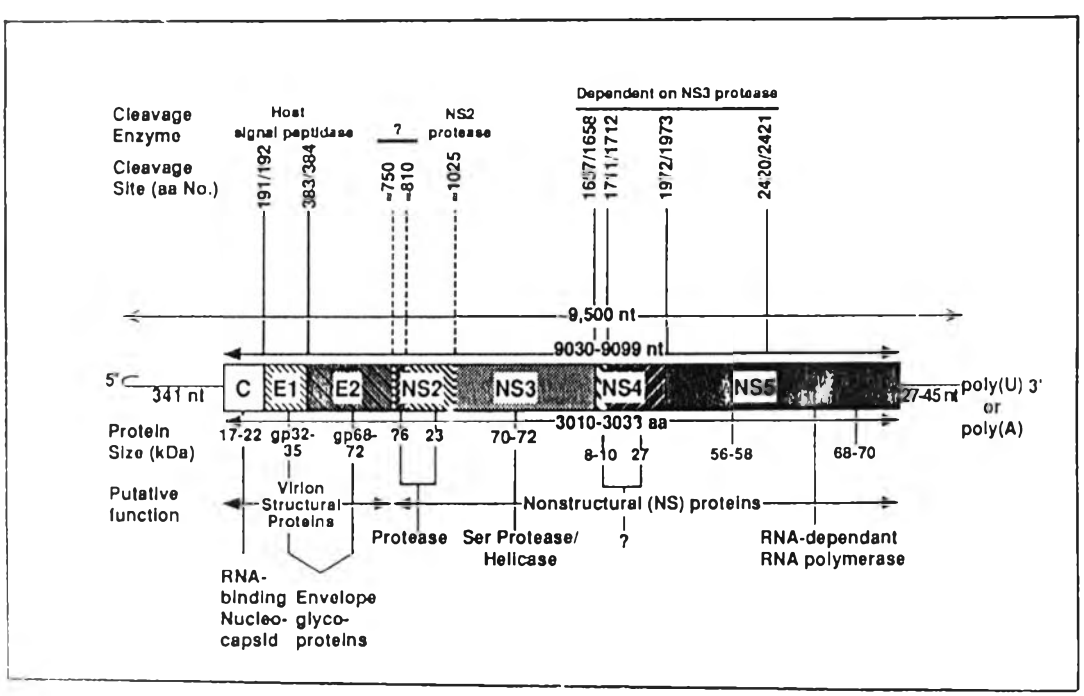


Figure 5. Genetic organization of the HCV genome.

The 3′ untranslated region of the HCV genome is very short (between 27-45 nucleotides; Fig.5). Interestingly, this short region is followed by either a homopolymeric tail of poly (rA) or poly(rU) depending on the viral isolate. Sequence heterogeneity has also been observed throughout the large HCV ORF region. Recent phylogenetic analyses indicate that there are at least six distinct genotypes of HCV occurring throughout the world along with many different subtypes. While such heterogeneity is not unusual for RNA viruses that produce error-prone replicases and which lack repairing mechanisms, this substantial heterogeneity of HCV could have important ramifications in the pathogenesis and control of HCV infections. For example, it appears that different HCV genotypes have different sensitivities to interferon (IFN) therapy.

Replication of HCV

The host range of HCV appears to be limited to humans and chimpanzees, suggesting a very stringent host species restriction. Very little is known of the replication cycle of HCV, largely due to the absence of a conventional *in vitro* cell culture system that is permissive for virus replication. Limited replication of the virus has been reported in primary hepatocyte obtained from chimpanzees (45), as well as cultured human T-cell lines. In both cases, however, the quantities of virus produced were very low. Han and co-workers at Chiron have recently reported the construction of an infectious full-length cDNA copy of the HCV genome: transfection led to persistent production of viral RNA in a continuous hepatocyte-derived cell line. If this result can be confirmed, it will represent a major breakthrough in the study of HCV.

