Hepatitis C virus: Why an Enigma?

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Abstract
Hepatitis C virus was identified as a distinct disease from Hepatitis A virus and Hepatitis B virus in 1989. Since then many studies have highlighted the complicated aspects of the HCV infection right from its worldwide prevalence to its clinical presentation to its management. Even after almost three decades and so much technological advancements our knowledge about HCV is still far from complete. Everything about the virus right from its evolution to its pathogenesis and management is an enigma. In this review we would like to provide insight into the currently known details about the origins and evolution of HCV and try to illustrate our understanding of the viral replication and pathogenesis of HCV. Lack of an alternative animal model beside the chimpanzees and inability of the virus to grow in cell cultures until recently hampered research on the therapeutic management of HCV. We would like to discuss the available treatment options and the recent developments in the field of HCV treatment and its likely future clinical impact. Also the latest hurdles for designing of vaccine against HCV will be discussed.

Introduction
Investigators from the Centers for Disease Control (headed up by Daniel W. Bradley) and Chiron (Michael Houghton) identified the Hepatitis C virus (HCV) in 1989. Even after more than two decades chronic Hepatitis C Virus (HCV) infection remains a cause of major concern worldwide. Hepatitis related to HCV is usually a progressive disease that may result in a spectrum of clinical disease ranging from chronic active hepatitis to cirrhosis to hepatocellular carcinoma (HCC) [1,2]. It is also one of the most important reason for liver transplantation as a result of HCC induced end stage liver disease.

The exact global prevalence of HCV largely remains unknown due to the silent nature of HCV infection. It has been estimated that almost 3% of the world's population is infected with HCV [3,4]. This large burden of disease is compounded by a number of factors such as incomplete data on exact prevalence of the disease, presentation of the patient late in the course of disease, inefficient diagnostic tests, suboptimal response to treatment in some patients and most importantly no successful vaccination against HCV.

There is no spontaneous resolution of virus even in immune competent individuals. For many years the research on HCV was hampered by lack of efficient cell culture system. First cell lines permissive to HCV infection has been successfully developed in 2005 [5]. Clinical trials on newer treatment modalities and vaccines are limited by the lack of smaller animal models. Recently resolution of the three dimensional structure of some HCV proteins have provided first insight into the genome and function of HCV. In spite of the considerable research there remains major gap in our complete understanding of the puzzle the HCV.

HCV genome
A member of family Flaviviridae, Hepatitis C Virus is a small, enveloped, positive sense single stranded RNA virus that belongs to the genus Hepacivirus. The genome has approximately 9600 nucleotides and contains one large open reading frame flanked by short highly structured non-translated regions (NTR) at the 5' and 3' ends that contains elements which regulate translation and
It is expected that almost 3% of world’s population is infected with HCV and there are more than 200 million chronic carriers [3,4]. But the actual prevalence is largely unknown because of a number of factors.

HCV remains undiagnosed in majority of infected individuals as they do not develop any signs and symptoms until advanced disease sets in. Hence the actual prevalence of HCV infection always remains under reported [13]. There is a long latent period and in most individuals HCV infection may first become apparent only on the development of liver failure or liver cancer. This usually occurs several decades after the acquisition of infection [13].

HCV is a highly heterogenous virus. The six genotypes of HCV differ in their worldwide distribution and response to treatment. Some genotypes are broadly disseminated worldwide (genotypes 1-3) whereas others have a limited geographical distribution (genotypes 4-6) [4,7,8]. Genotype 1 (subtypes 1a and 1b) is the most prevalent genotype in the world. It has been suggested that various regions of the African continent are endemic for genotypes 1, 2, 4 and 5, whereas South East Asia is an endemic region for genotype 6 [9,10]. Genotype 3 is more prevalent among i.v drug users.

In Europe, types 1b and type 2 are widely distributed particularly in older age groups, while those infected through drug use are more likely to be infected with genotypes 3a and 1a [10,11]. It is important to determine the genotypes as they markedly influence the response to treatment.

