The biology and pathogenesis of hepatitis viruses

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Viral hepatitis is caused mainly by infection with one of the five hepatitis viruses, which use the liver as their primary site of replication. Each of these, known as hepatitis A through E viruses (HAV to HEV), belong to different virus families, have unique morphology, genomic organization and replication strategy. These viruses cause similar clinical manifestations during the acute phase of infection but vary in their ability to cause chronic infection. While HAV and HEV cause only acute disease with no chronic sequelae, HBV, HCV and HDV cause varying degrees of chronicity and liver injury, which can progress to cirrhosis and liver cancers. Though specific serological tests are available for the known hepatitis viruses, nearly 20% of all hepatitis cases show no markers. Antiviral therapy is also recommended for some hepatitis viruses and a preventive vaccine is available only for hepatitis B.

Introduction

HEPATITIS, or inflammation of the liver can be due to a variety of causes of which viral infection is the most important, and leads to significant morbidity and mortality. Viral hepatitis is caused by infection with one of the five known viruses, which predominantly affect the liver – the hepatitis A, B, C, D and E viruses (HAV, HBV, HCV, HDV and HEV)1-5. Despite significant overlap in the clinical manifestations caused by them, the hepatitis viruses differ widely in their morphology, genomic organization, taxonomic classification and modes of replication. These viruses enter the host by one of the two major routes, enteral or parenteral. The HAV and HEV are enterally transmitted and cause an acute, self-limited infection, with complete resolution, except in rare situations in which fulminant disease with high mortality is observed. The associated clinical illnesses, named as hepatitis A and hepatitis E respectively, can each occur either as epidemics, or as sporadic cases in the absence of a recognized outbreak. The HBV, HDV and HCV are parenterally transmitted, cause acute infection with a high propensity to become chronic with long-term sequelae such as cirrhosis and hepatocellular carcinoma (liver cancer).

This article will review various aspects of the five known hepatitis viruses, which include their epidemiology, transmission, disease, biology, pathogenesis, approaches to vaccination and therapy.

Hepatitis A virus

Though hepatitis A virus (HAV) infection is distributed worldwide, its epidemiological characteristics vary with socio-economic development. In developing countries with poor sanitary and living conditions, such as those in Africa, Asia and parts of South America, transmission rates are high and most infections occur in early childhood. Since HAV infection confers strong life-long protection, infection and disease among adults is uncommon in these regions. In contrast, in developed countries, HAV transmission during childhood is much less frequent. The cases in these regions arise from travel to endemic areas, person-to-person spread or common-source contamination of food. Even within a country or population, rates of antibody prevalence may vary widely, depending on the socio-economic status, family size and hygienic practices. Humans are the only host, and hence the only source, of HAV. The virus is excreted in large amounts in faeces of infected persons, and is transmitted by the faecal-oral route. The most important mode of transmission is close contact with an infected person, usually in a household or a school. Contaminated water and foods such as seafood, farm products, milk, hamburgers, ice-slush beverages and salads are important modes of transmission. Although blood or blood products can also transmit HAV, such events are uncommon. Sexual transmission of HAV has been reported, especially among men having sex with men.

Infection with HAV may be asymptomatic or may result in acute hepatitis of variable severity, including fulminant hepatitis. The incubation period is 2–6 weeks. The illness usually begins with a prodromal phase of 1–7 days characterized by non-specific, systemic symptoms, such as fatigue, malaise, low-grade fever, headache, myalgias, arthralgias, loss of appetite, nausea and vomiting, altered taste-sensation and aversion to fatty foods and smoking. Hepatitis is first suspected with the appear-
ance of specific symptoms, such as dark urine, upper-quadrant pain, jaundice or light-coloured stools. The illness generally lasts a few weeks. In occasional cases, clinical relapses can occur weeks to months after the onset of the initial illness. Almost all cases recover completely with no chronic hepatitis or long-term sequelae. The likelihood of disease following HAV infection increases with age, with children below the age of 6 years remaining either asymptomatic or with mild and non-specific symptoms. In contrast, most older children and adults with HAV infection have liver-specific symptoms such as jaundice. The risk of complications and case fatality-rate also increases with age.

Elevation of aminotransferase (ALT, AST) levels is a sensitive, but non-specific indicator of liver damage. Specific diagnosis of hepatitis A is based on serology. Testing for anti-HAV IgM is the most appropriate since its levels rise early in the acute phase of disease and disappear in 4–6 months, unlike anti-HAV IgG, which persists for life. Patients with hepatitis A do not need any specific treatment, since the disease is self-limited. Management is thus primarily supportive. Hospitalization is needed only for patients with another serious medical problem. Patients with fulminant disease need treatment in an intensive care or specialized liver unit.

Improvements in hygiene, a safe water supply and adequate disposal of human waste are central to hepatitis A prevention. Passive transfer of antibodies, by intramuscular administration of human immunoglobulins, before or shortly after exposure to HAV provides protection against clinical disease. Two different vaccines are currently available against hepatitis A—Havrix™ (Glaxo Smith-Kline) and VAQTA™ (Merck). Both contain formalin-inactivated attenuated strains of HAV, are highly immunogenic and safe. For each vaccine, two doses separated by at least 4 weeks are recommended. Protective antibody levels persist for 10–20 years. A combined vaccine against both hepatitis A and B is also available—Twinrix™ (Glaxo SmithKline), for use in persons 18 years or older. The current high cost of these vaccines remains a major limiting factor.

