

## REVIEW

# Hepatitis C Virus - Proteins, Diagnosis, Treatment and New Approach for Vaccine Development

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### Abstract

**Background:** Hepatitis C virus (HCV) causes acute and chronic human hepatitis infection and as such is an important global health problem. HCV was discovered in the USA in 1989 and it is now known that three to four million people are infected every year. The WHO estimates that 3 percent of the 180 million people worldwide are chronically infected. Humans are the natural hosts of HCV and this virus can eventually leads to permanent liver damage and carcinoma. HCV is a member of the Flaviviridae family and *Hepacivirus* genus. The diameter of the virus is about 50-60 nm and the virion containing a single-stranded positive RNA with approximately 10,000 nucleotides in length and consists of one ORF which is encapsulated by an external lipid envelope and icosahedral capsid. HCV is a heterogeneous virus, classified into 6 genotypes and more than 50 subtypes. Because of the genome variability, nucleotide sequences of genotypes differ by approximately 31-34%, and by 20-23% among subtypes. The quasispecies of mixed virus populations make survival advantage for virus to create multiple variant genomes and a high rate of generation of variants to allow rapid selection of mutants for new environmental conditions. Direct contact with infected blood and blood products, sexual relationships and availability of injectable drugs have had remarkable effects on HCV epidemiology. Hundreds of thousands of people die each year from hepatitis and liver cancer caused by HCV virus infection. Approximately 80% of patients with acute hepatitis C progress into a chronic disease state leading to serious hepatic disorders, 10-20% of which develop chronic liver cirrhosis and hepatocellular carcinoma. The incubation period of HCV is 6-8 weeks and the infection is often asymptomatic so it is very hard to detect at early stages, making early treatment very difficult. Therefore, hepatitis C is called a “silent disease”. Neutralizing antibodies are produced against several HCV proteins during infection but the virus mutates to escape from antibodies. Some patients with chronic hepatitis C may have some symptoms such as fatigue, muscle aches, nausea and pain. Autoimmune and immunocomplex-mediated diseases have also been reported with chronic HCV infection.

**Keywords:** Hepatitis C Virus - treatment - vaccine - virus like particle

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### HCV Proteins

There are two UTRs located at the 5' and 3' termini called cis-active RNA elements which are essential for protein translation. The virus translation is cap-independent by IRES located at the 5' UTR. A single precursor polyprotein is approximately 3010 amino acids, (NH<sub>2</sub>-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) be cleaved into at least 10 different proteins. Structural proteins Core, E1, E2 and p7 are released from the polyprotein after cleavage by cellular proteases and some non-structural proteins such as NS2, NS3, NS4A, have been produced by virus-encoded proteases (de Vicente et al., 2009; Chatel-Chaix, et al., 2011). Core protein consists of first 191 amino acids and can be divided into three domains. Domain 1 (amino acids 1-117), Domain 2 (amino acids 118-174) is more hydrophobic and Domain

3 (amino acids 175-191) is highly hydrophobic (Honda et al., 2000). Domain 1 of Core protein can bind viral RNA. Core protein is involved in hepatocarcinogenesis and associated with the endoplasmic reticulum (ER), lipid droplets, mitochondria and the nucleus and also interacts with numerous cellular proteins and affects host cell functions such as gene transcription, lipid metabolism, apoptosis and cell signaling pathways (Counihan et al., 2011). Alternate reading frame proteins or ARFPs includes (1) frame shifting (2) form transcriptional slippage, or (3) from internal initiation in the +1 open reading frame (ORF) of the core protein coding sequence (Hsieh et al., 2012). Two “envelop proteins” E1 and E2 are highly glycosylated and play an important role in cell entry. E2 glycoprotein has 11 N-glycosylation sites acts as the receptor binding and E1 glycoprotein contains 4-5 N-linked glycans serves as the fusogenic subunit. E1 protein extends

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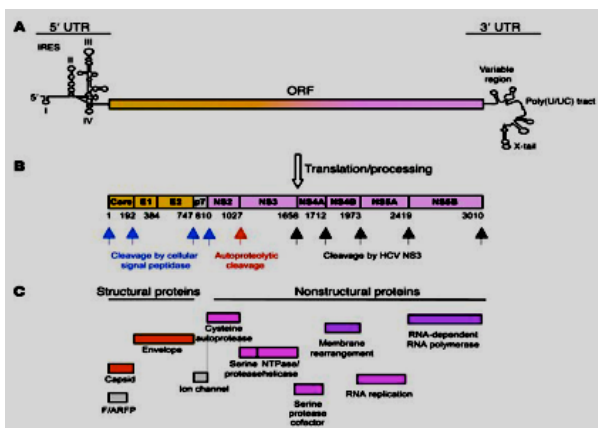
from 192-383aa and E2 from 384-746 aa, have molecular weights of 33-35 and 70-72 kDa, respectively. E1, E2 glycoproteins are processed by Golgi glycosidases and glycosyltransferases (Fouad et al., 2012). E1 is able to adopt a polytopic and has a heterodimeric noncovalent association with E2. E1 also interacts with Core, and this interaction being dependent on oligomerization of Core. According to E2 function, E2 can bind especially to immune receptor CD81 (Cluster of Differentiation 81) as a main receptor for this virus, high density lipoprotein (HDL) and also scavenger receptor (type B class 1) protein (SRB-1) but the interaction of E2 protein due to viral entry with Mannose binding proteins (DC-SIGN and L-SIGN) is not known. E2 has two hypervariable regions (HVR) which are under selection for mutation and they are targets for neutralizing antibodies (Fujiwara et al., 2012). The genetic heterogeneity of the HVR helps virus to evade the immune system. P7 is a membrane-spanning protein and with two transmembrane domains (TMDs) connected to ER lumen. This protein is 63-amino acid polypeptide located between HCV E2 and NS2 genes (Gupta et al., 2012). The cleavage of p7 is mediated by the ER signal peptidases between at the E2/p7 and p7/NS2 sites. P7 has been shown to be essential for virus particle assembly and release of virions. HCV P7 form is like ion channels, plays an essential role in virus infection and this form is similar to those groups of proteins called viroporins (Huang et al., 2010). NS2 interacts with itself is a 21-23 kDa transmembrane protein and is essential for viral replication cycle *in vitro* or *in vivo*. N-terminal of NS2 contains three or four transmembrane helices which insert into the ER and the C-terminal part of NS2 play an important role in NS2/3 auto protease activity together with the NS3 called metalloproteases. The C terminal domain of NS2 has two composite active sites. Moreover NS2 interact with other non structural proteins and to be involved in virus particle assembly (Kim et al., 2007). NS3 is a member of the superfamily 2 DExH/D-box helicases with multifunctional activity. It is 67 kDa with a serine protease in N-terminal, RNA helicase in C-terminal and NTPase activities. Serine protease catalytic activity is due to three amino acid residues His-1083, Asp-1107 and Ser-1165 with its cofactor NS4A involved in cleavage between NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B. NS3 protein also interacts with the catalytic subunit of protein kinase A

(PKA) and deregulates intracellular signaling. NS3 serine protease suppress innate cellular host defense by inhibition of RIG-I and TLR3 signalling (Yang et al., 2006; Wu et al., 2008; de Vicente et al., 2009). The NTPase/helicase domain is in the C-terminal of the NS3 protein which is responsible for RNA and DNA unwinding activity in a 3'-5' direction and the NS3 helicase is modulated by this interaction with NS5B polymerase. Other posttranslational modifications such as phosphorylation and acetylation are reported in NS3 (Ma et al., 2008).

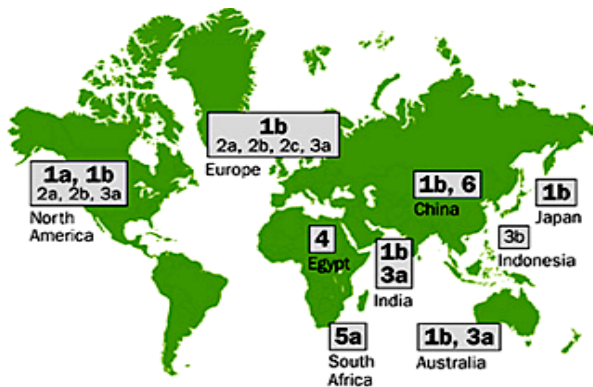
Non structural protein 4A acts as a cofactor for NS3 protein and this interaction is mediated between residues within the core of NS3 and the C-terminus of NS4A. NS4A has 54 amino acids and it was proposed that last 20 amino acids anchor the complex on the ER membrane. NS4A is also required for the phosphorylation of the centre of NS5A (amino acids 2135-2139) (Yamanaka et al., 2002; Wu et al., 2008). Non structural protein 4B is a small hydrophobic 27 kDa polypeptide. The C-terminus and N-terminus have a topology associated with membranes and ER lumen by co-localization with other non-structural proteins in the ER. NS4B interacts directly with NS4A and indirectly with NS3 and NS5A but failed to show cytopathic or oncogenic effects in the livers of transgenic mice (Wu et al., 2008). NS5A is phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) and this hydrophilic phosphoprotein has an important role in viral replication, modulation of cell signaling and interferon response. N-terminal of NS5 has an essential zinc motif, which play an important role in structural integrity. NS5A contains a region (amino acids 237-276) called interferon- $\alpha$  sensitivity-determining region (ISDR) which interacts directly with an IFN- $\alpha$  stimulated gene product, PKR protein kinase which is activated by binding to doublestranded RNA and modulates the IFN response (Tong and Malcolm, 2006; de Vicente et al., 2009). The NS5B is 65 kDa protein acts as RNA dependent RNA polymerase with a typical 'right hand' polymerase shape (finger, palm, and thumb sub domains) plays a role in new RNA synthesis and initiates synthesis of complementary negative-strand RNA using the HCV genome (positive polarity) as a template (Pratt et al., 2005).