Replication of HCV

Inspite of the rapid progress in research there are still unanswered questions regarding the nature of the infectious virus particle, pathway of virus entry and the assembly of structural proteins and RNA into new virus particles [14]. Hence for HCV usually an idealized life cycle is discussed based on the recent progress made in the field of HCV.

Viral entry is the most important step in the viral cycle. It is a highly coordinated but it is a complex process involving many unknown cellular receptors that trigger viral entry into the cell [15]. The enveloped virus attaches to the host cell by interacting with specific receptors. On hepatocytes, highly sulfated Glycos amino glycans (HS GAGs) serve as the first attachment sites [15]. The low density lipid receptors have been proposed as another potential attachment factor for HCV. Other extra hepatic receptors have been seen in peripheral blood lymphocytes, epithelial cells in gut and central nervous system. After attachment to the surface receptors virus is internalized into the cell by fusion of viral and cellular membrane and positive sense viral RNA is released into the cell cytoplasm [15].

The viral RNA now acts as a template for the RNA replication and translation of viral proteins. The translation of viral protein is a complex process as the HCV genome lacks a 5’ cap. Hence the translation depends on internal ribosome entry site (IRES) with in the 5’ NCR [16,17]. The product of translation is a large polyprotein which is proteolytically cleaved to form 10 viral proteins [17,18]. Almost one third of the polyprotein from the amino acid terminus encodes for the viral structural proteins that is highly basic core

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The core protein forms the viral capsids [6,14]. It regulates the activity of cellular genes and alters the transcription of viral promoters. The envelop glycoprotein E1 is thought to be involved in intra cytoplasmic viral membrane fusion and E2 has an important role in the early steps of viral infection by helping the attachment of virus to cellular receptors [6,16]. It also contains hypervariable regions with amino acid sequences differing up to 80% between HCV genotypes. After this region comes the integral membrane protein P7 which probably functions as ion channel and has a role in viral assembly [18,19]. Mutation or deletion in the P7 protein is associated with decreased infectivity of the virus [6,14].

The rest of the genome encodes the non-structural proteins such as NS2, NS3, NS4A, NS4B, NS5A and NS5B [16]. The role of all the gene products of HCV is not clear. These non-structural proteins are thought to coordinate the intra cellular processes of viral life cycle [18,19]. The non-structural proteins NS2 and NS3 are viral protease required for processing of HCV polyprotein. NS3 is a multifunctional enzyme having a protease and helicase activity. NS3 protease along with NS4A cleaves the junction between NS3/4A, NS4A/4B, NS4B/5’, and NS5A/5B. NS3 helicase helps in the unwinding of HCV genome and thus helps in HCV replication [16,19]. NS3 is also seen to induce malignant transformation of cells and may be involved in hepatocarcinogenesis though the exact mechanism is not known. NS4B is the main inducer for intra cellular membrane rearrangements. NS5A is a RNA binding phosphoprotein and it helps in regulating the switch from replication to assembly of infectious viral particles. NS5B is a RNA dependent RNA polymerase required for viral replication. These non-structural proteins are the major targets for antivirals against HCV.

Little is known about the process of RNA synthesis within the replication complex. It is thought to be semi conservative and asymmetrical [19]. The HCV RNA polymerase lacks proof reading and there is an error rate of 10⁻⁴ to 10⁻⁵ nucleotide per base pair [6,14]. Chronically infected patients have high viral loads ranging from 10⁷ to 10⁸ genomes per ml of serum. It is seen that about 10¹² viruses are produced per day in an infected person with an average half-life of 3 hrs [19,20]. This high turnover along with high error rates virtually allows mutation in every single position of the genome. Thus many quasispecies exist within the same patient.