HCV heterogeneity

Comparison of the genomic nucleotide sequences from different HCV isolates around the world indicates the HCV is highly heterogenous. Phylogenetic analyses performed on the nucleotide sequences of the 5′ leader, C, NS3 and NS5 gene regions from different isolates indicate the existence of multiple HCV genotypes. Based on this plylogenetic analysis, HCV isolates can be separated into types 1 to 6 containing subtypes a, b or c. This level of sequence diversity may have several ramifications. First, it is conceivable that the course and severity of disease may depend on the infecting genotype, and preliminary data are consistent with this

notion. Second, there is evidence that HCV strains vary in their responsiveness to interferon therapy. Third, such heterogeneity has important connotations for vaccine development because it is likely that vaccine antigens from multiple serotypes will be necessary for global protection. The current geographic distribution of HCV variants has been mapped, using a classification based on restriction fragment length polymorphism (RFLP) in the 5′ non-coding region, primer specific probe, or sequencing. Both blood donors and patients with chronic hepatitis from countries in Westren Europe and the USA all show frequent infection with genotypes 1a, 1b, 2a, 2b and 3a, although the frequencies of each may vary. There is a trend for relatively more frequent infection with type 1b in southern and eastern Europe. In this connection, HCV type 1, 2, and 3 appear to be widely distributed around the world (46). Type 1a and 3a are more common in intravenous drug users in Europe. Within Japan and Taiwan and probably parts of China, genotypes 1b, 2a and 2b are the most frequently found. Type 3 is only rarely found in Japan, and is also infrequent in Taiwan, Hong Kong and Macau. Infection with type 1a in Japan appears to be confined to haemophiliacs who have received commercial (US produced) blood products and concentrates. However, this genotype accounts for the majority of infections in Thailand, Singapore, and possibly in Bangladesh and Eastern India. A striking geographical change in genotype distribution is apparent between Europe, the Middle East and parts of North and Central Africa, where infection with genotype 4 is highly prevalent, this genotype actually comprises an array of subtypes, which may have resulted from long-term versus more recent introduction of HCV (46). In Egypt and elsewhere in the Middle East, at least three other subtypes of type 4 are identifiable, which differ from those found in Central Africa. Thirty to 50% of anti- HCV positive blood donors are infected with type 5a in South Africa. Type 6a was originally found in Hong Kong. Epidemiological differences between the age distribution of major types, and risk factors associated with particular genotypes are becoming apparent. For example the prevalences of different genotypes may change with age; in Europe, type 3a and 1a are relatively more common in young individuals than 1b, which accounts for most infections in those aged 50 or more (46).

Clinical features

The mean incubation period of hepatitis C is 6-12 weeks. However, with a large inoculum , such as cases following administration of factor VIII , the incubation period is reduced to 4 weeks or less. The acute course of HCV infection is clinically mild, and the peak serum ALT

eleivations are less than those encountered in acute hepatitis A or B. Only 25% of cases are icteric. Subclinical disease is common; such patients may first present decades later with sequelae such as cirrhosis or HCC. During the early clinical phase the serum ALT levels may fluctuate and may become normal or near normal, making the determination of true convalescence difficult. Fulminant hepatitis is a relatively rare outcome of HCV infection, and no correlation between antigenic variants of HCV and severe acute infection has been reported. The disease has been reported in patients after withdrawal of immunosuppression. The diagnosis of such cases requires confirmation by HCV RNA testing. Arthritis, rashes, glomerlonephritis, vasculitis, neurological syndromes and aplastic anaemia have been reported.

Chronic hepatitis C

Most patients with chronic hepatitis C are asymptomatic , or only mildly symtomatic. In symptomatic patients, fatique is the common complaint. Many patients do not give a history of acute hepatitis or jaundice. Physical findings are generally mild and variable, and there may be no abnormalities. With more severe disease, spider angiomata, and hepatosplenomegaly may be found. Serum aminotransferases decline from the peak values encountered in the acute phase of the disease, but remain 2-8 times of normal. The serum ALT concentrations may fluctuate over time, and may even intermittently be normal. Many patients have a sustained elevation of the serum aminotransferases. A characteristic histologic pattern of mild chronic hepatitis with portal lymphoid follicles and varying degrees of lobular activity is found in many patients.