### HCV Epidemiology

HCV is divided into six major genotypes and more than 80 subtypes. 30-50% variation among genotypes and 15-30% among subtypes while 1-5% variation in nucleotide sequence from a single HCV infected patient. The hypervariable region is in E1 and E2 and the lowest variability is found in the 5' untranslated region (UTR) containing RNA secondary structures which are required for replication and translation functions. The lack of proofreading activity of RNA-dependent RNA polymerase causes sequence variability and the nucleotide misincorporation rate is approximately 10-3 base substitutions per genome site per year (Bhatti et al., 1995; Fujiwara et al., 2012). All HCV genotypes are pathogenic and hepatotropic and influence on the rate of progression to cirrhosis and HCC. According to World Health Organization data, 3% of the human population,



**Figure 1. Genome and Proteins Structure for Hepatitis C Virus**

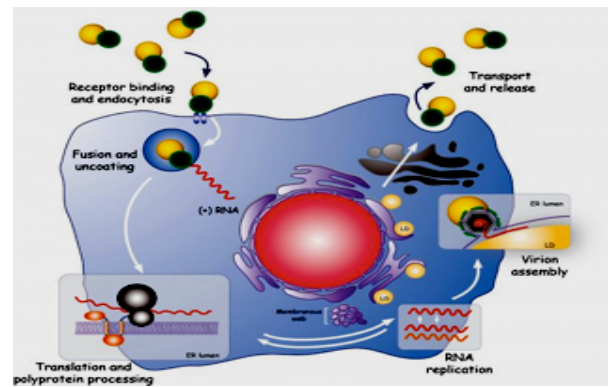


**Figure 2. Distribution of HCV Genotypes in the World**

(approximately 170 million people), is infected with HCV and the prevalence of chronic HCV is 0.1-26% and varies in different regions (Bassouini et al., 2012). There are geographic and temporal differences in the patterns of HCV infection. Different countries, including the United States, Australia, Turkey, Spain, Italy, and Japan, the average prevalences of HCV infection is (1.0-1.9%), but have different patterns of age-specific prevalence (Carreno et al., 2012). Genotype 1 is the most common type and presents 40-80% of HCV chronic infection (Roffi et al., 1998). In USA Genotype 1 is common, but in Europe and Japan genotypes 2 and 3 are more common. In Egypt and the Middle East Genotype 4 is the most common genotype and in southern Africa genotype 5 is most prevalent, genotype 6 is in Hong Kong and other countries of south eastern Asia; genotypes 4, 5 and 6 are rarely found outside of those African or Asian regions (Wang et al., 2009). About 80% of patients in Poland are infected with genotype 1b. Genotype 3 is most frequent in India, Nepal and Pakistan. Genotype 4 is a common genotype in Africa and the Middle East. Genotypes 1a, 1b and 3a are the most prevalent genotypes in Iran and in Pakistan the major HCV genotype is 3a. The prevalence rate is: in Africa 5.3%, in the Eastern Mediterranean 4.6%, in the West Pacific 3.9%, in Southeast Asia 2.15%, in the Americas 1.7%, in Europe 1.03%. For many developing countries, including Iran HCV prevalence are not available. A recent study on blood donors indicated a seroprevalence of 0.13% during the period of 2004-2007 and the male to female ratio 10:1 in such reports. Prevalence of HCV in Iran is as high as 11-25% for patients on hemodialysis and 11-52% for intravenous drug abusers and 15-76% for hemophilia and thalassemia patients (Mizui et al., 1994; Gelberg et al., 2012; Kamili et al., 2012; Kleven et al., 2012).

## HCV Entry into the Host Cell

HCV entry is the first step in a virus life-cycle which is a slow and complex interaction between a receptor on the cell surface and a viral attachment protein. HCV receptors or coreceptors in host cell are glycosaminoglycans, CD81, scavenger receptor class B type I (SRBI), members of the claudin family (CLDN1, 6 and 9) and mannose-binding lectins DC-SIGN and L-SIGN and their interaction is associated to HCV virion proteins (Dreux, Peitschmann et al., 2006). Initial attachment is with GAGs and the LDL-R and followed by interaction with SR-BI and CD81 with



**Figure 3. HCV Entry to the Host Cell and Replication**

binding of HCV E2 with high affinity to the large external loop of CD81 and CLDN1. HCV may enter to the cell by binding to low-density lipoprotein (LDL) receptors (Piver et al., 2010). The role of E1 is less clear but the presence of hydrophobic amino acids tentatively called the E1 fusion peptide, displaying similarities to the fusion peptides and suggests that E1 is involved in membrane fusion. E1 and E2 form heterodimers that represent the native form of HCV envelope (Tan et al., 2003; Steinmann et al., 2004; Zhang et al., 2008; Velazquez et al., 2012; Ye et al., 2012; Zeng et al. 2012).

## Transcription, Replication and Translation

After virus internalizing, nucleocapsid is released into the cytoplasm and the virus is decapitated. Then genomic HCV RNA is used for translation and replication in the cytoplasm. Translation of the viral RNA is cap-independent mechanism and HCV IRES binds to the 40S ribosome subunit (Ye et al., 2012). The single polyprotein is translated at the rough endoplasmic reticulum (ER) and cleaved co- and posttranslational by cellular and viral proteases to produce structural and non structural proteins. A proteinase and NTPase/helicase activity is found NS3 and this protein has some corporation with NS4A and NS5 for their actions (Ma et al., 2008).

RNA-dependent RNA polymerase replicates the genome by the synthesis of negative strand RNA. Negative strand RNA serves as a template for the synthesis of positive strands. NS5B RdRp is the key player catalysing the synthesis of minus and plus strand RNA and NS5A is involved in regulation of replication. Replication and post-translational processing take place in a membrane located in close perinuclear membranes. HCV E proteins are in the ER compartment and the viral nucleocapsids acquire their envelope by budding through ER membranes. Genome encapsidation take place in ER and nucleocapsids get envelop and be matured in Golgi apparatus before produced virions are released in the pericellular space by exocytosis (Bhatti et al., 1995; Pan et al., 2007; Huang et al., 2010; Nakabayashi 2012).

## HCV Transmission

### Intravenous drug use

Transmission of Hepatitis C virus is associated with intravenous and percutaneous drug and using needle. Most countries with a young population have reported HCV



transmission with intravenous drug using as the main cause of virus spread and the interesting point is many of these intravenous drug users do not know they have been infected. In the District Buner study in Pakistan, all 751 anti-HCV patients had a history of injections. Another study in London, England took 428 intravenous drug users and found that 44% had antibodies to hepatitis C. Many studies in Iran reported that the rate of intravenous drug users (IDUs) with HCV infection is between 38% and 47% (Bevilacqua et al., 2009).

**Blood Transfusions**

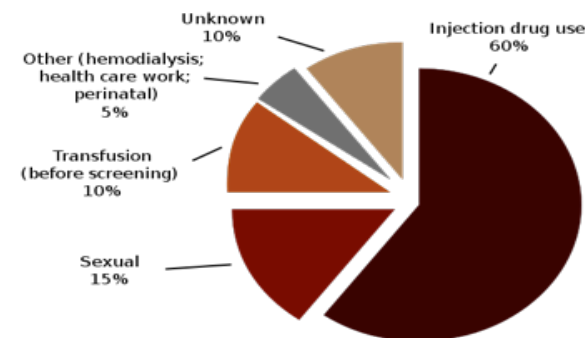
HCV infection may result from blood transfusion and blood products however, with improved screening, HCV transmission through transfusions has decreased in most developed countries. In the US, incidence of post-transfusion hepatitis C dropped from 3.84-0.57% per patient after HCV screening in 1990 (Gunson et al., 2003). In England, the frequency HCV infected donations dropped from 1 in 520,000 (1993-98) to 1 in 30 million (1999-2001) when donations were tested for HCV RNA. There are approximately 105-107 viral particles in 1 ml of blood of an infected patient with the chronic form of the disease (Ruzzenenti et al., 2000; Januszkiewicz-Lewandowska et al., 2003).

**Sexual activity**

Sexual transmission of HCV reported in 2-27% of patients and the prostitutes, intravenous drug users and homosexuals are being the most commonly affected. A study among spouses in Egypt, it reported that wife to husband transmission was 34% with detectable HCV RNA and 10% without detectable HCV RNA among women and transmission of husband to wife was 3%. Sexual transmission of HCV is still rare but it is higher in high-risk sexual activity (Zeremski et al., 2012).