The mechanism of assembly of viral gene products with viral RNA into new infectious particle and their secretion are inadequately in eradicating HCV infection [13,21]. This is because of large diversification of the virus and existence of quasispecies with in an infected patient [6,13]. This large genetic variation and rapid replication of HCV helps the virus to adapt to the immune response and even antiviral treatment [6,13]. How exactly HCV is able to evade the immune response of the host is not entirely clear. It is probably by multiple mechanism such as inhibition of IFN-α production, inhibiting NK activity, HCV has a long term asymptomatic carriage and a variable course in the development of liver disease. HCV infection acquired in infancy and childhood remains poorly characterized and the long-term outcome of the disease is still a matter of debate [2]. The spread of virus in these populations is also poorly understood. Before blood screening was made mandatory transfusion of blood and its products was one of the major routes of its transmission. It is believed that majority of people acquired infection from blood and blood products before HCV screening was made mandatory [2]. Recently there is evidence of its spread parenterally in a new risk group the injecting drug users (IDU’s) [2,12].

The incubation period after acquisition of HCV on an average ranges between 6 to 8 weeks. Most patients who develop acute HCV infection remain asymptomatic. A minority of patients develop non specific symptoms such as easy fatigability, anorexia, nausea, vomiting, abdominal discomfort and fever. Jaundice is seen in only about 20% of the patients. The acute episode resolves on its own after some time [12,13]. But the point of concern is failure of 70-90% of the patients to spontaneously clear the virus in spite of being immunocompetent. These patients become chronic carriers.

HCV causes a progressive disease that may result in chronic active hepatitis, cirrhosis, and hepatocellular carcinoma [12,13]. Chronic hepatitis is a complex clinico-pathological syndrome with varying stages of necro-inflammatory and sclerosing liver damage, different prognoses and responses to treatment. The mechanisms leading to liver cell injury, inflammation, steatosis and fibrosis are still under study.

Liver cirrhosis develops in up to 20-25% of patients with chronic HCV infection and HCC usually develops in about 1-5% of these cirrhotic patients [12,13]. In many patients the HCV infection becomes first apparent at this stage of liver failure or liver cancer many years after the initial infection. The long asymptomatic stage of HCV infection and its slow disease progression make estimation of the future clinical impact of HCV difficult to assess.

Pathogenesis and Immunity

In spite of the initial high viral load in HCV infected patients it is seen that inflammatory process leading to liver injury occurs late in the course. This finding supports the fact that HCV is relatively non cytopathic and the liver injury is usually immune mediated [21].

Of concern is the fact that HCV infection becomes persistent even in immunocompetent people. In HCV infected patients both the innate and adaptive immune response are seen. This shows that the humoral and cellular immune response generated by the host are inadequate in eradicating HCV infection [13,21].

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and producing escape mutants from antibody and CD8+ T cell recognition [21,22].

The innate immune response is not able to clear the virus as HCV down regulates interferon receptors and also blocks its signaling pathway. It also impairs the function of natural killer (NK) cells [21]. Humoral immunity is also not effective as the antibodies which develop are not able to recognize all HCV quasispecies. Moreover neutralizing anti HCV antibodies have not yet been identified and to what extent they neutralize the virus is not very clear. Moreover there is presence of interfering antibodies which decrease the function of neutralizing antibodies. Two important epitopes located at E2 region generates antibodies. Epitope I located at residue 412–426 is an important neutralizing site and conserved between different genotypes, epitope II at 434–446 varies among different genotypes and generates antibodies interfering with the antibody to epitope I of E2. These interfering antibodies are produced much earlier than the neutralizing antibodies in the patients [21,22]. A vaccine targeting the production of antibodies against this epitope II would be critical in protecting against HCV [22].

Effective cell mediated immunity plays a crucial role in viral clearance [21,23]. In patients with favorable outcome of HCV infection a polyclonal, multispecific cross genotypic CD8+ T-cell response along with a coordinated CD4+ T-cell response is present [23,24]. In patients with self-limited disease proliferative CD4+ T-cell response is seen against many HCV antigens in the E2, NS3, NS4 and NS5 region [21,23]. These CD4+ T cells also plays an important role in shaping adaptive immune effectors such as CD8+ T cells and B cells [21,23]. In one of the study it was seen that loss of virus specific CD4+ memory T-cell response led to recurrence of previously controlled HCV infection [24].