The spectrum of chronic disease varies. Most patients appear to have an indolent, only slowly progressive course with little increase in mortality after 20 years. However, cirrhosis develops in approximately 10-20% of patients with chronic disease within 10 years, albeit that the cirrhosis remains indolent and only slowly progressive for prolonged period. The disease is not necessarily benign however, and rapidly progressive cirrhosis can occur. Older age of infection, concomitant alcohol abuse, concurrent HBV or HIV infection or other illness may be important aggravating co-factors. With the development of cirrhosis, weakness, wasting, oedema, and ascites become progressive problems. Older patients may present with complications of cirrhosis or even HCC.

Course of HCV infection

Following primary HCV infection, the first viral marker detected in serum is HCV RNA, which is usually found by PCR within 1 to 2 weeks of exposure. Antibody to C100-3 was detected in no patient before 10 weeks after exposure to HCV but was present in 60 percent of patients within 15 weeks and in 90 per cent by 6 months of exposure. Second-generation tests that detect antibody to multiple HCV antigens are positive at an earlier time and are positive in almost all patients by 15 to 20 weeks after exposure to virus. The incubation period of hepatitis C [time from exposure to elevation of alamine aminotransferase (ALT)] has been shown to be between 6 and 12 weeks; anti-HCV can be detected by second-generation tests within a few weeks of disease. Post-transfusion hepatitis C can be asymptomatic and is generally less severe than hepatitis B. A majority of patients after primary HCV infection develop chronic hepatitis and have persistence of anti-HCV. Such patients appear to have persistent HCV infection. Anti-HCV commonly becomes undetectable within 12 months of the time hepatitis resolves. This suggests that most patients with anti-HCV in serum have active HCV infection. Patients with chronic infection may develop cirrhosis and hepatocellular carcinoma (HCC) usually over the course of many years, but development of cirrhosis can be more rapid.

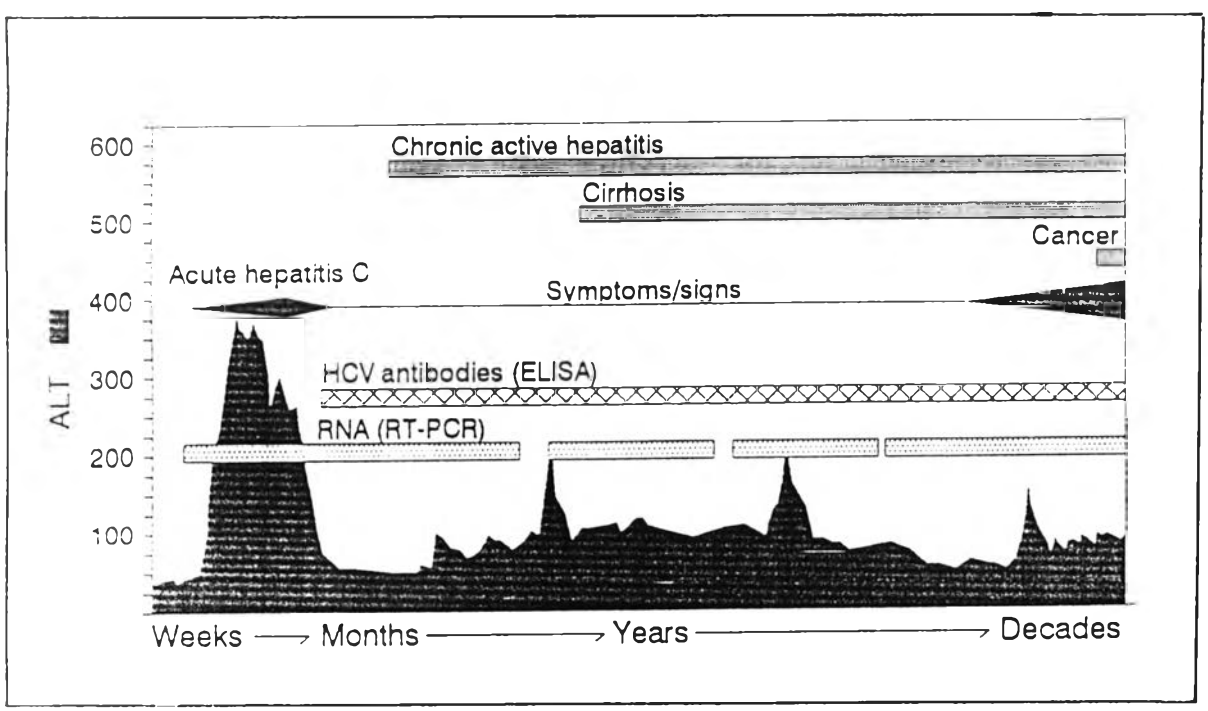
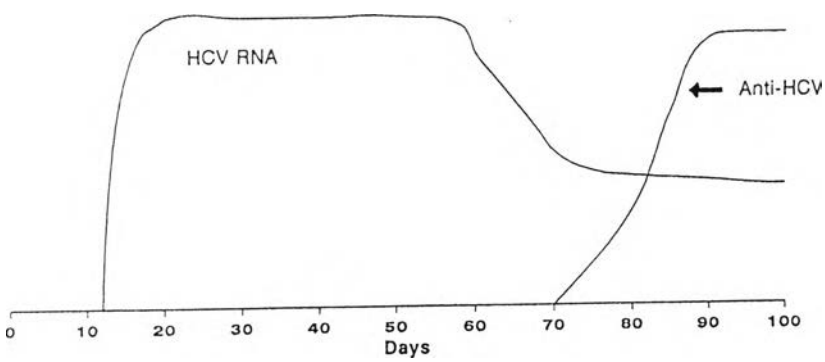