**Hemodialysis**

Dialysis patients have a higher rate of HCV infection and the rate of seroconversion among hemodialysis patients with no other risk factors has been reported 1.38-1.9% per year. The prevalence of HCV infection in Iranian hemophilic patients is from 15.6% in Fars, a southern district of Iran to 76.7%, in North-West of Iran and the prevalence rate of thalassemic patients with HCV infection is between 15.7% and 63.8%. Prevalence rates of HCV in Ireland (1.7%), in Europe 20-30% and in Saudi Arabia showed to be 9.24% (Ruzzenenti et al., 2000). Transmission of the HCV virus to hemodialysis patients



**Figure 4. Frequency of HCV Transmission Routs**

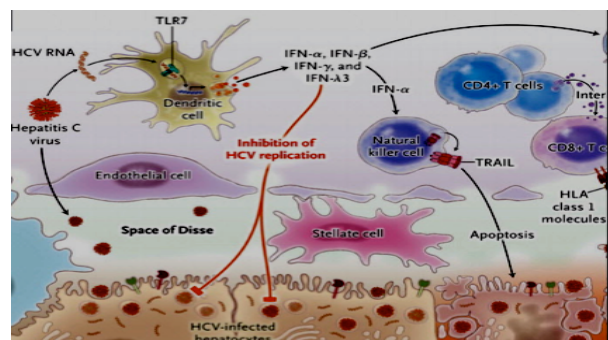
happens with possible risk factors such as disinfect devices between patients, sharing of vials for infusions, lack of sterile technique and poor cleaning of dialysis machines (Januszkiewicz-Lewandowska et al., 2003).

**Special Populations**

Hemophiliacs and patients receiving hemodialysis are 2 groups of patients prone to the infection and 50-90% of hemophiliacs patients are infected with HCV and this are caused by numerous blood transfusions and blood product infusions. For organ transplant, 50% of patient’s transmission of the virus occurs when a donor is an HCV infected person and also it may occur during invasive diagnostic procedures (e.g. organ biopsies, endoscopic examination). In Italy the prevalence was reported 33.3% among kidney transplants and most of these kidneys transplant patients have done dialysis as well. The HCV infection can be acquired through perinatal exposure and it is not clear that the disease could occur during pregnancy, at birth, after delivery or while breastfeeding (Delamare et al., 1999; Huët and Dabis, 2000; Januszkiewicz-Lewandowska et al., 2003; Quesnel-Vallières et al., 2008; Bevilacqua et al., 2009; Jorgensen et al., 2012).

**HCV Evasion Strategy**

HCV evades from innate and adaptive immune responses in several ways. NS3/4A proteins of HCV can block RIG-1 activation and translation of IRF-3 and impair the interferon signaling pathway. JAK/STAT pathway is interfered with HCV core protein by activating SOCS-3 which is a JAK-STAT signaling inhibitor and increases the degradation of STAT1 (Iken et al., 2006). Protein kinase receptor (PKR) activity can be blocked by HCV E2 protein and also E2 inhibits cytotoxicity or IFN production by NK cells when E2 crosslinks the HCV receptor CD81 (Merquiol et al., 2010). HCV escapes from antiviral action of interferon with NS5A. Interferon sensitive region (ISDR) in NS5A plays an important role in response of interferon therapy. Moreover, protein kinase receptor is blocked and PKR expression is down-regulated by NS5A and also NS5A induces IL-8 which inhibits the antiviral actions of IFN (Nguyen et al., 2006). HCV escapes from adaptive immune response with several different mechanisms. One of the major HCV virus evasion strategies is mutational escape of HCV which is due to lack of proofreading activity of RNA polymerase and high replication rate (10<sup>12</sup> virions per day) of HCV and also HCV can escape from B cell with Sequence changes in



**Figure 5. Immune Response to HCV Infection**

the hypervariable region of the E2 envelope glycoprotein (Cao et al., 1996; Yoo et al., 2010).

## HCV Infection Diagnostics

### Serological assays

The first generation of anti-HCV test widely used for c100-3 epitope from the nonstructural NS4 region. Third-generation enzyme immunoassays (EIAs) with 99% specificity including core, NS3, NS4, and NS5 regions of the HCV genome can be used to detect antibodies in plasma or serum against various HCV epitopes and the optical density (OD) ratio of the reaction shows the amount of antibodies in the serum or plasma samples (Cao et al., 1996; Coppola et al., 2009). Moreover, Immunoblot tests are clinically obsolete given the good performance of third-generation anti-HCV EIAs. An HCV diagnosis can be confirmed with Recombinant immunoblot assays (RIBA) and the second, third-generation RIBA HCV strip immunoblot assays (SIAs) (HCV 2.0 and 3.0 SIAs) are established methods and since 1992, the third-generation RIBA system (RIBA HCV 3.0 SIA) been widely used in Europe. Serological determination of the HCV genotype can be reported by seeking for antibodies directed to genotype-specific HCV epitopes with a competitive EIA (Masalova et al., 1998; Lee et al., 2007; Schmeding et al., 2010; Kamili et al., 2012).

## Molecular diagnosis

### Qualitative HCV RNA detection.

The HCV RNA in the plasma in active infection can be detected 1-3 weeks post-exposure. Qualitative assays are based on target amplification using either polymerase chain reaction (PCR), "real-time" PCR or "transcription-mediated amplification" (TMA). After HCV RNA extraction and cDNA synthesis, Double-stranded DNA copies of HCV genome are synthesized in PCR assays, whereas single-stranded RNA copies are generated in TMA. In "real-time" PCR, the number of fluorescent signals per cycle is proportional to the amount of HCV RNA in the sample (Abacioğlu et al., 1996; Bossi and Galli, 2004).

## HCV RNA Quantification

The World Health Organization (WHO) has established an international standard for universal standardization of HCV RNA quantification units (IU) which is used in all of the commercial HCV RNA quantitative assays. HCV

RNA quantity can be used in competitive PCR, real-time PCR and branched DNA (bDNA) assay. bDNA method uses solid phase oligonucleotide probes that capture target RNA, after hybridization of branched secondary probe, the bDNA bind to enzyme conjugated probes and after substrate is added the chemiluminescence produced is proportional to the amount of target RNA. Five standardized assays are commercially available, two of them are based on competitive PCR [Cobas Amplicor HCV Monitor® v2.0 (Roche Molecular Systems), and LCx HCV RNA Quantitative Assay (Abbott Laboratories)]; one is based on bDNA technology [Versant® HCV RNA 3.0 Assay (Siemens)]; and two are based on real-time PCR amplification [Cobas TaqMan HCV Test and Abbott RealTime™ HCV assay (Abbott Diagnostics)] (Bossi and Galli, 2004; Tanaka et al., 2004; Mavromara et al., 2005).

## HCV genotype detection

HCV genotype determination method is direct sequencing of the 5' UTR, NS5B or E1 regions of HCV genome and alignment with prototype sequences and phylogenetic analysis and using various commercial kits for direct sequence analysis of the 5' noncoding region (Trugene® 5'NC HCV Genotyping Kit, Bayer Health Care) or reverse hybridization analysis using genotype-specific probes located in the 5' noncoding region commercialized as INNO-LiPA HCV, Innogenetics, Ghent, Belgium, or Versant® HCV Genotyping Assay, Bayer HealthCare) (Abacioğlu et al., 1996; Albertoni et al., 2010; Ciotti et al., 2010). For HCV genotyping base on Serology techniques, the different genotypes can be determined by antibodies directed to genotype-specific HCV epitopes with a competitive EIA. In restriction fragment length polymorphism (RFLP) analysis, a certain region of the HCV genome is amplified with universal primers and restriction enzyme recognition sites present in the DNA fragment usually show type-specific distribution. Thus, vary lengths of restriction fragments are created after cutting the PCR fragment with restriction endonucleases. The mobility of these fragments in the gel shows the approximate lengths of the restricted fragments and identifies the genotype (Monis et al., 1994; Mizui et al., 1994; Mangia et al., 1997; Zheng et al., 2003; Safi et al., 2012).

## Liver Biopsy

Serum alanine aminotransferase (ALT) measurements, when combined with Liver biopsy, can be useful in determining the virus activity in liver. So the stage of fibrosis and liver biopsy is recommended for treatment to know disease stage and distinguish other forms of liver disease. Immunohistochemistry with the TORDJI-22 monoclonal antibody is a very specific and sensitive diagnostic test for hepatitis C virus in fixed liver tissues. Increasing of Serum hyaluronic acid (HA) during fibrogenesis is a useful marker in chronic HCV patients in and can be monitored at progressive fibrosis (Erensoy, 2001; Al Swaff, 2012; Carreno et al., 2012).