How these memory T cells persist after viral clearance is not completely understood. It is presumed that low grade HCV infection persists in liver cell thus providing continuous antigenic stimulus to the immune cells [25,26]. Alternatively it is also hypothesized that the trapped residual HCV antigens in the dendritic cells of the regional lymph nodes may keep activating the CD4+ T cells [25,26].

Compared to CD4+ T cell our understanding regarding the cytotoxic CD8+ T cells (CTL) is limited. These CTLs are seen to reach the site of HCV infection, recognize these infected cells presenting the viral peptides along with MHC class I and lyse these infected cells with the help of antiviral cytokines [21,23].

Cytokines are produced both locally within the liver and systemically and may play an important role in controlling viral replication. These cytokines also contribute to hepatocellular damage through amplification of a nonspecific immune response [21,22]. These CTLs are the most important effectors for viral clearance but they can act as a double edged sword. If the activation of these CTLs is not effective it can lead to liver cell injury by way of cytokines without HCV elimination. These ineffective CTLs also provide selection pressure for the emergence of HCV mutation [21,22].

In patients responding to antiviral treatment a more robust CD4+ T cell response was observed. Antiviral therapy in general leads to enhanced immune response but sustained virological cure and histological improvement is seen only in patients developing multispecific and persistent cellular immune response. A single nucleotide polymorphism at the upstream of IL28B is associated strongly with sustained virological response. Thus detection of the IL28B polymorphism can help guide prognosis in HCV infected patients [21].

**Diagnosis**

The main purpose of diagnosis is for detection of acute infection, chronic infection and assessing the progression of chronic liver disease as measured by liver fibrosis or damage to hepatocytes. Diagnosis is difficult as most patients are asymptomatic during the acute infection and present when chronic liver disease has set in.

There are no assays which can differentiate acute HCV from chronic HCV infection. Positive anti HCV IgM antibodies are seen in 50-90% of acute HCV and 50-70% of chronic HCV. Thus it cannot be used as a reliable marker of acute HCV infection.

The first step in the detection of HCV infection is to look for the presence of anti HCV antibodies via screening assays [27,28]. These screening assays doesn’t indicate whether the infection is acute or past HCV infection [27,28]. The newer assays have high degree of specificity but false positives can occur in pregnant females, patients with immunological or hematological disease or even in population with low risk of infection [28]. Hence the current recommendation is to confirm the result of anti HCV screening assays. One time HCV screening test for adults born between 1945 to 1965 is also recommended in certain areas especially if there are other associated risk factors such as patients on hemodialysis [29]. All the patients who test positive by this method should be additionally tested for HCV RNA to confirm the presence of active viremia. Nucleic acid testing for HCV RNA should also be done for non-reactive anti HCV test if the person is immune compromised or there is a history of HCV exposure [27,28].

In persons with confirmed HCV infection quantitative PCR should be done to measure the baseline viremia before initiating treatment. Genotyping may be done to predict the outcome of therapy. It is also advisable to assess the degree of liver fibrosis with either liver biopsy or indirect serum markers to determine the urgency of treatment [27,28]. Various test available for detection of HCV infection and disease are listed in Table 1.

Viral diagnostic tests have continuously improved since the first anti HCV assay was made available in 1990. The first generation anti HCV assays used epitope from C100-3 region of NS4 [29,30]. These assays could identify 80% of HCV positive patients but there was a long window period from 6 weeks to 6 months. The diagnosis of HCV infection significantly improved with the introduction of newer generation multi antigen Enzyme immunoassays and recombinant immunoblot assays (RIBA) [30]. These tests contained recombinant polypeptides from the immunodominant regions of core, NS3 and NS4. These assays had improved sensitivity and specificity besides having a short window period.
Highly sensitive and specific assays capable of detecting HCV antigens either alone or in combination with antibodies are now available [28,30]. It is thought that these can be used as an alternative to HCV nucleic acid testing for confirmation of screening test result. Now the assessment of severity of HCV disease can be done by indirect markers in serum as compared to liver biopsy. Thus marked technological advancement in the field of diagnosis has been made with more rapid, sensitive, specific and non invasive test being available to work up the HCV patient. This has helped clinicians in better management of the patient by guiding treatment protocol, predicting response to treatment and detecting relapse or non responders much earlier.