HAV is a member of the family Picornaviridae (pico = small; rna virus), which also includes other important human pathogens such as poliovirus, rhinovirus, coxsackie virus, etc. It is a non-enveloped virus of about 27–32 nm (Figure 1), made up of a capsid of three or four proteins and a single-stranded, positive-sense polyadenylated RNA genome of about 7.5 kb (ref. 1). The HAV genome is linear and includes 5′ and 3′ non-coding regions (NCR), the former of about 750 nucleotides, and a single open reading frame (ORF) that encodes all the viral proteins (Figure 2). Translation of the HAV ORF is driven by an internal ribosome entry site (IRES), a highly structured region of RNA in the 5′NCR, which acts as a site for ribosome assembly in the absence of a 7-methylguanine (cap) at the 5′ end of the viral RNA. The 5′ end of HAV RNA is linked to the viral protein VPg, which aids in genome replication. The ORF is divided into three regions—P1, P2 and P3. The P1 region encodes the four viral structural proteins—VP1 of
33 kDa, VP2 of 27 kDa, VP3 of 29 kDa and VP4 of 17 amino acids. The P2 and P3 regions encode the non-structural proteins with biochemical activities that support viral genome replication and protein processing (Figure 2).

Various primary and continuous cell lines of primate origin support the culture of HAV. These include primary African green monkey kidney (AGMK) cells, primary human fibroblasts and MRC5 human diploid lung cells. Many HAV strains have been characterized, of which HM175 has been adapted to different cell types to yield a range of attenuated, persistent, cytopathic and neutralization-resistant viral variants. The HAV genomic RNA was molecularly cloned as a cDNA copy and RNA transcripts produced in vitro from this copy were found to be infectious when transfected into cultured cells.

Following infection, HAV presumably replicates in the small intestine, from where it reaches the liver through portal circulation. The major site of HAV replication is the hepatocytes, but this tissue tropism is not fully understood as HAVcr-1, a cell surface glycoprotein with immunoglobulin (Ig) and mucin-like domains, which acts as the entry receptor is not specific to hepatocytes. Following entry and uncoating, the entire ORF on the genomic RNA is translated into a polyprotein, which is subsequently processed into 11 different proteins through the actions of the viral 3C protease and unidentified cellular proteases (Figure 2). The viral 2C (helicase) and 3D (RNA polymerase) proteins replicate the genomic plus-stranded RNA through an amplification cycle that involves an antigenomic minus-stranded RNA intermediate. Late in the replication cycle, the capsid proteins package the genomic RNA and the newly formed virions are secreted across the apical surface of hepatocytes into liver sinusoids and bile canaliculi from where they enter the small intestine and are excreted in faeces. Unlike other picornaviruses, which are cytopathic, HAV is not cytopathic and liver injury is possibly due to the host immune response.

During acute infection, viraemia and faecal shedding of the virus appear within days of infection; while viraemia typically persists till the appearance of symptoms, stools remain infectious for another 1–2 weeks. The host immune response to HAV infection is marked by the appearance initially of anti-HAV IgM, followed by anti-HAV IgG, both of which can neutralize the virus in vitro. While anti-HAV IgM titers wane off in 4–6 months following exposure, anti-HAV IgG persists throughout life and confers protection against reinfection. Virus-specific and HLA-restricted CD8+ cytotoxic T cells have been described in the liver during acute hepatitis A. These cells secrete interferon-gamma, which is likely to recruit non-specific inflammatory cells to the sites of viral replication to aid in viral clearance, but this may also cause liver injury. During the incubation period, HAV replicates to high titers in the liver, with viraemia and faecal shedding, suggesting that HAV may regulate host pathways for virus recognition and clearance. Innate immunity to limit the spread of viral infection is based primarily on interferon production by infected cells. During the replication of RNA viruses, double-stranded RNA (dsRNA) is produced, which triggers interferon production following its recognition by toll-like receptor 3 (TLR3) or a cytoplasmic sensor RNA helicase, retinoic acid-inducible gene I (RIG-I). In vitro, HAV was shown to attenuate the interferon response by inhibiting the RIG-I-mediated signalling pathway, thus potentially evading innate immunity. This would be crucial for the establishment of a self-limited viral infection.

**Hepatitis B virus**

The hepatitis B virus (HBV) chronically infects 350 million people worldwide, causing maladies ranging from acute hepatitis to chronic hepatitis, cirrhosis and hepato-
cellular carcinoma (HCC), and is responsible for nearly 1 million deaths annually. The virus is not cytopathic and the hepatocyte necrosis is probably a consequence of cytotoxic T-cell response directed against HBV or the membrane-bound viral antigens on hepatocytes. Chronic HBV infection with cirrhotic liver has been considered as the single most important factor associated with the development of HCC, which is one of the most malignant cancers and the third leading cause of death among men.

The diagnosis of chronic HBV infection is made by monitoring biochemical, virological and histological parameters. Routine liver function tests and serological assays for the detection of HBV antigens (HBsAg and HBcAg) and antibodies (anti-HBs, anti-HBc and anti-HBe) are performed to assess the phase of chronic hepatitis B. The HBsAg is considered as a good marker of active HBV replication. Presence of viral DNA in serum by PCR or DNA hybridization is used for monitoring disease activity, antiviral treatment and response to such treatments. Liver biopsy is used to confirm the diagnosis, to know the stage of fibrosis and to grade the necroinflammation.

The epidemiology of HBV resembles that of HIV in many ways. Transmission occurs primarily through parenteral, sexual or faeto-maternal routes. The faeco-oral route of transmission is relatively non-important. HBV is present in nearly all body fluids of infected individuals including blood, saliva, semen and urine. Viral transmission generally involves transfusion of blood products, injection with a contaminated needle or intimate personal contacts (between sexual partners; mother and infants). The high-risk groups for HBV infection include intravenous-drug users, sexually promiscuous individuals, health workers and transfusion patients. Besides, certain geographic regions of the world have been identified with high rates of HBV carriers; these regions include sub-Saharan Africa, Southeast Asia, Oceania and the Mediterranean region. The high rates of HBV infection in these regions are thought to be due to vertical transmission of the virus from mother to neonate.