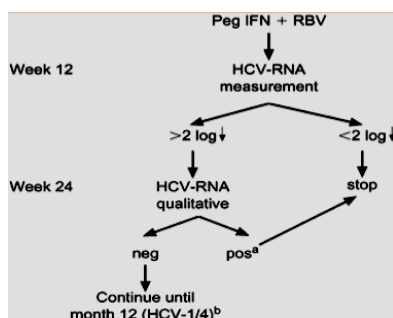


Figure 6. Treatment Strategy of HCV Infection

## Protective Immunity to HCV

HCV clearance could be related to host genetic background such as HLA types, cytokine and chemokine expression (e.g., IL-10, IL-28B, and CCR5). Many studies published recently HCV clearance is associated with HLA-B27 allele in MHC class I locus and MHC class I is associated with CD8+ T cells, so this shows that cytotoxic T is very important in HCV eradication. Many studies reported that when patients with chronic HCV infection were investigated, the lack of cytotoxicity in CD8+T cells against HCV epitopes was observed and patients with resolved HCV infection contained stronger CD8+ T cell responses (Feinstone et al., 2012). CD4+ T cells are vital in HCV recovery and important in adaptive immunity. For example; CD4+ T helper cells are effective in B cells and CD8+ T cells functions or CD4+ T cells activity induce IL-2 and IFN- $\gamma$  secretion that seemed to correlate with HCV clearance during acute HCV infection and also CD4+ T cells are specific to many different HCV epitopes in chronic infected individuals (van de Laar et al., 2010). Neutralizing antibodies are responsible for immune protection against HCV infection. If E1 and E2 proteins are present on the surface of HCV virions, specific antibodies, mostly IgG isotype, have the capacity to neutralize them. Generally with third generation of EIA anti-HCV assays, anti-HCV antibodies to HCV core, NS3, NS4, and NS5 proteins can be measured (Alter et al., 2011). Different studies have showed that the polymorphism at the upstream of IL28B is associated strongly with sustained virological response and Patients who express more IL28 mRNA have a better response to standard IFN therapy. IFN- $\lambda$ 3 together with IFN- $\lambda$ 1 (IL-29) and IFN- $\lambda$ 2 (IL-28B), have a complex connection with IL-28R $\alpha$  and IL-10R $\beta$  to produce JAK/STAT pathway, has an anti-viral effects like interferon type I (IFN- $\alpha$  and IFN- $\beta$ ) (Eisenbach et al., 2006; Bassyouni et al., 2012; Zeremski et al., 2012).

## Management of Antiviral Therapy

The presence of HCV RNA in the absence of anti-HCV antibody indicates the acute HCV infection, so patients with acute hepatitis C should be tested for anti-HCV antibodies which will be confirmed by seroconversion a few days later and HCV RNA with a lower limit of detection of 50 IU/mL or less with sensitive techniques (Ampurdanés et al., 1996). If both anti-HCV antibodies and HCV RNA are absent or anti-HCV antibodies are present without HCV RNA, the occurrence of acute hepatitis C is very unlikely and in patients who have recovered from a past HCV infection, anti-HCV antibodies is seen in the absence of HCV RNA (Arase et al., 2001). Treatment of acute HCV patients with genotype 1 is important because therapy is much more efficacious compared with therapy in chronic phase, so molecular diagnosis is critical for defining treatment responses. In this way the risk of major adverse effects of therapy is reducing with (1) optimal time after infection to initiate therapy (8-12 weeks); (2) treatment duration (24 weeks) and (3) Ribavirin is not required for optimal responses

during acute infection (Clarysse et al., 1995; Carrión et al., 2009). On the other hand chronic hepatitis C is certain when both anti-HCV antibodies and HCV RNA are present and clinical signs of chronic liver disease are reported. In immunosuppressed, hemodialysis or gammaglobulinemic patients HCV replication can be detectable in the absence of anti-HCV antibodies (Guo et al., 2012). Treatment of chronic HCV infections is the combination of pegylated interferon (IFN) alfa and Ribavirin which recommend 48 weeks of peg-interferon plus 1000-1200 mg of ribavirin for genotype 1 infection and 24 weeks of peg-interferon plus 800 mg of Ribavirin for genotypes 2 or 3 infection and 24% of the patients receiving combination therapy achieved a rapid and sustained virological response rates were equivalent for 24-48 weeks of therapy using weight-based Ribavirin (88-91%) (Vermehren et al., 2011). Thus, defining the treatment course for patients with genotype 1 with rapid virological response (undetectable RNA by week 4) may be very useful for shortening treatment in individuals. Studied of genotype 2 or 3 infections reported that impact of shortening the treatment course is from 24 weeks to 12-16 weeks. It is generally believed that if HCV RNA level develops to undetectable level after 24 weeks, The HCV infection has been eradicated and the patient's therapy is completed. Sometimes in occult hepatitis C, patients have positive result for HCV antibodies but negative for HCV RNA with normal serum ALT levels, in this condition HCV RNA can be detected with low level in liver biopsies or peripheral blood mononuclear cells (Kanto et al., 1995; Ide et al., 2002; Idobe-Fujii et al., 2006; McCaughan et al., 2012).

## Antiviral Agents

The aim of future HCV treatment is to make antiviral drugs which are less toxic and allow shorter duration of therapy (12-24 weeks) than current standard. The HCV life cycle includes (1) Binding to the cell (2) uncoating (3) translation into a single large polyprotein (4) processing by cellular and viral proteases (5) RNA replication (6) packaging and assembly (7) Virion maturation (8) Release from the host cell and each of these steps can be a target for antiviral therapy (McCaughan et al., 2012).

## Interferon and Ribavirin Therapy

Some drugs effecting on human body differently has been shown to have anti-viral activity against HCV. The first treatment against HCV was INF- $\alpha$  which was discovered in 1957. After years it has been improved for HCV treatment and pegylated interferon (PEG-INF) developed which has longer half-life (Buckwold et al. 2007). Ribavirin (1-beta-D-ribofuranosyl-1, 2, 4- triazole-3-carboxamide) is a purine-analog was discovered 30 years ago and is effective against HCV when used with interferon or PEG-INF-alfa-2a in combination therapy and increases SVR rate. Ribavirin is not very effective in monotherapy and pegylated interferon (PEG-INF) and Ribavirin combination is a golden standard treatment for hepatitis C (Koev et al., 2007; Lacolla et al., 2010). Some mechanisms such as modulation of interferon



gene expression, inhibition of Inosine monophosphate dehydrogenase, regulation of immunity, inhibition of the viral RNA polymerase and lethal mutagenesis of genomes, caused by Ribavirin triphosphate can suppress HCV infection and life cycle. Unfortunately, INF and Ribavirin are toxic and some side effects such as headache, fever, severe depression, myalgia, arthralgia and haemolytic anaemia have been reported. In summary, development of cheaper and improved treatment is required and also in some genotypes such as 1 and 4 the response to treatment is weak (Ampurdanés et al., 1996; Ikejiri et al., 2007; Koev et al., 2007; Korba et al., 2007; Jouan et al., 2010; Lawitz et al., 2011).

### NS3/4A Serine Protease Inhibitors (PIS)

NS3 protein acts as a serine protease and RNA helicase with its cofactor NS4A together cleave HCV polyprotein and help to HCV polymerase (NS5B) function. So NS3/4A serine protease has a vital role in viral replication. PI blocks NS3/4A serine protease and prevents viral replication (Chase et al., 2009). Two classes of PI molecules have been developed; (1) noncovalent inhibitors, such as Ciluprevir and ITMN-191/R-7227 and (2) covalent reversible inhibitors such as Telaprevir (TVR) and Boceprevir. TVR with slow binding and dissociation kinetics showed a reduction of viral RNA levels and eventually eliminated HCV RNA from the replicon cells. Many clinical trial studies reported that triple therapy (pegylated IFN, TVR, and RBV) for 12 weeks provided more-potent antiviral efficacy than did standard pegylated IFN and RBV therapy but some side effects have been reported. Boceprevir gained FDA approve In January 2006, is safe and well tolerated which is more effective when combined with pegylated IFN. In 2003 a new recombinant Human antibody produced that have an inhibitory action on HCV helicase in carboxy terminal of NS3 (Chase et al., 2009; 2012; Furusyo et al., 2012).

### NS5B RNA-Dependent RNA Polymerase Inhibitors

HCV RNA-dependent RNA polymerase (NS5B) is an enzyme responsible for replication. Many antiviral drugs against viruses have focused on the viral polymerase and two types of HCV polymerase inhibitors (1) nucleoside analogues and (2) nonnucleoside analogues have been developed. The nucleoside analogue (R-1626) has potent antiviral activities which bind to NS5B nucleotide-binding site inhibits replication by acting as a chain terminator and stopping further elongation of RNA strand. R-1626 can be given together with pegylated IFN or pegylated IFN plus RBV to patients and no resistance mutations were observed but some adverse effects have been reported. On the other hand nonnucleoside inhibitors block the polymerase by interacting with outside of catalytic site. BMS-824 is a small molecule which binds to NS5A inhibits hyperphosphorylation and decreases virus replication. Zinc mesoporphyrin is another molecule causes NS5A protein downregulation by polyubiquitination and proteasome-dependent degradation (Wegzyn and Wyles,

????; Zuo et al., 2007; Zhao et al., 2008; Shi et al., 2012).