Figure 6. The natural history of HCV infection.

HCV window period

Three sources of data have been used to understand the HCV window period: HCV-seroconverting plasma donor panels, cases of transfusion–transmitted HCV infection from posttransfusion hepatitis studies of the 1970s and early 1980s, and chimpanzee infection studies. When a plasma donor seroconverts, the preceding serial donations can be tested to determine the duration and levels of HCV RNA before liver enzyme elevation and antigen formation occurred. Unlike HIV, in which the RNA-positive and antibody-negative stage is brief, with HCV there is a prolonged high-titer viremic phase before an increase in alanine aminotransferase and antibody seroconversion (47). In addition, the transition from a negative to a positive RNA NAT result is extremely abrupt with HCV, with an extraordinarily rapid virus-doubling time, estimated at approximately 0.1 day. In one study, the duration of HCV RNA positivity before seroconsion averaged 41 days (47). The number of RNA copies during this period averaged over 100,000 per mL. With this high-titer viremia phase, HCV is very amenable to detection by pooled-specimen NAT, even with pools as large as 100 to 500 donations. The second source of data on the HCV window period is posttransfusion hepatitis cases from the late 1970s. The findings from analysis of these cases were very similar to those based on seroconverting plasma donors, with a prolonged, high-titer viremia preceding seroconversion. Because the date of exposure (i.e., transfusion) is known, it can be shown tha' most recipients develop high-level viremia within 10 days of infection. This development is followed by an average 40-day period of high-level viremia before alanine amino-transferase elevation and, approximately 10 days later, antibody seroconversion. Third, chimpanzee infectivity studies are consistent with these observations in humans. RNA is detected by NAT an average of 9 days after inoculation, with antibody detected an average of 73 days after inoculation.



Infection Day 0, HCV RNA Day 12, HCV Antibody Day 70

Figure 7. HCV markers during early infection.