Initially, HBV strains were classified into four serotypes, viz. adw, adr, ayw and ayr based on two pairs of mutually exclusive serotype determinants d/y and w/r in the surface antigen (HBsAg), along with the main antigenic determinant ‘a’. These subtypes can be further classified into nine subtypes (ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adwr and adrq). Now based on phylogenetic analysis of complete viral genome sequences and 8% genetic variability among the viral strains observed, the HBV strains have been classified into eight well-established genotypes named A through H, some of which have their distinct subtypes. The HBV genotypes and sub-genotypes show a distinct geographical distribution pattern. For example, genotypes B and C are most prevalent in East Asian countries where vertical transmission is common whereas genotypes A and D are generally found in Africa, Europe, Middle-East and the Indian subcontinent which relate to horizontal transmission.

The seroprevalence of HBsAg in different studies from India ranges from 2% to 10%. Thus, India falls in the group of intermediate to high endemicity for HBV infection. Assuming an average HBsAg carrier rate of 5%, the total number of HBV carriers in the country is estimated to be about 50 million which is ~15% of the total world pool of HBV carriers.

HBV transmission can be prevented by screening of donated blood and plasma, by virus inactivation in plasma-derived products and by implementation of infection-control practices. However, the single most effective prevention measure is routine immunization for infants and high-risk individuals. Infants born to HBsAg carrier mothers can be protected against perinatal transmission by passive immunization.

The only antiviral agent that is currently FDA approved for use in the treatment of HBV infection is alpha interferon (IFNα). However, due to a low efficacy rate of around 35% cases and many side effects, the search for a more effective treatment still continues. Recombinant subcutaneous IFNα (10 MU thrice weekly) and oral Lamivudine (100 mg once a day) are used in many countries. Adefovir, Entecavir, Telinevidine and Tenofovir are other licensed nucleoside analogues for human use. Response to therapy includes measurement of serum levels of HBV DNA (<10^5 copies/ml), sustained absence of HBeAg from serum and improvement in liver architecture and function.

The sera of HBV patients carry large numbers of 22 nm spherical and filamental empty particles (Figure 1). These particles are composed of envelope proteins and lipids derived from the host hepatocytes. The infected sera may also contain small numbers of 42 nm particles, also known as Dane particles, which represent the intact virus (Figure 1). Present within the viral envelope of the 42 nm particle is a nucleocapsid, which is predominantly made up of the core protein. The core protein is important for packaging of the viral genome during viral assembly. The viral genome is a partially double-stranded circular DNA of about 3.2 kb, with four ORFs that encode the viral core (HBCAg), envelope (HBsAg), polymerase (P) and HBx proteins. Interestingly, all regulatory signal sequences reside within protein-encoding sequences and proteins are encoded from overlapping translation frames. The HBx is a multifunctional regulatory protein required for viral infection and has been implicated in the development of HCC.

The mechanisms of viral entry are not fully understood. Several candidate receptors for HBV have been described whereby endocytosis represents a putative mechanism for viral entry. The polymerized human serum albumin is known to facilitate the viral entry process. The other candidate receptors include HBV binding protein, carboxypeptidase D (gp180), glycine carboxylase...
(p120), asialoglycoprotein receptor and endonexin II, which were characterized using either cell culture or duck hepatitis virus model. For lack of proper information about the HBV receptor, it has not been possible so far to develop a suitable animal model for viral replication and disease. The orthotopic nude mouse models of human hepatocytes hold promise in future.

The life cycle of HBV is complex but unique. Although it is a non-retrovirus, it still uses reverse transcription as a part of its replication process. After gaining entry into cells, the viral genomic DNA is transferred to the nucleus where the partially double-stranded DNA is made fully double-stranded covalently closed-circular DNA (cccDNA). The cccDNA serves as a template for transcription of four viral mRNAs with the help of host RNA polymerase. The largest mRNA also called pre-genomic RNA is longer than genomic length (3.5 kb), is used as a template to make new copies of the genome and to make the capsid protein and the viral DNA polymerase. The pregenomic RNA is then transported back to the cytoplasm where the viral DNA polymerase makes a DNA copy [L(–) strand] via its reverse transcriptase activity. As the viral polymerase copies the L(–) strand to generate the S(+) strand, the viral particles begin to assemble at the hepatocyte surface and are released by budding off.

Significant advances have been made towards the prevention of HBV infection. These include development of an effective recombinant vaccine composed of purified HBsAg as well as Ig containing high-titer anti-HBsAg.

The strategy for the control of HBV infection, as outlined by the World Health Organization (WHO) and endorsed by the Advisory Committee on Immunization Practices (ACIP), is the introduction of hepatitis B immunization at birth. This strategy is designed to reduce the risk of childhood acquisition of HBV and reduce the number of chronic carriers in endemic populations. In lower-risk populations where transmission of HBV primarily occurs in older individuals, newborn immunization is also used to prevent the small number of cases transmitted in early childhood. The Indian Academy of Paediatrics (IAP) also endorses the recommendations of WHO on the HBV vaccination at birth whether the mother is a carrier or not. The duration of hepatitis B vaccine protection has not been firmly established. Long-term protection of 10–12 years appears to occur for those infants at high risk. The necessity of booster doses to extend protection through adulthood needs to be established.

### Hepatitis C virus

Since its discovery in 1989, hepatitis C virus (HCV) is estimated to have infected almost 200 million people, representing almost 3% of world population. In 20–30% of patients HCV causes acute infection and the virus is naturally cleared, but in the majority of patients, it causes a long-term chronic infection. Persistent infection with HCV is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis and HCC. Worldwide, an estimated about 27% of HCV-infected people develop cirrhosis and about 25% develop HCC.