## HCV Vaccines

### *Envelope protein vaccines*

The recombinant proteins vaccine candidates induce an immune response to a limited number of viral epitopes which are cloned in bacteria, yeast or mammalian cells is sufficient to develop protective immunity. Some recombinant proteins are enough to induce immune response but others require adjuvant therapy (Duan et al., 2010). According to the genetic variability of the HCV viral envelope glycoprotein, this protein is the main target for anti-HCV antibodies. The earliest preventive vaccine candidate includes the envelope gpE1/gpE2 proteins in an oil water adjuvant MF59 together with a CpG oligonucleotide and all cases developed neutralizing antibodies and T-cell lymphocyte proliferation (Frey et al., 2010). The first candidate therapeutic vaccine was a recombinant HCV-E1 protein in alum adjuvant which was administered to humans in 2003 and induced HCV-specific antibody and T-cell responses in patients. In 2003 Vaccination with HCV E1 protein (Innogenetics/GenImmune) started with a 135aa C-terminally of the E1 protein formulated on alum and humoral and cellular immune responses were produced but failed to get improvement in fibrosis scores and in 2007 stopped (Alvarez-Obregón et al., 2001; Zeng et al., 2009).

### *Core proteins*

Tarmogens (GlobeImmune GI-5005) are a novel vaccines taken up by dendritic cells and stimulate both innate and specific cellular immune responses are Core-NS3 fusion protein which expressed in yeast cells (*Saccharomyces cerevisiae*) have been trialed as a therapeutic vaccine candidate (GI5005) and is in a Phase II, placebo-controlled trial and combined with standard therapy (PEG-IFN/ Ribavirin). Vaccinated mice exhibited strong Th1 with IL-2 and IFN- $\gamma$  production and cytotoxic activity to NS3 and core proteins. Core protein with an adjuvant composed of Saponin, cholesterol and phospholipid, called ISCOMATRIX, has been evaluated in a Phase I trial (Alvarez-Obregón et al., 2001; Krishnadas et al., 2010).

### *Peptide vaccines*

Peptide-based vaccines induce HCV-specific T-cell immunity with the presentation of peptide to T-cell receptor by HLA molecules. IC41 (Intercell AG, Vienna, Austria) consists of five peptides from core, NS3 and NS4 proteins, which are CTL epitopes (core35-44 and 132-140, NS3 1073-1081, NS4 1764-1772) and CD4+ T cell epitopes (core23-44, NS3 1248-1261, NS4 1767-1786) combined with the adjuvant poly-L-Arginine is an example of peptide vaccine. In 2009, a peptide of core region (C35-44) was evaluated in a Phase I, dose-escalation in a Japanese study in 26 patients (23 nonresponders to PEG-IFN/Ribavirin and three who had declined standard therapy) (Firbas et al., 2010). In December 2006 Pevion Biotech started phase I clinical trial for virosome-based hepatitis C virus with the combination of the PeviPRO

and PevITER (PEV2A and PEV2B) that utilize synthetic HCV peptide antigens. Synthetic lipopeptides containing both T helper and CD8+ epitopes induce strong T cell responses by increasing their uptake by antigen presenting cells (Firbas et al., 2006; Schlaphoff et al., 2007; Klade et al., 2012).

#### DNA vaccines

The first DNA vaccine was licensed in 2001 and research efforts have been aimed an effective hepatitis C DNA vaccine. Several methods were developed to improve naked DNA delivery such as using a gene gun for DNA vaccination, electroporation with electrical impulses which creates pores in living cells and puts DNA through the cell membrane (Desjardins et al., 2009). The first DNA vaccine evaluated in Cuba in Phase I trial, which is (CICGB-230) combining plasmid expressing HCV structural antigens (core/E1/E2) with recombinant core protein (Co.120) (Memarnejadian and Roohvand, 2010; Huret et al., 2012). Another HCV DNA-based vaccine is a T cell vaccine based on HCV non-structural 3/4A which expressed under the control of the Cytomegalovirus immediate early promoter (ChronVac-C has recently been developed by TripepAB Sweden) used electroporation to inject plasmid expressing HCV antigens NS3/4a. CD8+ T cells could be eliminated against NS3/4A expressing hepatocytes and tumor cells in mouse model. No side effect was observed and two patients had viral load reductions of up to 1.2 and 2.4 log<sub>10</sub> and the development of HCV-specific T cell reported (Ma et al., 2002; O'Hagan et al., 2004; Park et al., 2008; Memarnejadian and Roohvand, 2010).

#### Vector vaccines

Using viral vectors for the delivery of HCV RNA is the aim of vaccine choice. Adenoviral vectors are inducers of HCV-specific T-cell responses in the chimpanzee model and reduce HCV viremia during primary infection. Synthetic peptides are able to deliver a limited number of epitopes (Papa et al., 1998). However, Adenoviral vectors have complete viral genes, so can deliver HCV proteins intactly and do not be limited by HLA class restriction. Specific CD8+ T cell responses were induced by vaccination and were vital for HCV clearance of 4 of the 5 challenged chimpanzees. Adenovirus vectors are used in a Phase I trial to deliver NS HCV proteins (NS3, NS4 and NS 5B) to 36 healthy volunteers and because of pre-existing anti-adenoviral antibodies which can limit vector efficacy, two Adenoviral vectors Ad6 and simian vector AdCh3 to which humans are rarely exposed are used and have been shown to induce CD4+ T cells and specific CD8+ T cells to make IFN- $\gamma$  (Ma et al., 2002; Blanchard et al., 2003; Hourieux et al., 2007; Desjardins et al., 2009). A therapeutic vaccine (TG4040) is a recombinant poly-antigenic T cell vaccine base on highly attenuated poxvirus strain, Modified vaccinia Ankara (MVA) which expresses NS3/4/5B proteins. Six of 15 patients received 3 weekly injections while the remaining 9 patients received a 4th injection at 6 months. T cell responses were detected in all patients as early as one week after the first vaccination and were maintained during the 6-month and the vaccination

reduced HCV viral loads by up to 1.5 log<sub>10</sub> (Zhang et al., 2008)

## Future Vaccines

#### Virus like particles approach (VLPs)

Virus-like particles (VLPs) produced by the structural proteins of many viruses which have self assembly ability in the absence of other viral components and these particles (VLPs) can be isolated directly after expression in prokaryote and eukaryotic cells, or assembled in vitro from protein subunits purified from any recombinant hosts. VLPs recognized in the common size range of viruses (22–150 nm) and their exact properties depending on the viral proteins incorporated, but because they assemble without DNA or RNA or genetic materials, VLPs are non-infectious (Acosta-Rivero et al., 2001).

#### Spherical VLPs

VLPs have either helical or icosahedral symmetry and have a single layer of viral proteins but some VLPs are double-layered with outer and inner layer, whereas triple-layered VLPs have been recognized. They are models to study assembly of macromolecules and virus assembly (Acosta-Rivero et al., 2002). The VLP assembly pathway consist of protein–protein, metal ion or disulfide bond interactions and in some viruses, assembly can be mediated by proteolytic maturation. Structural proteins like coat proteins or capsid proteins can make VLPs, even in heterologous expression systems.

Expression of Norwalk virus (NV; family Caliciviridae) capsid protein (VP1) in insect cells showed that a single histidine residue at position 91 (S) domain at the N-terminus of VP1 can initiate VLP assembly and produced empty VLPs. The minor structural protein VP2 may be encapsidated when coexpressed in insect cells. This protein increases the stability of VP1 from disassembly and protease degradation (Acosta-Rivero et al., 2004).

The Adeno-associated virus 2 (family Parvoviridae) has VP1, VP2, and VP3 capsid proteins and only VP2, VP3 are necessary for capsid formation and also after expression in insect cells VP2 and VP3 produce VLPs (Chin et al., 2008).

The Rice dwarf Reovirus (RDV) contains six structural proteins, P1, P2, P3, P5, P7, and P8. Expression of P3 and P8 in insect cells, leading to VLPs formation. Double-shelled VLPs were produced by coexpression P3 and P8 in vitro. Sindbis alphavirus (SINV) was used to explain the role of a coiled coil structure. Viral capsid protein helix I has three conserved leucine residues L38, L45, and L52 which are crucial for VLP assembly (Ait-Goughoulte et al., 2006).

#### Rod-Shaped VLPs

Two types of rod-shaped viruses are reported. The rigid rod-shaped in Tobamoviruses (Tobacco mosaic tobamovirus; TMV) and the flexible filamentous in Potyviruses (Johnsongrass mosaic potyvirus; JGMV). Nonhelical native TMV and JGMV were expressed in *E. coli* (Desjardins et al., 2009).



### *Viral Scaffolding Proteins*

Scaffolding proteins play an essential role in correct assembly of the outer shell of the capsids in some viruses. Herpes simplex virus (family Herpesviridae) capsid have four outer (VP23, VP5, VP26, and VP19C) and three inner (VP21, VP24, and VP22a), and VP22a is a scaffolding protein in particle assembly. In the absence of VP23, VP5, or VP19C VLP assembly is not produced and lack of VP26 has no effect on VLP formation (Thuman-Commike et al., 1999; Newcomb et al., 2001; Fane and Prevelige, 2003).

Rotavirus is a triple-layered particle and has four major structural proteins VP2, VP4, VP6, and VP7 and two minor proteins VP1 and VP3. VP2 makes Core like particles and its N-terminal is responsible for encapsidation of VP1 and VP3 (Attoui et al., 2009). An interaction of VP2 with VP6 is sufficient to form double-layered VLPs. Bluetongue virus (BTV; family Reoviridae) has four major structural proteins VP2, VP3, VP5, VP7 and three minor structural proteins VP1, VP4, VP6. Interaction between VP3 and VP7 are necessary to form single-shelled VLPs in a Baculovirus expression system (Bellamy and Both, 1990). In infectious bursa disease virus (family Birnaviridae), VP2 and VP3 structural proteins expressed in a heterologous system and showed that VLPs formation depends on the immature VP2 and C-terminal residue of VP3 (Delgui et al., 2009).