Laboratory diagnosis of HCV infection

Serological diagnosis

Antibodies to HCV

HCV probably circulates in the serum at a concentration of between 10^2–10^7 particles per ml , so far, it has proved impossible to detect viral antigens by conventional methods. Therefore, the detection of antibodies to HCV by Enzyme immunoassay (EIA) has become important as an indication of past or present infection. Sensitivity was low (70-80%) and false positive results in patients with high immunoglobulin concentrations were frequent. Second generation enzyme immunoassays utilize structural proteins (core-antigen or c-22) as well as nonstructural proteins of the NS3 (c-33) and NS4 (C-100) region for the detection of HCV-specific antibodies. These assays detect up to 95% of HCV-infected persons with much higher specificity than the first generation assays. Furthermore, second generation EIAs detect HCV seroconversions much earlier than first generation EIAs and become positive within 4 to 6 weeks after infection. Reducing this window period by testing for HCV-specific IgM-antibodies is of marginal benefit and restricted to a few cases. Anti-HCV core -IgM-antibodies do not develop in all patients with acute hepatitis C and may persist in patients with chronic disease. They can be found in more than 50% of patients with severe ongoing chronic infection. Usually IgG antibodies persist in chronically infected HCV-patients but may vanish after resolution of acute hepatitis C virus infection over years. Anti-HCV antibodies positivity is strongly correlated with persistent viremia. However, antibodies may not be present in patients with low viremia levels. Third generation antibody tests are characterized by the addition of recombinant proteins derived from the NS5 gene region. Their increased sensitivity results from a higher reactivity of the NS3 derived antigens. Most likely, the NS5 antigen is responsible for an increased rate of false-positive results. For the confirmation of positive EIA results diverse immunoblot / dot techniques have been developed to detect monospecific anti-HCV antibodies against nitrocellulose bound recombinant proteins (RIBA, Chiron; Matrix HCV, Abbott) (48). These assays are technically based on a variation of the EIA principle. It has to be taken into account that up to 20% of anti-HCV EIA- positive blood donors which are negative or indeterminate in the immunoblot may be positive for HCV RNA by RT PCR. These antibody confirmation tests are of (limited) value only

in those populations with low HCV prevalence (e.g. blood donors) and are usually dispensable in the diagnosis of patients with liver disease. Hepatitis C virus core antigen can be detected in serum after the enrichment of viral particles by centrifugation and subsequent disintegration of the viral envelope. Due to a low concentration of viral particles in serum, the sensitivity of the assay is low. Moreover, the sensitivity may be negatively influenced by the specificity of the monoclonal antibody used and which may fail to bind to variant core proteins present in the sera.

Molecular diagnosis

HCV Polymerase Chain Reaction (PCR)

Diagnostic approaches that rely on the direct detection of protein or nucleic acid components of a virus must be highly sensitive because of the typically low levels of such viral components in serum or tissue. Exquisitely sensitive procedures for detecting viral proteins have been developed over many years, and more recently, the analogous procedures for detecting viral nucleic acids are *ultra*sensitive even when compared to the most sensitive immunoassays. The benchmark for this new generation of nucleic acid assays has been the polymerase chain reaction or PCR. Detection of HCV RNA requires prior reverse transcription (RT) into cDNA by so-called RT-PCR. Due to low viral titers, the unpredictability and apparent incompleteness of the immune response to HCV, RT-PCR has become an indispensable tool in the diagnosis of hepatitis C. The extreme sensitivity of RT-PCR for HCV RNA was ±10 molecules, viral RNA has been detected as early as one week after infection. The major indication for nucleic acid based diagnosis of HCV infection, and the advantages and disadvantages of PCR in comparison to serologic testing are summarized in Table 2. The most sensitive diagnosis primers for HCV PCR are conplementary to highly conseved 5 ′- non coding region of the HCV genome (49,50). In order to further increase sensitivity, many laboratories utilized two primer sets ("nested" PCR) for the amplification of viral sequences. However, a similar sensitivity can be achieved with only one primer set after stringent optimization of primers and reaction conditions. A major drawback of the nested procedure is the necessity to open reaction tubes between the two rounds of PCR with the different primer sets. This manipulation is prone to contamination via aerosol thus generating false positive results.