A combination of serological (RIBA test) and molecular methods (RT-PCR), as well as determination of viral loads by quantitative RT-PCR techniques are generally used to assess HCV infection. Screening for antibodies against HCV indicates exposure to the virus but cannot confirm an active HCV infection. Detection of HCV RNA in blood confirms the presence of virus and the nucleotide sequencing precisely identifies the actual genotype of the infecting strain.

HCV is transmitted through large or repeated percutaneous exposures to blood from transfusion, transplantation of infected organs, injecting drug use, etc. In fact, transfusion-associated HCV infection was the highest risk before HCV testing became available and mandatory for blood donors. Transmission is not so efficient upon mucosal exposure to blood or serum-derived fluids. Perinatal transmission takes place at lower rate and the possibility of sexual transmission is controversial.
Both geographic and temporal differences in the pattern of HCV infection have been observed. The highest prevalence is reported from Egypt (15–20%), whereas UK and Scandinavia have reported least prevalence of HCV infection (0.01–0.1%).

There are six reported genotypes of HCV, which differ in their pathogenicity, efficiency of translation/replication and responsiveness to antiviral therapy. Genotypes 1, 2, 3 are the major types observed in Japan, Western Europe and North America. Type 4 has been found in Central and Northern Africa and in the Middle East. Type 5 has been described in South Africa and type 6 in Southeast Asia. HCV infection in India accounts for 1% of the total population. Unlike other parts of the world, genotype 3 has been found to be the predominant genotype in India followed by genotype 1. Earlier, HCV type 1a, 1b, 2a, 3a, 3b and 3g were reported in the northern regions of India, whereas genotype 1 was shown to be prevalent in the southern part of India.

Currently, no vaccine or effective therapy is available for HCV. A combination therapy of interferon-α (IFNα) with the nucleoside analogue Ribavirin is usually given to patients. However, its efficiency varies with the HCV genotype and the viral loads at the start of therapy. The sustained virological response (SVR) with IFNα monotherapy in HCV genotype 1 patients was found to be poor. However, combination therapy with pegylated interferon (peg IFNα) and Ribavirin, which is the current strategy, is associated with significantly higher SVR. The response to therapy still varies with the viral genotype, apart from potential host and treatment efficacy factors. In India, patients infected with genotypes 2 and 3 achieve SVR of up to 95% to a combination of daily IFNα2b and Ribavirin. Worldwide, patients infected with genotypes 2 or 3 achieve a SVR of up to 72% whereas genotype 1 shows relatively low response rates (13%) after 24 weeks of treatment. Though the mechanism of resistance to interferon treatment in infected patients is not thoroughly understood, it is believed that both host and viral factors including several viral genomic regions are important for the effective response to interferon therapy.

HCV is a small, enveloped virus (Figure 1), a member of the Flaviviridae family and the lone example of the genus Hepacivirus. The genome consists of a single-stranded positive sense RNA molecule approximately 9.6 kb long encoding a large ORF, which is flanked by highly structured 5′ and 3′ untranslated regions (UTR) (Figure 4). The 5′ UTR (341 nt) harbours the IRES that mediates cap-independent internal initiation of HCV RNA translation. The 3′ UTR varies between 200 and 235 nt, which includes a polyU/UC tract of variable length (~80 nt) and a conserved 3′ X tail region (98 nt) that helps in RNA replication.

The viral proteins are translated as a single polyprotein, which undergoes a series of co- and post-translational cleavage with the help of both host cell signal peptidases and viral proteases to generate the structural and non-structural proteins (Figure 4). The structural proteins include the core, E1, E2 and p7 proteins. The E1 protein has been proposed to serve as a fusion protein during infection, whereas the E2 protein may be involved in binding to host cell receptors. The N-terminal 27 residues of the E2 protein are considered as the hypervariable region I (HVR1). The p7 protein belongs to the family of viroporins, which is essential for the production of infectious virions in vivo and thought to form ion channel in the lipid bilayer. However, its exact function in HCV infection is not clear at this stage.

The non-structural proteins include the NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins (Figure 4). The NS2 protein together with the amino-terminal region of the NS3 protein constitutes the NS2–NS3 proteinase that catalyses a single autocatalytic cleavage to release NS2 and NS3 proteins. Later, the N-terminal region of NS3 serves as a serine protease and cleaves at different sites within the polyprotein to release other non-structural proteins. The C-terminal part of NS3 binds to HCV RNA.
and serves as a RNA helicase. The NS4A protein acts as a cofactor of NS3 activity, while the NS4B protein appears to interact with the viral replicase. The NS5A protein has been shown to be involved mostly in HCV pathogenesis causing ER and oxidative stress. It also confers resistance to IFN therapy by binding to a subunit of the PKR enzyme. The NS5B protein is a RNA dependent RNA polymerase (RdRp) and helps in the replication of HCV RNA.

HCV enters its target cells in a highly coordinated process involving components of the virus particle and numerous cellular factors. Interestingly HCV entry is restricted to only human and chimpanzee cells, suggesting a block at the level of virus entry in other cell types. Although replication of HCV RNA has been shown in mice, the virus cannot enter murine cells due to lack of specific receptors. So far several putative receptors have been demonstrated to be involved in HCV entry into the permissive human hepatocyte cell line Huh7.5. These include CD81, SR-B1 (the scavenger receptor class B type 1) and Claudin 1 (CLDN1). Recently human Occludin (OCLN) has also been shown to be an essential entry factor that contributes to HCV susceptibility of human cells. More importantly, over expression of human Occludin and CD81 in the presence of murine SR-B1 and CLDN1 can render murine cells permissive to HCV entry. Identification of the receptors is a big jump towards developing small animal models for HCV infection. Recently, the genetic variation in the regulatory region of the co-receptor CLDN1 has been shown to influence the susceptibility to HCV infection.