### *Protein–Nucleic Acid Interactions*

The role of viral structural proteins is genetic material protection from degradation and the interaction between nucleic acids and VLP formation has been reported (Bourne et al., 2001). Hepatitis C flavivirus (HCV; family Flaviviridae) VLPs have been showed in the body and cell expression systems such as insect, bacterial, yeast. The first report on HCV VLPs assembly with a bilayer envelope indicated that 5'UTR interaction with highly basic N-terminal 120 or 124 amino acids of the core protein is necessary for VLP formation and presence of core, E1, E2 might enhance the assembly and formation of VLP process (Desjardins et al., 2009). Rous sarcoma virus (RSV) can assemble into VLPs in vitro in the presence of RNA. Some studies showed that the dimerization of Gag protein and nucleic acid binding domain of Gag proteins is crucial for formation of VLPs (Poranen and Tuma, 2004).

### *Cell Membranes*

The formation of enveloped viruses happens by budding through cell membranes and two mechanisms can be distinguished. The first mechanism depends on the presence of the capsid protein which was showed in Retroviruses whereas Togaviruses require both capsid protein and envelope proteins. The second mechanism is called capsid independent and budding occurs by formation of envelopes (Blanchard et al., 2002). The Pr57gag precursor of Simian immunodeficiency virus and Gag precursor (Pr55gag) of Human immunodeficiency retrovirus 1 (family Retroviridae) have been expressed in a Baculovirus system and assembled into 100 to 120 nm VLPs that bud from the cell membrane and also expression of Gag-Pol protein by Vaccinia virus expression vector causes the immature and mature Retrovirus-like particles

formation with budding from the cell surface (Affranchino and González, 2010). Rubella virus (family Togaviridae) capsid protein and the two envelope glycoproteins E1 and E2 form VLPs by a budding mechanism. The E2 transmembrane and cytoplasmic domain contains retention signals which are required for interaction with the capsid and VLP secretion (Ivanova et al., 1995).

Unlike retrovirus, members of Coronaviridae assemble their viral envelopes into VLPs without the capsid protein. The membrane glycoprotein (M) and envelope protein (E) are required for assembly of Coronavirus VLPs. Some study on VLP formation showed that M and E proteins of Infectious Bronchitis virus (IBV; family Coronaviridae) targeted to the Golgi complex need their cytoplasmic tails to assemble into VLPs (Liu et al., 2012).

Influenza virus (family Orthomyxoviridae) has M1 protein which is required to induce efficient formation of VLPs budding from the cell membranes and also human Parainfluenza virus type 1 (hPIV-1; family Paramyxoviridae) has matrix protein M which induced budding of VLPs from the plasma membrane but in Paramyxovirus, Simian virus 5 (SV-5) Hemagglutinin-Neuramidase (HN) and fusion protein (F), together with the nucleocapsid protein (NP) are critical for VLP budding process (Hechtfisher et al., 1999; Barman et al., 2001; Fane and Prevelige, 2003). Ebola virus and Marburg virus (family Filoviridae) has (VP40) protein which leads budding and formation of filamentous spikeless VLPs by membrane-bound formation and corporation of the GP and matrix proteins in mammalian cells also cause filamentous formation (Lee et al., 2009).

### *Disulfide Bonds and Metal Ions*

Disulfide bonds between interacting domains of viral structural proteins or ions are corresponding elements which leads to the VLP structure. Polyomaviruses, Murine polyomavirus, Simian virus 40 (SV 40), Human BK polyomavirus, and JC polyomavirus (JCV) have three proteins VP1, VP2, and VP3, of which VP1 is the major structural protein. Some study showed that the minor capsid proteins are not essential for VLP formation and the major structural protein VP1 forms disulfide linkages that stabilize dimeric and trimeric interactions. In addition calcium ion binding are important for assembly of the capsid (Chen et al., 2001). Expression of the major late 1 (L1) structural protein and minor late 2 (L2) structural protein of Human papillomavirus (HPV; family Papillomaviridae) in prokaryote, Baculovirus, yeast, or mammalian systems showed that L1 self-assembly produces VLP and L2 is not strictly required and disulfide bond formation could be essential for capsid assembly (Mukherjee et al., 2008).

### *Proteolytic Maturation*

Expression of immature precursor viral envelopes proteins are followed by their processing seems to be an important strategy in formation of viral particles. The Thosa asigna virus (TaV; family Tetraviridae) pre-CP is cleaved at two positions to produce the L and S proteins and VLPs were formed when the cleavage between the L and S happens after expression (Speir and Johnson, 2008;

### Host Factors

Beside the viral factors, the host factors and cell condition are important players in the efficiency of VLP production (Blutt et al., 2006). RSV Gag protein in insect cells failed to produce VLPs but the same Gag protein expressed in mammalian and avian cells makes VLPs without any modifications and it shows that chaperone in vertebrate cells may cause proper folding of Gag protein (Yoo et al., 2010). Human T-cell leukemia virus type I (HTLV-I) Gag and Gag-pro polyprotein show differential budding efficiencies. The expression of Pr53Gag polyprotein in Baculovirus in insect cells was unable to drive budding of immature VLPs (Desjardins et al., 2009). On the other hand, Gag particles were secreted into the medium as enveloped VLPs in human 293 cells. Specific cell factors are necessary for the maturation of the Gag protein and HIV VLP synthesis (Mizukoshi et al., 2009).

### Principles of Virus-Like Particle-Based Vaccines

Vaccines for viral disease are attenuated or chemically inactivated live viruses or recombinant subunit vaccines with a single viral protein or peptide. Virus-like particles (VLPs) are a type of subunit vaccine which mimics the structure of virus particles and many of them completely lack the DNA or RNA genome. They can stimulate B-cell-mediated immune responses and also has been shown that VLPs are effective at stimulating CD4 proliferative responses and cytotoxic T lymphocyte (CTL) responses (Liu et al., 2012).

## Vaccines Based on VLPs

### Clinical trials in humans and animal models

Antigens composing to VLP induce strong B cell as well as T cell responses. These vaccines are well tolerated and safe because they are noninfectious and are immunogenic. The immunization of young women with HPV16 VLPs composing major structural protein L1 induced high titer neutralizing antibodies and protected the patient from HPV and reduces the incidence of cervical cancer and cervical dysplasia. According to this therapy, T-cell response increased in T-cell proliferation (CD4+>CD8+), Th1-(T helper 1) and Th2-specific cytokines (Radaelli, Bonduelle et al., 2007).

Norwalk virus (NV)-like particles tested in humans with oral vaccination protocol and induction of serum IgG in all vaccine recipients and 30-40% of the volunteers developed mucosal IgA (Blazevic et al., 2011). Linkage of adjuvant molecules to VLPs was shown to increase the immunogenicity of the particles. The nontoxic subunit B of cholera toxin (CTB) was bound to VLPs made specific serum IgG1, secretory IgA antibodies and cellular immune responses against Simian immunodeficiency virus Gag and Env proteins in mice. Virus like particles can interact with dendritic cells (DCs). In a study on EBOV mouse bone-marrow-derived DCs were activated and surface expression of CD40, CD80, CD86, major histocompatibility complex (MHC) class I and II and secretion of interleukin 6 (IL-6), IL-10, macrophage

inflammatory protein-1a (MIP-1a) and tumor necrosis factors enhanced (Song et al., 2010; Hamdy et al., 2011).

Using yeast-derived HIV-1 p55gag VLPs induces human DCs production and makes IL-12 secretion, through yeast membrane-triggered Toll-like receptor 2 signaling. These DCs can activate memory CD8+ T cells to become effector cells in chronically HIV-infected patients (Mizukoshi et al., 2009). By the ability of HBsAg to self-assemble into particulate structures, the particles were released from recombinant yeast cells allowing high-yield production and safer purification of the vaccine. In the same way HIV-1 Pr55Gag polyprotein self-assembles into particulate spheres provided a new rationale for generating a Gag-based VLP vaccine and Gag is a common antigen expressed for vaccine approaches, and its VLPs can be produced in mammalian or insect cells and induces Th1-biased humoral and cellular immune responses in mice and macaques (Mizukoshi et al., 2009; Sistigu et al., 2011). A second class of VLPs with a wide range of applications is virosomes, representing virion-like liposomebased complexes with integrated surface glycoproteins for endocytosis. Virosomes are becoming more popular since they combine with VLP and have been established for many viruses such as HIV, EBV and Sendai virus with the aim of vaccination and delivery of nucleic acids, drugs or heterologous antigens (Buonaguro et al., 2007). Virus-like particles are famous as a recombinant protein vaccine, because they mimic the properties of native virions. Synthesis of HCV like particles (HCV-LPs) with Baculovirus which contains HCV structural proteins (core/E1/E2) showed that HCV-LPs have biophysical, ultrastructural, and antigenic properties similar to the native virions. In some studies BALB/c mice and HLA-A 2.1 transgenic (AAD) mice immunized with HCV-LPs and strong humoral and cellular immune responses against HCV structural proteins induced (Frey et al., 2010). Furthermore, adoptive transfer of lymphocytes from HCV-LP-immunized mice to native mice provided protection against recombinant HCV-Vaccinia challenge in AAD mice. Adjuvants have been used with vaccines to elicit an immune response, and they can modulate different T-helper (Th1 versus Th2) response. To maximize HCV-LPs ability to elicit immune responses, 2 vaccine adjuvants tested with HCV-LPs in mice (Provided by Coley Pharmaceutical Group, Wellesley, MA) is an immunostimulatory oligodeoxynucleotide optimized for immune stimulation and AS01B contains monophosphoryl lipid A (MPL) and QS21, a naturally occurring Saponin molecule (Harris et al., 1996; Roohvand et al., 2007; Hamdy et al. 2011). The combination of HCV VLP and these adjuvants enhanced the speed of the immune responses by improving antigen presentation to T cells and the interaction between immunogen and macrophage (Qiao et al., 2003).