Table 2. Comparision of antibody detection and nucleic acid amplification for HCV

	Antibody detection	Nucleic acid amplification
Acute infection	After 2-6 months	After 1-2 weeks
Resolved vs. chronic infection	+/-	+
Immune compromised patients	+/-	+
Neonatal infection	-	+
Re-infection post-transplantation	-	+
Virus load determination	-	+
Organ-specific detection	-	+
Specificity	95-99%	100% possible
Hands-on time	Low	High
Cost	Low	High

At the end of 1993, the first standardized RT-PCR test kit for detection of detection of HCV RNA (Amplicor™HCV, Roche Diagnostic Systems) was introduced in the market. Several important improvements to this test system have been made since. The DNA-polymerase of *Thermus thermophilus* which replaces the *Taq*-polymerase has both reverse transcriptase and DNA-polymerase activity under the appropriate buffer conditions, enabling reverse transcription with subsequent amplification without opening tubes and changing buffer (51). Pre-incubation of the PCR reaction in the presence of uracil-N-glycosylase (UG, Amperase™) and the replacement of dTTP by dUTP enables the selective enzymatic digestion of potentially contaminating dUTP containing amplification products from previous PCR reactions (52,53).

A recent quality control study of HCV RT-PCR organized by Eurohep, revealed that 29% (9/31) all participating laboratories produced false positive results. Only 5 (16%) laboratories performed faultlessly with the entire panel of samples (53). The introduction of the qualitative Amplicor in several laboratories participating in the second round of the Eurohep quality control study did not contribute to a significant improvement in sensitivity and specificity. There was still a high proportion of false positive and false negative results in HCV RT-PCR, most probably due to handling difficulties in laboratories inexperienced in the use of molecular techniques. The Amplicor™HCV RT-PCR kit performed well with a lower detection limit

ranging between 100 to 1000 RNA copies per ml. In comparative evaluation against in-house PCR, the Amplicor test achieved sufficient sensitivity and specificity on a test panel of 100 clinical samples. However, 1-3 false positives. Of which 2 were resolved on re-test, were observed during routine performance (54-56).

Recently, the first competitive principle based PCR assay for sensitive quantity (±100 copies per ml) of HCV viremia has been commercialized (Amplicor™HCV Monitor™, Roche Diagnostic Systems). In the Monitor™ assay target HCV RNA and an internal synthetic HCV RNA quantity standard are both reverse transcribed and amplified in a single tube reaction employing rTth DNA polymerase in the presence of Mn^{2+}. Amplification products are then serially diluted and detected via hybridization in separated microwells for target and internal standard HCV RNA. The number of HCV RNA copies per ml in the sample are calculated from a computer program. The utility of Amplicor™HCV Monitor™assay for the monitoring of viral load in patients with chronic hepatitis C treated with interferon is currently under evaluation.

Even with the sensitive HCV ELISA there remains a gap of several weeks between infection and first appearance of antibody and in the latter part of this interval HCV RNA can be detected. Donors presenting at such a time will be ELISA negative and the blood processed for use. Transmission from such donations is known. In countries with low HCV prevalence such transmissions will be rare but in high prevalence countries their frequency could be sufficient to justify a PCR approach, e.g. by screening pools of sera. One of the major contributions of genome amplification technology to knowledge of HCV has been the identification of 6 major genotypes of the virus, numerous subtypes and their worldwide distribution (57). This extensive variability in the HCV genome raises the question of whether the current HCV ELISA available for blood donor screening are detecting all HCV infected donors. The present HCV ELISA and also the supplementary tests are based on genotype 1 antigens. This genotype is by far the most frequent found in the USA and Japan with type 3 and others being rare in the USA and not reported in Japan. Type 3 however has a significant frequency in NW Europe (Scotland, Finland, Netherlands, Eire) and is the most common genotype in Thailand, Bangladesh, Pakistan and the Indian sub-continent. In addition, more diverse genotypes such as type 5 and type 6 have significant frequency in South Africa and Hong Kong, respectively. If ELISA and supplementary tests are contained only type 1 antigens. Then these have to be carefully chosen so that either the

amino acid sequence variability is minimal or that the variability between genotypes is such that it has no effect on the sensitivity of the ELISA for other genotypes.