The translation of HCV RNA is mediated through the IRES element located in the 5′UTR, which also extends up to 40 nt within the core protein coding sequences. Translation of the input viral RNA is the initial obligatory step; however the mechanism of switching from translation to viral RNA replication is not clear. Four highly structured stem-loop domains (SLI-IV) along with a pseudoknot structure constitute the IRES element where several protein factors bind. The RNA–protein interaction largely influences the function of the HCV IRES. Ribosome assembly onto HCV IRES is unique and fundamentally different from the host cell RNA. The 40S ribosomal subunit has been shown to bind directly to HCV IRES (through the ribosomal protein S5) without any help from initiation factor, analogous to prokaryotic RNA. Subsequently, canonical initiation factors (eIF2 and eIF3) and non-canonical IRES trans acting factors (ITAFs) bind to the HCV IRES and help in the formation of functional initiation complex. Earlier, human La autoantigen was shown to bind to HCV IRES RNA and help in ribosome assembly during internal initiation of translation. The central RRM (112–184) of human La protein was shown to interact with the GCAC motif near the initiator AUG and trigger a conformation change, which facilitates 40S binding during internal initiation.

It appears that La proteins binding near the AUG help in the interaction of ribosomal protein S5 that is an integral part of the 40S interaction with the HCV IRES. The La protein has also been shown to bind to the 3′UTR of the HCV RNA and might assist in its replication. Recent reports suggest that some HCV proteins also interact with the HCV IRES and negatively regulate its function.

Research on the mechanistic details of HCV replication in cell culture received a major boost after the availability of the replicon systems. Several replicon constructs were in use in the past few years, which includes a bicistronic replicon containing IRES of HCV type 1b (Con1) that mediates synthesis of the selection marker for neomycin resistance, followed by the IRES element of EMCV, which mediates translation of the non-structural proteins NS2 to NS5. Transfection or electroporation of the in vitro transcribed RNA from this construct into Huh7 cells followed by selection with the antibiotic G418 can lead to detectable levels of replication of the viral RNA in transfected cells. Other than this, monocistronic replicons of different genotypes (1a, 2a) of HCV RNA have been generated containing reporter genes (Luciferase or GFP), which has allowed screening for HCV replication inhibitors. Furthermore, a full-length infectious HCV genome has been developed, transfection of which leads to production of infectious HCV particles in cell culture and animal models. The efficiency of the HCV cell culture (HCV cc) system was also enhanced by using the cured cell lines Huh7.5, Huh7.5.1 and Huh7-Lunet. Use of a chimeric construct (J6/JFH1) with the core-NS2 region of genotype 2a (J6 clone) in the background of JFH1 strain has improved the infectivity further. The analysis of HCV replication complex revealed that the viral RNA and proteins exist within detergent resistant lipid raft membranes. Although HCV non-structural proteins exist both in the ER and the Golgi compartments, the RNA replication occurs primarily within the Golgi.

In an attempt to study HCV replication in a near natural system in the microenvironment of the hepatic tissue, 3D cultures of Huh7 have been used. A dicistronic HCV genome (type 1b) has been shown to produce and secrete infectious HCV particles in this system using a radial flow bioreactor and a thermoreversible gelation polymer (TGP), which is a major advance towards understanding the mechanism of HCV replication and cellular pathogenesis in the context of liver tissue.

Results from different laboratories suggest that the nucleocapsid-like particles size varies from 30 to 80 nm. The core protein interacts with HCV RNA and is largely involved in nucleocapsid assembly, oligomerization of the capsid protein and encapsidation of the viral genomic RNA. Once the nucleocapsid is formed in the cytoplasm, it acquires the envelope proteins E1 and E2 and the HCV particles are released through a secretory pathway.

About a third of patients spontaneously clear a primary HCV infection, while others have persistent infection. It
has been shown that CD4+ T cell response appears to be critical to coordinate effective immunity. Reinfection with both homologous and heterologous viruses has been shown. In fact, HCV reinfection and superinfection occur frequently among the intravenous drug users (IDUs). Some patients who have initially cleared the virus may develop persistence following reinfection but others may not, suggesting that early immune responses and genetic factors might contribute to protection.

HCV infection is regulated by hepatic immune defenses triggered by the cellular RIG-I helicase. Innate immune defenses are triggered through host recognition of viral macromolecular motifs, known as the ‘pathogen associated molecular patterns (PAMP)’. The RIG-I protein has been shown to bind HCV polyU/UC PAMP motif at the 3’UTR and trigger the hepatic immune response by signalling through activation of IRF3 to induce the expression of IFNα–β and interferon-stimulated genes (ISG). Together, these limit the infection.

At present no vaccine candidate is available. Several initiatives to develop prophylactic vaccines used recombinant envelope protein E1/E2 to demonstrate up to 80% protection against challenge with homologous and heterologous strain of HCV 1a in chimpanzee model. A T-cell vaccine candidate also showed similar protection against different genotypes of HCV. Similarly several initiatives are currently on for developing therapeutic vaccine against HCV infection. A recombinant HCV E1 protein produced in mammalian cells showed significant T cell responses and is currently being tested in clinical trials. DNA vaccines could also be an effective strategy for chronic HCV infection.

Specifically targeted antiviral therapy for HCV (STAT-C) is a major initiative due to the lack of selective and effective anti-HCV treatment options. A majority of antiviral research at this moment is focused on protease and polymerase inhibitors. Telaprevir, a peptidomimetic inhibitor of the NS3-NS4A protease, has shown promising results in trials and is now in an advanced stage of clinical development. Other than this, several studies have reported the use of immune modulators, cyclophilin inhibitors, novel inhibitors based on RNA interference, etc.