### VLP production

The Baculovirus Expression Vector System (BEVS) is one of the most powerful eukaryotic expression systems, available to express heterologous genes from many different sources which has been used including fungi, plants, bacteria and viruses, in insect cells

(Futatsumori-Sugai and Tsumoto, 2010). Baculoviruses (family Baculoviridae) is a member of large group of double stranded DNA viruses which infect many different species of insects and its DNA (between 80 and 200 kb) is packaged into rod-shaped nucleocapsids. The Baculovirus used for VLP production is the *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Roldão et al., 2007). In this system several nonessential genes replaced by heterologous genes and because the Baculovirus genome is large to insert foreign genes, heterologous genes are cloned into transfer vectors. Co-transfection of the transfer vector and AcNPV DNA into *Spodoptera frugiperda* (Sf) cells allows recombination between homologous sites, transferring the heterologous gene from the vector to the AcNPV DNA (Lenhard et al., 1996). With strong polyhedrin promoter (P10), AcNPV infection of Sf cells causing high rate of Recombinant proteins and VLP production produced at range between 0.1% and 50% of the total insect cell protein. Over than 20 years the Baculovirus–insect cell expression system has been used for routine production of recombinant proteins. This system has eukaryotic protein processing capabilities and insect cells can fold, modify, traffic and assemble polypeptides to produce products. A weak point of the Baculovirus expression system is its inefficiency at processing heterologous proteins that are synthesized as larger precursor proteins (Huang et al., 2002).

Baumert et al. (1998; 1999; 2000), showed that expression of the core, E1 and E2 proteins by recombinant Baculoviruses resulted in the formation of recombinant virus particles. These recombinant HCV particles induced antibodies and cell-mediated immune responses after immunization of rabbits. Kunkel et al. 2001 expressed HCV core protein in the recombinant Baculovirus system, and reported the generation of nucleocapsid-like structures. The insect cell expression system has been used for production of virus-like particles of papillomavirus, Rotavirus, human immunodeficiency virus, Norwalk virus, and hepatitis E virus (Baumert et al., 1999; Matsuo et al., 2006).

Recombinant Baculovirus (rBV) infected insect cells also is a useful system to investigate the viral particle assembly processes. For example the rBVs encoding HSV-1 capsid proteins used to infect insect cells and purified capsid proteins from infected cells have been tested for the ability of assembling into procapsids (Belyaev et al., 1995; Palomares et al., 2012). Human Polyomavirus JC virus causing progressive multifocal leukoencephalopathy has a major structural viral glycoprotein (VP1) which has been produced recombinant VLPs with the morphology of empty JC virus particles by using rBVs and this purified VLPs are immunogenic when used with adjuvant (Goldmann et al., 2000; Zielonka et al., 2006). VLPs containing L1 structural proteins of the Human Papilloma Virus types 16/18 produced in Baculovirus-infected insect cells in clinical trials can prevent cervical infections with HPV-16 and HPV-18, together and block associated cytological abnormalities (Ault et al., 2004; de Witte et al., 2008). Recent studies have also showed promising approach for the development of an HIV/AIDS vaccine candidate (Tobin et al., 1995). Recently the Coronavirus

S, E and M structural proteins of severe acute respiratory syndrome (SARS) have been expressed in Baculovirus-infected insect cells and assembly of virus-like particles has been described and a new candidate VLP based vaccines against human Corona virus disease provided (Lai et al., 2006).

Baculovirus transduction has been studied for hepatitis C virus (HCV) by placing the entire HCV cDNA under the control of the CMV promoter in recombinant Baculovirus. Transduction of HuH7 cells with rBVs-HCV virus expressed HCV polyprotein and processed into structural and non-structural gene products. HCV VLPs have been produced in Baculovirus–insect cell system. Recombinant VLPs containing HCV core, E1 and E2 proteins have been shown to induce HCV-specific humoral and cellular immune responses in baboons (Scott, 2003; Matsuo et al., 2006; Gal-Tanamy et al., 2009). According to the lack of cell culture to study hepatitis B virus (HBV) and HCV virus, this problem was overcome by using a Baculovirus to deliver the HBV genome to hepatoma cells and produce HBV infection or deliver a cDNA copy of the HCV genome to cells and HCV polyprotein was produced and properly processed (Zeisel and Baumert, 2006). The initial heterologous gene expression in yeast developed on *Saccharomyces cerevisiae* which can make hyperglycosylate recombinant proteins and N-linked carbohydrate chains are terminated by  $\alpha$ 1, 3 mannose attached to the chain (Acosta-Rivero et al., 2001). This system has been applied to produce insulin and HBsAg of HBV virus. Alternative wide ranges of yeast-based systems are available: Two methylotrophic yeast species *Hansenula polymorpha* (H.p) and *Pichia pastoris* (P.p), and the two dimorphic organisms *Arxula adenivorans* (A.a) and *Yarrowia lipolytica* (Y.l). Some of yeast species such as *H. polymorpha*, *P. pastoris*, *Candida boidinii* and *P. methanolica* are able to use methanol as a source of energy and carbon. For two methylotrophic species (*H. polymorpha* and *P. pastoris*) a range of integration and expression vectors employ promoter elements such as AOX (alcohol oxidase), derived from methanol pathway genes. All *P. pastoris* strains used for heterologous gene expressions are from strain NRRL-Y-11430. Yeast derived vectors are available as circular plasmid or linearized and targeted to a specific genomic locus (Acosta-Rivero et al., 2003).

In 1970s, material and methods for supporting *P. pastoris* and the condition of growth on the methanol were created by Phillips Petroleum Company. In the early 1980s, Phillips Petroleum Company with the Salk Institute Biotechnology/Industrial Associates, developed heterologous gene expression system of *P. pastoris* (Falcón et al., 1999).

*P. pastoris* is a single-celled microorganism that is easy to manipulate and culture. The first step in the methanol metabolism is methanol oxidation to formaldehyde, generating hydrogen peroxide in the process by two enzyme alcohol oxidase (AOX1 and AOX2) but the AOX1 gene is responsible for the vast majority of alcohol oxidase activity in the cell (Lunsdorf et al., 2011). Like other eukaryotic cells, it can be able to make posttranslational modifications, such as proteolytic



processing, folding, disulfide bond formation, and O- and N-linked glycosylation. The length chain of glycoproteins expressed by *P. pastoris* is only 8-14 mannose residues, whereas that by *S. cerevisiae* is 40-150 residues. Thus, inactive proteins that are produced in bacterial systems are biologically active in *P. pastoris*. The *P. pastoris* system is faster, easier, and less expensive than other eukaryotes expression systems such as insect and mammalian cell systems. High-density fermentation of *P. pastoris* is vital for secreted proteins, so with cell density the concentration of culture medium be increased. Because the foreign genes insertion into the expression vector is carried out in *Escherichia coli*; therefore, all expression vectors of *P. pastoris* have been made as *E. coli*/*P. pastoris* shuttle vectors and they also have an origin of replication for plasmid maintenance in bacteria, with selectable markers for transformation of the vector in both organisms. For foreign proteins secretion, expression vectors have a secretion signal which is in frame with the foreign gene. *E. coli* is the first host for recombinant gene expression which has been used more than 40 years but *Pichia pastoris* within the past 15 years, has been the second most-used host for recombinant gene expression. From 1995 to 2009, using of *P. pastoris* has been increased from 4% to 17% but in the same time usage of *E. coli* remained 60% constantly (Falcón et al., 1999; Acosta-Rivero, Aguilar et al., 2001; Acosta-Rivero et al., 2003; Haller, Lauer et al., 2007; Mizukoshi et al., 2009; Lunsdorf et al., 2011). Yeast can secrete the protein in a form that mimics its native conformational structure and immunogenicity and in this condition the majority of antigenic epitopes with neutralization activity of antibodies be preserved. Bovine herpes virus-1 (BHV-1) gD gene, expressed in *P. pastoris* as a secretory protein while keeping its native conformation, can be used as a subunit vaccine against BHV-1. The recombinant gD protein of Equine herpes virus-1 (EHV-1) being expressed in *P. pastoris*, used in a boost injection program. This protein induced high EHV-1 ELISA and virus neutralizing antibodies and also protected BALB/c mice which had been challenged with infection (Balamurugan et al., 2003; Dummer et al., 2009). L1 protein of Human papilloma virus (HPV) type 6 expressed in *P. pastoris*, has helped the HPV vaccine development and structure and function study (Hanumantha et al., 2011). The expressions of recombinant polio and Dengue viral vaccines in *P. pastoris* have also been reported. The recombinant envelope glycoprotein (E4) of Dengue-4 virus produced in *P. pastoris* and elicited neutralizing antibody and haemagglutination inhibition antibodies as well as specific memory T cell against expressed protein when it was injected to BALB/c mice (Balamurugan et al., 2003; van Oers 2006; Roldão et al., 2011). The recombinant hepatitis E virus (HEV) ORF2 protein has been expressed in *P. pastoris* and high immunogenicity reaction against ORF2 protein was showed by inducing strong immune response in mice. The new fusion protein composing HBsAg with epitope-containing HEVAg was expressed in *P. pastoris* and this chimeric HBV/HEV virus like particles has been used as a recombinant HBV/HEV vaccine candidate (Lal et al., 1997; Patil and Khanna, 2012).