**Treatment**

Patients with HCV infection usually present late in the course of disease. It has been seen that suppression of virus does improve the outcome even late in the course of chronic liver disease such as for cirrhosis and hepatocellular carcinoma. It also benefits patients with extra hepatic complications such as type 2 diabetes mellitus and rheumatological disease. Hence there is a rationale for treating HCV infected patients whenever they present to the clinicians [28,31].

The antiviral properties of interferon (IFN) were discovered in 1957 much earlier than the discovery of HCV. Interferon was approved for HCV treatment in 1991 by FDA. The general treatment protocol which was followed was to inject 3 million units of interferon 3 times a week for 48 weeks. In spite of this the benefit seen from this treatment was less as measured by sustained virological response (SVR) [31]. Sustained virological response is defined as negative viral load 6 months after treatment.

The treatment with interferon usually led to suboptimal response and the side effects were high. For almost a decade the standard of care for HCV remained PEG IFN in combination with Ribavirin. This again was associated with many side effects and suboptimal SVR. In the past few years tremendous progress has been made in the field of HCV treatment [28,31,32]. Newer drugs with improved dose and formulations and more effective combination therapies have been developed (Table 2). This has largely changed the course of treatment. The treatment is much more effective and safe. The regimens are easily tolerated and the drugs have to be taken once daily. The duration of treatment has been shortened and SVR of more than 90% could be achieved. The newer treatment is effective independent of prior treatment, level of fibrosis or comorbidities such as HIV co-infection.

A major milestone in the treatment of HCV occurred with the approval of first direct acting antivirals (DAA) such as telaprevir.
and boceprevir as this lead to a high SVR in treatment naive and previously treated patients [28,32]. The next major breakthrough was the approval of Sofosbuvir the first available once-daily NS5B polymerase inhibitor in combination with PEG-IFN/RBV for just 12 weeks with 89% SVR in treatment-naive patients with genotype 1 infection and 83-100% in treatment-experienced patients with genotypes 2/3 [28,32]. Research for newer antiviral agents is ongoing and approval for some of these newer DAAs is imminent.

Since IFN free regimes are costly and it would be difficult to incorporate them in the health care budget focus should be on cost effective regimes.

The goal to develop an ideal therapeutic regimen would be a cost effective, all oral regimen with pan-genotype coverage having minimal side effects and high virological cure rates in all patient groups.

### Vaccine

Although the newer drugs are promising they may induce side effects and may cause suboptimal response in few patients. The treatment is lengthy, costly and many a times resistance may develop to these drugs causing treatment failure. A better alternative approach would be HCV vaccination. There are two major approaches for HCV vaccination that is prophylactic and therapeutic vaccination [33,34].

A prophylactic HCV vaccine can halt the spread of HCV infection, and therapeutic vaccines can help in the treatment of chronically infected patients [34]. The prophylactic vaccination must be capable of inducing and sustaining broad humoral and cellular immune response against various HCV components. It would be of great benefit in the high risk group especially in high prevalence areas.

It is seen that the prophylactic vaccine induced immunity may not prevent HCV infection but stops the persistence of HCV. This might be an acceptable goal, because chronic persistence of the virus is the main cause of pathogenesis in case of HCV [34,35].

A therapeutic vaccine approach is also required for either replacing or supplementing the current standard of care treatment. If the patients could be treated with 2-3 doses of such vaccine major benefits in terms of economics and logistics would be gained as opposed to several months of costly combination drug therapy [36].