HCV RNA translation is unique and fundamentally different from host cell RNA translation and thus can be exploited to develop selective antiviral agents. IRES mediated translation of viral RNA is largely influenced by cis acting RNA elements and trans acting factors. Thus targeting the ribosome–RNA interaction using small-structured RNAs or using sh/si or DNAzyme can really block the viral proliferation in cell culture systems. Targeting the host factors by using peptides corresponding to the RNA binding region of the ITAFs (such as La protein) has also been shown to selectively inhibit viral RNA translation and replication. Since in positive-strand RNA viruses, translation of input viral RNA is the initial obligatory step, the inhibition of translation has drastic consequences on the viral RNA replication. Thus inhibitors of HCV IRES can be developed into an effective alternative strategy for developing selective therapeutic intervention against HCV infection.

**Hepatitis D virus**

Like other hepatotropic viruses, hepatitis D virus (HDV) also causes liver inflammation and produces symptoms similar to the other acute viral hepatitis diseases, just probably more severe. However, it is considered to be a sub-viral satellite because it can propagate only in individuals who are HBsAg positive or who have evidence of recent HBV infection. The first evidence for the existence of HDV was presented in the mid-1970s when a previously unrecognized nuclear antigen was shown in hepatocytes of patients with chronic type hepatitis B (ref. 69). Hepatic cell death has been reported due to the cytotoxic effect of HDV or via a host-mediated immune response. Transmission of HDV can occur either via simultaneous infection with HBV (co-infection) or via super-infection of HBV infected individual. Co-infection is usually acute and resolves by itself. Fulminant liver failure is seen in ~1% of patients. Super-infection with HDV is in less than 5% cases of which 80–90% patients develop chronic HDV infection. These patients progress more rapidly to develop cirrhosis and may even develop HCC. In combination with HBV, hepatitis D has the highest mortality rate of all the hepatitis infections of 20% (ref. 70). The course of HDV infection is determined by the HBV infection and thus, it cannot progress any further once hepatitis B infection is resolved.

The diagnosis for hepatitis D infection is made following serologic tests for the virus. Total anti-HDV antibodies are detected by specific immunoassays. The markers of HDV infection include IgM and IgG antibodies against hepatitis D antigen (HDAg), which disappear within months after recovery. In chronic infection however, HDV RNA, HDAg, IgM anti-HD antibodies and IgG anti-HD antibodies persist. There is increasing use of RT-PCR to monitor the ongoing HDV infection; this can detect 10–100 copies of the viral genome in patient sera.

Approximately 20 million people worldwide have HDV infection. Areas of higher prevalence include southern Italy, sub-Saharan Africa, the Middle East, the Amazon Basin and the American South Pacific islands. However, HDV infection is uncommon in the large populations of HBsAg carriers in Southeast Asia and China and is relatively rare in developed countries. The spread of HDV infection is similar to hepatitis B, i.e. via blood transfusion, sexual contact, sharing needles and from mother-to-child. The infection is found most commonly in IV drug users and hemophiliacs. It also relates to poor hygiene.
Three known genotypes (I, II and III) of HDV are described which are divergent from each other by as much as 20–35% and have different geographical distributions.

Genotype I has a worldwide distribution, genotype 2 is found in Taiwan, Japan and northern Asia, and genotype 3 is found in South America.

Prevention of HDV infection is based on prevention of HBV as the former requires HBsAg for its propagation. In order to prevent HDV–HBV co-infection, the HBV vaccine or post-exposure prophylaxis (hepatitis B immune globulin) can be used. It is equally important to educate the chronic HBV carriers about transmission and risky behaviours of HDV. Direct contact with infected blood should be avoided.

There is no specific treatment for HDV infections; it usually involves interferon therapy. The antiviral treatments for hepatitis B, such as Lamivudine or Acyclovir, Ribavirin, Lamivudine and thymosin analogues do not work. Liver transplantation has proven useful for treating fulminant acute and advanced chronic hepatitis D infections.

The HDV virions are 36-nm particles and are roughly spherical with an envelope made up of HBsAg but without a distinct nucleocapsid structure. The nucleocapsid is made up of ~70 copies of large and small delta antigens. The HDV genome exists as a negative-sense, single-stranded, closed circular RNA genome of ~1.7 kb – the smallest of known human pathogens. As the nucleotide sequence is 70% self-complementary, the viral genome forms a partially dsRNA structures a part of which can assume the catalytically active functions of a ribozyme. This activity is required during viral replication to produce unit length copies of the genome from longer RNA concatamers.

HDV genome encodes only two highly basic proteins called the small delta antigen (HDAg-S or p24) and large delta antigen (HDag-L or p27) which can bind nucleic acids. These two proteins are produced from a single ORF and have 195 common amino acids. The HDAg-L protein (215 amino acids) carries an additional 19 amino acids at the C-terminus. Despite sharing 90% identical sequences, the two proteins play different roles during the course of viral infection. While HDAg-S is produced during the early stages of infection and is essential for viral replication, HDAg-L is produced during infection and functions as an inhibitor of viral replication but facilitator of viral assembly.

HDV resembles viroids in many ways including non-coding of its own polymerase and similar replication strategy. Viral replication occurs in the nucleus of primary hepatocytes with the help of host cell machinery using a double-rolling circle mechanism. The host RNA polymerase II is used for replication – a rare usage of DNA-dependent polymerase as an RNA-dependent polymerase. The ribozyme activity associated with viral genome is utilized for cleavage and self-ligation of unit lengths of genome. New virions are produced only in the presence of HBsAg.

There is no vaccine for hepatitis D. However, as HDV needs HBV for its propagation, the HDV infection may be controlled by hepatitis B vaccination.