Epidimology of HCV infection

Parenteral transmission

The parenteral route of HCV transmission is responsible for almost two thirds of hepatitis C cases and constitutes the most commonly recognized and characterized transmission mechanism of HCV. Anti-HCV testing has largely confirmed that HCV is responsible for the vast majority of hepatitis cases in which transfusion of blood or blood components or obvious percutaneous exposure to blood are involved (58).

Transfusion recipients (59,60)

Before the implementation of mandatory anti-HCV screening in 1990, there was a wide range of transfusion-associated hepatitis C (TAH-C) incidences indifferent geographic areas, ranging from 0.5% in England, 1% in Australia, 3 to 4% in the United States, 8% in Japan, 11% in Spain 12% in Taiwan, to 13% in Greece. As expected, patients requiring multiple transfusions have been found to have very high prevalences of HCV infection. Among more than 1000 transfusion – dependent Italian thalassemics 80% had confirmed anti-HCV; as well as 47% of Egyptian thalassemic children and 75% of multitransfused patients in long-term remission from leukemia with evidence of liver disease. Screening of blood donors for anti-HCV has practically eradicated TAH-C so that transfusion of screened blood should no longer be considered a primary risk factor for HCV infection. The risk of acquiring HCV infection from transfusion of screened blood is currently extremelly small. In a recent study, it has been estimated that, in the United States, the current risk of receiving blood from a donor in the window period of infectivity, i.e. before seroconversion, would be 1/100,000. At least three such cases have been reported in Europe during the past two years.

Plasma product recipients

The prevalence of HCV among hemophiliacs correlates with the amount and type of product transfused. Virtually all hemophiliacs exposed to untreated commercial clotting factor concentrates have evidence of HCV infection, while prevalence of about 65% are seen among those treated with cryoprecipitates. In contrast, hemophiliacs who have exclusively received appropriately inactivated coagulation components or single-donor cryoprecipitates are generally anti-HCV negative.

Hemodialysis patients

Among patients on maintenance hemodialysis prevalence of HCV infection averages 20%, although there are wide grographical variations ranging between less than 5% in the UK, Australia, New Zealand, and South Africa, to 30-40% in Taiwan, Japan, Saudi Arabia, South America and Eastern Europe. Intermediate prevalences, between 5 and 30%, have been reported from the U.S. and Western Europe (61). The high prevalence of HCV infection in hemodialysis patients has attributed not only to the frequency of blood transfusion among these patients but also to increasing years on dialysis, suggesting that HCV may be transmitted between patients in the dialysis unit probably as a result of poor infection control practices.

Organ transplantation

Organ transplant recipients are at high risk of acquiring HCV infection. Infection in this setting can derive from recurrence of HCV infection already present prior to transplantation, to transfusion –associated transmission during transplantation, or to the presence of HCV infection in the organ donor (62).

Nosocomial transmission

Recently, surgeon-to-patient transmission of HCV during cardiac surgery has been documented (63), although the frequency of such transmission remains to be established. It is

likely that with the decrease of transfusion–associated hepatitis C, nosocomial transmission becomes the predominant mode of health care-associated spread of HCV.

Intravenous drug use

Intravenous drug addiction carries an extremely high of HCV infection. Data from the Centers for Disease Control (CDC, Atlanta, USA) showed that from 1986 to 1988, drug abuse was reponsible for 42% of cases of acute hepatitis C acquired in the community (64), and worldwide studies have found anti-HCV in 70 to 90% of intravenous drug users.

Health care

Health care workers have a higher prevalence of anti-HCV than blood donors. Reported rates in the U.S. have been 1.4% in general staff, 2% in dialysis staff, 10% in drug addict assistance staff, and 0.9% among hospital surgeons.

Nonparenteral transmission

Perinatal transmission

Recent reports (65), have shown that, even in the absence of HIV coinfection, perinatal transmission of HCV occurs in 3 to 5% of newborns to carrier mothers, and that the risk of transmission correlates with HCV RNA levels in the mother. No transmission seems to occur when HCV RNA levels are below 10^6 genome equivalents/ml. Breast-feeding carriers have no further risk of transmission.