A successful HCV vaccine should be able to tackle high mutagenicity of HCV virus, provide protection against all genotypes and quasispecies of HCV, and must incorporate epitopes from HCV structural proteins in their correct three-dimensional conformations, to induce the production of high titers of neutralizing antibodies. It should also generate strong cellular responses against HCV-specific T-cell epitopes from HCV nonstructural proteins.

Many different approaches have been undertaken towards developing a therapeutic/prophylactic vaccine against HCV infection. These include; peptide-based vaccines, DNA-based vaccines, recombinant protein-based vaccines, virus like particles-based vaccines, viral vector-based vaccines, and dendritic cells(DCs) vaccines [33,35].

There are many challenges towards a successful vaccine development such as lack of small animal models, inability to grow HCV in vitro, great genetic variability and mutation rate of HCV, antibody responses always lag behind the rapidly evolving quasispecies population. Till date no therapeutic vaccine has achieved sustained virological response. No successful approach has been finalized for prophylactic vaccination. However, promising results for several types of HCV vaccination in clinical

### Table 2 Treatment modalities for HCV.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Year approved (FDA)</th>
<th>Treatment effectiveness for different genotypes</th>
<th>Advantage/ disadvantage</th>
</tr>
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<tbody>
<tr>
<td>IFN-α</td>
<td>1991</td>
<td>SVR : 9% (genotype 1) and 30% (genotype 2 &amp; 3)</td>
<td>Effect short lasting required multiple injections, Side effects high</td>
</tr>
<tr>
<td>IFN-α+Ribavirin</td>
<td>1998</td>
<td>SVR : 29% (genotype 1) and up to 62% (genotype 2 &amp; 3)</td>
<td>Synergistic effect with combination treatment. Ribavirin decreased the level of liver enzymes</td>
</tr>
<tr>
<td>PEG IFN</td>
<td>2001</td>
<td>SVR : 14% (genotype 1) and 47% (genotype 2 &amp; 3)</td>
<td>Pegylation leads to increased concentration of interferon over longer period of time</td>
</tr>
<tr>
<td>PEG IFN+Ribavirin</td>
<td>2001</td>
<td>SVR : 41% (genotype 1) and 75% (genotype 2 to 6)</td>
<td></td>
</tr>
<tr>
<td>PEG-IFN-2α</td>
<td>2002</td>
<td>SVR : 28% (genotype 1) and 56% (genotype 2 &amp; 3)</td>
<td></td>
</tr>
<tr>
<td>First generation protease inhibitors (Bocepravir and telaprevir)</td>
<td>2011</td>
<td>SVR upto 60-86% in genotype 1</td>
<td>Approved for use only in genotype 1 as triple combination therapy</td>
</tr>
<tr>
<td>Second generation protease inhibitor (Simepravir)</td>
<td>2013</td>
<td>SVR : 80% in genotype 1</td>
<td>Approved for treatment in combination with PEG IFN and Ribavirin, Oral dose (once a day), duration of treatment: 24 weeks</td>
</tr>
<tr>
<td>NS5B polymerase inhibitor (Sofosbuvir)</td>
<td>2013</td>
<td>SVR : 90% (genotype 1) and 83-100% (genotype 2/3)</td>
<td>Approved for treatment in combination with PEG IFN and Ribavirin, Oral dose (once a day) duration of treatment: 12 weeks</td>
</tr>
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trials, suggest that it should be possible to develop HCV vaccine candidates in the near future.

**Conclusion**

Hepatitis C virus still remains an enigma and many aspects of the virus from its genome to its replication and its pathogenesis need to be deciphered further. With the availability of complete cell culture system it is an exciting time in HCV research, and rapid progress can be expected. Once cellular determinants of HCV tropism are better understood, it might be possible to reveal new and surprising aspects of HCV genome, its replication, the cellular receptors and provide us with better treatment and vaccination strategies to combat HCV infection and eradicate HCV-associated disease.
References