Hepatitis E virus

A large epidemic of waterborne hepatitis in New Delhi, India during 1955–56 is the first reported outbreak of hepatitis E. Stored sera of patients from this epidemic and another outbreak in Kashmir (1978–79) showed the absence of serological markers for hepatitis A and hepatitis B, suggesting the existence of a new viral agent, the enterically-transmitted non-A, non-B hepatitis virus. It was later named the hepatitis E virus (HEV), the E representing enteric transmission, propensity to cause epidemics and being the fifth hepatitis virus to be discovered.

The transmission of HEV is by the faecal-oral route from contaminated drinking water; food-borne transmission is also possible. Vertical transmission from mother to newborn and through blood transfusion has been documented from endemic areas, but the contribution of such transmission to the overall disease burden may be small. Hepatitis E is endemic in several parts of the world, which are similar to those where hepatitis A is endemic. These areas experience sporadic infections as well as medium to large localized outbreaks that have all been linked to faecal contamination of drinking water supplies. In HEV-endemic regions, though all age groups are affected, the disease most commonly affects young adults. The infection seems to have worse consequences in pregnant women, and is associated with a high attack rate, increased severity of illness and a 15–25% mortality rate. Unlike HAV, person-to-person transmission of HEV...
is uncommon\textsuperscript{82}. While zoonotic transmission of HEV is reported from non-endemic areas, its role in endemic regions may be limited. Genotype 1 HEV, which is the most prevalent outbreak virus in endemic areas, has not been isolated from animals, but genotypes 3 and 4 viruses reported in pigs and deer have been positively linked to human transmission in non-endemic areas. The most convincing evidence has come from a cluster of Japanese cases that developed hepatitis E a few weeks after consumption of deer meat; genomic sequences of HEV isolated from these cases and the leftover frozen meat were identical\textsuperscript{83}. Demonstration of HEV genotype 3 in pig-liver meat sold for human consumption in several developed countries suggests that consumption of pig meat or contact with pigs may be responsible for cases in non-endemic regions\textsuperscript{84}. Anti-HEV IgG antibodies, generally taken as evidence of prior exposure to HEV, have been found in healthy persons from all geographical areas. Though the prevalence rates are generally higher in HEV-endemic regions, anti-HEV prevalence rates ranging from 1\% to >20\% have been reported from developed countries. This suggests a global presence of HEV.

The incubation period of hepatitis E varies from 2 to 10 weeks, with a mean of around 40 days. The clinical consequences range from asymptomatic infection, through typical acute viral hepatitis, to fulminant hepatic failure. In endemic areas, HEV infection occurs in patients with pre-existing chronic liver disease of any etiology may present with acute-on-chronic liver disease\textsuperscript{86}. Cases reported from non-endemic areas either have icteric illness resembling that seen with other hepatitis viruses or are anicteric with non-specific symptoms\textsuperscript{87}. Though HEV infection is generally self-limiting, some recent reports of organ transplant cases showed persistent HEV viraemia, prolonged liver enzyme elevation, and biopsy showing portal hepatitis, dense lymphocytic infiltrate and variable degrees of piecemeal necrosis and fibrosis. Thus, chronic HEV infection may lead to cirrhosis. There are, however, no reports on whether genotype 1 HEV, the predominant disease-causing strain, can cause persistent infection in otherwise healthy persons.

An anti-HEV IgM response, viraemia and faecal shedding of HEV are associated with acute hepatitis E; these markers are used for its diagnosis. Serological tests for anti-HEV antibodies utilize parts of the viral ORF2 and ORF3 proteins. Whereas in an endemic area, the IgM test is of more value in ascertaining the cause of acute hepatitis, in non-endemic areas, the IgG test has also been used together with clinical evaluation to diagnose hepatitis E. No specific treatment for hepatitis E is available. Since the disease is self-limited, general supportive measures are used, except in fulminant hepatitis cases, which require measures to control cerebral oedema. As in the case of HAV, prevention is linked to proper hygiene, safe drinking water and proper sewage disposal. A recombinant vaccine against HEV has recently been tested in humans and was found to have good efficacy. This is based on an ~56 kDa truncated HEV capsid (ORF2) protein expressed as virus-like particles (VLPs) in insect cells using recombinant baculoviruses\textsuperscript{88}. The vaccine showed good protection against experimental HEV infection in pre-clinical challenge studies in monkeys. In the human trials, 20 μg of alum-adjuvanted VLPs were given as intramuscular injections at 0, 1 and 6 months and the recipients followed for over two years\textsuperscript{89}. The vaccine showed no adverse effects and protective efficacy of 88\% or 95\% after one or three doses respectively. This HEV vaccine is not yet commercially available.

The HEV genome was first cloned in 1990 (ref. 90). The subsequent cloning and sequencing of multiple distinct geographic isolates of HEV reveal it to be a novel virus, which does not fit into an existing family. Though initially classified in the family Calciviridae, HEV is now a tentative species in the genus Hepeviridae of family Hepeviridae. Besides human HEV, closely related viruses that infect pigs (swine HEV) and a more distantly related virus that causes splenomegaly in chickens (avian HEV) have also been characterized.