Sexual and household transmission

Transmission of HCV through sexual intercourse seems extremely uncommon, although concomitant HCV infection may increase the risk somewhat.

Sporadic hepatitis

Acute hepatitis C with no apparent risk factor, continues to occur. The mechanism of transmission of sporadic hepatitis C cases is possibly a combination of intravenous drug use which is not revealed in the history, inapparent or covert, nosocomial, percutaneous exposure, nonpercutaneous mechanisms, including rare cases of sexual transmission and, perhaps, as yet unidentified modes of virus dissemination. Recently cocaine snorting has been suggested a significant risk factor for HCV infection when it involves sharing of blood contaminated straws.

HCV infection in patients with chronic liver disease

HCV markers are detected in 70 to 80% of patients formerly classified as cryptogenic chronic hepatitis and cirrhosis or sporadic chronic NANB hepatitis. Anti-HCV antibodies are found in patient with hepatitis B surface antigen (HBsAg) positive chronic liver disease from Southern Europe, the United States and Far East with seroprevalence ranging from 10 to 20%. The highest rates of HCV and HBV co-infection (30-50%) have been detected in the Mediterranean, some areas of China, India, Indonesia, Japan, and New Zealand, most usually among patients with severe chronic hepatitis or cirrhosis and inactive HBV infection, due to the suppressive effect of HCV core antigen on HBV replication. HCV plays also an important role in alcoholics with chronic liver disease. Several studies on this association have been reported (66).

Prevalence of HCV infection among blood donors and the general population (67)

Very low prevalences of HCV have been reported in the U. K. and Scandinavia 0.04-0.09 %, and also in some areas of Japan. Low prevalence ranging from 0.15-0.5 % have been described in the U.S., Western Europe, and Israel. Moderate prevalences between 0.6-1% have been found in some areas of Southern Europe, Kenya, and Thailand. Prevalences between 1 and 1.5 % have been reported from India, China, Cuba, and Ethiopia. High prevalences between 1.6-3.5 % have been found in Japan, Indonesia, Turkey, some area of Russia, Brazil, and the Middle East. Extremely high prevalences have been reported in some parts of Cameroon (6.4%), and in Egypt (14%). The highest prevalence thus far reported was in Cario (26%).

Prevalence of hepatitis C in Thailand

Hepatitis C virus, infecting about 1.5% of the general population, is the second major cause of chronic chepatitis and liver cirrhosis, but is less common in hepatocellular carcinoma (40). The proportion of hepatitis C cases in male and female was 1.3: 1. The most of hepatitis B cases were found in persons 30 to 60 years of age. Recent studies have demonstrated risk factor of persons with HCV infection in Thailand. Injecting –drug use accounted for 4.8 % of HCV transmission, household member who had a history of hepatitis for about 6.3%, homosexual for about 1.6%, post transfusion for about 42.9% and no recognized source of infection for about 44.4%(68). There was another report that HCV infection associated with IDVU (52%), multiple transfusion (14%), post transfusion hepatitis (63%), non HBV cirrhosis (42%), sexual partner (7.7%), and prostitute (4%), (69). The prevalence of HCV infection among blood donors was 1.4%(data from National Blood Center. 1995-1999). The HCV and HBV co-infection rates in hepatitis patient was 6% (68) and was 0.03% in blood donors.

Multiplex PCR

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988 (70), this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse-transcription PCR. This technique is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. Multiplex PCR often requires extensive optimization because primer-dimers and other nonspecific products may interfere with the amplification of specific products. Especially important for a successful multiplex PCR assay are the relative concentrations of the primers at the various loci, the concentration of the PCR buffer, the cycling temperatures and the balance between the magnesium chloride and deoxynucleotide concentrations. Figure 8 illustrates a rational approach for developing efficient multiplex PCRs (71).

Figure 8. Step-by-step protocal for the multiplex PCR.