The 27–34 nm non-enveloped HEV particle includes a capsid composed of a single protein and a single-stranded, positive sense RNA genome of about 7.2 kb (Figure 1). The linear HEV genome includes a short 5′ NCR of about 25–30 nucleotides, with a 7-methylguanine (cap) at its 5′ end, a protein-coding region with three ORFs (ORF1, ORF2 and ORF3), and a short 3′ NCR terminating in a poly(A) tract (Figure 6). The ORF1 non-structural protein is proposed to contain methyltransferase, papain-like protease, RNA helicase and RdRp activities, based on the presence of homologous motifs and to aid in viral genome replication\textsuperscript{91}. Of these, only the methyltransferase activity is biochemically characterized and the putative polymerase shown to bind viral RNA 3′ ends. The ORF2 protein is proposed to encapsidate the viral RNA genome and was shown to bind it\textsuperscript{92}. The protein is glycosylated\textsuperscript{93} and this modification was found to be important for virus infectivity\textsuperscript{94}. In some expression studies, a truncated capsid protein self-assembles into VLPs, which have also been explored as a recombinant subunit vaccine against hepatitis E\textsuperscript{95}. The structure of these VLPs was recently determined and showed an inner shell domain or scaffold that adopts a jelly-roll fold commonly found in small RNA viruses, and three-fold protrusions and two-fold spikes, both adopting β-barrel folds\textsuperscript{96,97}. These sites are likely to interact with the cellular receptor, which remains unidentified. Recent studies have implicated a role for cell surface heparan sulphate proteoglycans in the binding of HEV
Figure 6. The hepatitis E virus genome. The positive sense HEV genomic RNA and a subgenomic RNA produced during replication are shown. The genomic RNA has three ORFs, which encode ORF1, ORF2 and ORF3 proteins. MT, methyltransferase; Pro, protease; Hel, RNA helicase; Pol, RNA-dependent RNA polymerase; NCR, non-coding region; ◆, phosphorylation; ◆, glycosylation.

and its infection of HuH7 liver cells. The ORF3 protein of HEV is required for virus infection in vivo, but not for replication in cultured cells. A number of in vitro studies suggest a multi-tasking role for this protein. It is proposed to optimize the host cell environment for viral replication through its interaction with various cellular proteins and intracellular pathways.

Though all HEV strains correspond to a single serotype, there are at least four genotypes. Genotype 1 includes human isolates from Asia and Africa, genotype 2 includes human strains from Mexico and Africa, genotype 3 includes human and swine strains from industrialized countries, and genotype 4 includes human and swine strains from Asia. Viruses of genotypes 1 and 2 are associated with outbreaks due to efficient human-to-human faeco-oral transmission, while viruses of genotypes 3 and 4 are maintained in animal species and show inefficient cross-species transmission. Some reports suggest that compared to genotype 3, the genotype 4 viruses are more virulent and show higher viral loads in infected patients.

The replication of HEV has not been characterized, but a model is proposed based on analogy to other positive-stranded RNA viruses. Following entry and uncoating, the ORF1 on genomic RNA is translated into a polyprotein, which also includes the viral replicase. From the genomic RNA template, this replicase produces a negative-stranded RNA intermediate, which in turn produces the genomic RNA as well as a subgenomic RNA (Figure 6); the latter is translated into the ORF2 (and possibly also the ORF3) proteins. In vitro transcripts of full-length HEV cDNA clones are infectious for cell lines, non-human primates, and pigs, suggesting that the subgenomic RNAs are not required to initiate an infection, and are synthesized as part of the replication process. The subgenomic RNA and negative-stranded replication intermediates have been observed in cell culture as well as animal models. Only limited success has been achieved in propagating HEV in vitro. Recently, HEV genotype 3 was successfully passaged for multiple generations in PLC/PRF/5 cells, and these cells were also used to assess the infectivity of HEV shed in patient stools.

The route of HEV infection is faecal-oral. On entry, the virus probably replicates initially in the intestines, from where it reaches the liver through portal circulation. The major site of HEV replication is the liver, where it replicates in hepatocytes. Analogous to HAV infection, HEV viraemia precedes the elevation of serum aminotransferases and histopathological changes in the livers of experimentally infected animals. In infected animals, liver injury coincides with decreasing HEV antigens in hepatocytes and increasing anti-HEV titers. Further, cytotoxic lymphocytes have been found to infiltrate the liver tissue of infected animals. These observations suggest that HEV is not directly cytopathic and liver injury may be mediated by the host immune response.

The host immune response to HEV infection is marked by the appearance initially of anti-HEV IgM, which is followed by anti-HEV IgG. While the IgM titers wane off in 4-6 months following exposure, IgG persists for longer periods, but its protective efficacy for longer periods is in question. Unlike HAV, the highest prevalence of hepatitis E in endemic areas is in young adults (15-40 years). This suggests that following subclinical infection in childhood, protection wanes off with time. Studies in experimentally infected animals show that high-titer antibodies are required for protection. The cellular immune responses to HEV infection are poorly characterized. One recent study suggests that natural killer (NK) cells might be involved in HEV pathogenesis, and there may be an inherent defect in T cell activation in HEV-infected persons. ELISPOT assays were used to estimate B and T cell memory in individuals living in an HEV-endemic area. Even in anti-HEV IgG-negative individuals, B and T cell memory could be detected, suggesting that subclinical HEV infection is more widespread than predicted from the antibody studies (S. Naik, pers. commun.). The pathogenesis of hepatitis E during pregnancy is not understood. A role for endotoxin-mediated injury to hepatocytes has been pro-
posed, and an increased T-helper type 2 response was observed in pregnant women with hepatitis E compared to non-pregnant women\(^\text{110}\).

**Other hepatitis viruses**

A number of other ‘hepatitis viruses’ have been proposed. These include the hepatitis F virus (HFV)\(^\text{111}\), the hepatitis G virus (HGV) and GB virus C (GBV-C), which were independent isolates of the same virus\(^\text{112}\). Later, two other viruses were characterized from patients with transfusion-transmitted hepatitis and designated as TTV\(^\text{113}\) and SEN-V\(^\text{114}\). HFV has failed to be confirmed as a real virus, and the others have not passed the tests for hepatitis viruses, which include (i) using the liver as the primary site of replication, and (ii) causing liver disease in the absence of any other known hepatitis-causing agent. It is still estimated that about 20% of clinically established acute viral hepatitis presents with no markers of hepatitis A to E viruses. This may be due to problems of detection sensitivity of the existing tests and markers, variants of known viruses that escape detection, or novel hepatitis viruses. The availability of metagenomic sequencing methods should make this search possible, providing appropriate clinical materials are available.


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