#### *HCV like-Particles*

HCV-like particles (HCV-LP) produced by recombinant Baculovirus, vesicular stomatitis virus (VSV), an RNA replicon system of Semliki forest virus (SFV), and pseudotype viruses based on VSV or Retroviruses which have been used to examine the binding and entry receptors for HCV (Akazawa et al., 2011). The structural proteins expression by various viral vectors leads to the formation of virus-like particles (VLP). Its production by recombinant Baculovirus expression system in insect cells has been successful and this usage is not only for subunit vaccine but also for study of virus-cell interactions. HCV VLP based vaccine strategy makes it possible to elicit long lasting neutralizing antibody and core-specific T-cells against to vaccine construct which mimics nature HCV virion antigenicity. The similar investigations on human papilloma virus and hepatitis B virus have been reported (Mihailova et al., 2006). Insect cell-derived HCV VLPs have produced E1, E2 antigens as a Vaccine. These noninfectious 40-50 nm HCV-LPs consist of a lipid envelope containing E1 and E2. In addition Immunization of primates and chimpanzees with heterodimers of recombinant E1 and E2 proteins to stimulate a protective antibody response has been reported.

HCV VLPs are superior in both mice and non-human primates in immunity response compared to DNA or recombinant envelope protein vaccines and also it is an advanced vaccine candidate because can induce humoral and cellular immunity (CTL) (T-helper) against HCV structural proteins and stimulate the maturation of human dendritic cells (Acosta-Rivero et al., 2001; 2002; 2003). HCV-LPs are produced by self-assembly of HCV structural proteins in some cells and are detected by morphologic, biophysical, and antigenic properties similar to original virions from infected humans. HCV-LPs can induce humoral and cellular immunity by cytotoxic and helper T lymphocyte cells. Dendritic cells (DCs) can interact with viral particles and causes the immunopathogenesis of HCV infection. Because of virions synthesis or purification limitation, HCV like particles (HCV-LPs) has been used to study HCV with human DCs interaction. DCs show an envelope-specific and binding of HCV-LPs. Some studies reported that C-type lectins such as DC-SIGN (DC-specific intercellular adhesion molecule 3-grabbing nonintegrin) were not enough for HCV-LP binding and this interaction followed by DC activation. Activated DC cells stimulate specific CD4 T cells by antigen processing and presentation with major histocompatibility complex (MHC) class II molecules (Kim et al., 2012; Velazquez et al., 2012). In insect cells, the HCV structural proteins have assembled into virus-like particles (HCV-LPs). The E1-E2 heterodimer glycoprotein are presented in HCV-LPs in insect cells. E2 recombinant glycoprotein binds to human cell lines and this interaction has been used as a model for binding of virus to host cells. This assay showed that envelope glycoprotein E2 interacts with the large extracellular loop of cellular membrane protein CD81 as a receptor candidate which may play a role in T-cell activation (Ciccaglione et al., 1998). HCV structural proteins which expressed in insect cells, assembled trough

the large cytoplasmic cisternae into enveloped virus like particles (40-60 nm in diameter) derived from the endoplasmic reticulum (ER). Evaluation of virus like particles by CsCl and sucrose gradient centrifugation revealed biophysical information similar to natural HCV virions isolated from infected patients. Some study results suggested that HCV core and envelope proteins without p7 are enough for viral particle assembly and also production of HCV-like particles in insect cells may help scientists to know the structural requirements for HCV particle assembly as well as to study viral genome encapsidation and virus-host interactions (Falcón et al., 2003). The presence of a green fluorescent protein within HCV pseudo-particles allowed reliable determination of HCV infectivity by the E1, E2 glycoproteins which are required for pseudo-particles infectivity and neutralized by sera from infected patients and anti-E2 monoclonal antibodies. Primary hepatocytes as well as hepatocarcinoma cells are the major targets of infection in vitro and E1, E2 glycoproteins are necessary for pseudo-particle interaction (Katsarou et al., 2009). Recombinant Baculovirus are able to deliver foreign genes not only into insect cells, but also into mammalian cells without extensive cytopathic effects. Recently a modified recombinant Baculovirus having the VSV envelope G protein (VSVG) enhanced gene transfer efficacy in a variety of cell lines. The HCV-LP was made in a human hepatoma cell line, FLC4, by using the recombinant baculovirus system (Matsuura et al., 2001). Weijia et al. 2010 used *Pichia pastoris* to express truncated HCV E1E2 protein, which consists of E1 residues 187-346 and E2 residues 381-699 and made high level of recombinant HCV E1E2 protein. The protein is glycosylated and can bind to the putative HCV receptor CD81 and the purified protein can induce anti-E1E2 antibodies in rabbits, which can neutralize two kinds of HCV pseudotype particles derived from HCV genotype 1a and 1b, as well as HCV virions derived from HCV genotype 2a. These findings indicate that the recombinant E1E2 glycoprotein is effective in inducing neutralizing antibodies, and is a potent HCV vaccine candidate (Matsuo et al., 2006; Kouvatzis et al., 2007). Hepatitis C virus-like particles (HCV-LPs) from HCV H77 strain (1a genotype) was used to study virus-cell interaction. HCV-LPs showed a buoyant density of 1.17 to 1.22 g/cm<sup>3</sup> in a sucrose gradient and formed double-shelled particles 35-49 nm in diameter. Some results indicated that HCV-LPs can be used to characterize the mechanisms of early steps of HCV infection. With the indirect method (detection with anti-E2 antibody) and the direct method (use of dye-labeled HCV-LPs) showed that HCV-LPs bind to several human hepatic (primary hepatocytes, HepG2, HuH7, and NKNT-3) and T-cell (Molt-4) lines. Using anti-E1 or anti-E2 antibodies showed that E1 and E2 proteins mediate HCV-LPs binding and entry into cells. Hepatitis C virus (HCV) antigens are reported by transmission electron microscopy in *Pichia pastoris*. The core protein (aa 1-191) and 148 aa of the E1 envelope antigen (aa 192-339) was cloned to express the first 339 NH<sub>2</sub>-terminal amino acids of the HCV polyprotein (C-E1.339 polypeptide) and Virus-like particles (VLP) with diameters ranging from 20 nm to 30

nm were observed. The VLP stayed in the endoplasmic reticulum and also localized in vacuoles, either free or inside autophagic bodies. Chains of particles, high-density reticular structures, and crystalloid bodies were also detected. Both kinds of particles, the VLP formed after treatment with NP-40 and the crystal-associated particles were core protein-positives. Like mammalian cell lines, the *P. pastoris* yeast could be a suitable host for the analysis of HCV polyprotein processing and virus assembly (Falcón et al., 1999; Roingard et al., 2004). HCV HCVAg was also recognized by serum from chronic patients. The electron microscopy analysis for purified HCcAg showed aggregated virus-like particles (VLPs) with an average diameter of 30 nm. The HCVAg obtained from *P. pastoris* assembled into HCV nucleocapsid like particles. Such HCVAg aggregates could be a valuable tool to evaluate the mechanisms of HCV nucleocapsid assembly. The core (C) protein of hepatitis C virus (HCV) is a multifunctional protein which is involved in many viral and cellular processes (Matsuo et al., 2006; Kouvatzis et al., 2007). Several studies have described the formation of nucleocapsid-like particles (NLPs) when HCV C protein is expressed in *E. coli* (Lorenzo et al., 2001), yeast (Acosta-Rivero et al., 2002), insect cells (Baumert et al., 1998) or mammalian systems (Shimizu et al., 1996; Blanchard et al., 2002). The in vitro study developed with purified recombinant proteins from *E. coli* (Kunkel et al., 2001) reported the first 600 nt of the HCV genome or structured tRNA could trigger the formation of NLPs when mixed with purified C protein. Also, it was shown that the N-terminal 120 aa of the C protein were enough for self-assembly into particles (Baumert et al., 1999; Kunkel et al., 2001; Lorenzo et al., 2001; Acosta-Rivero et al., 2002; Roingard et al., 2004). The in vitro assembly of several deleted versions of recombinant HCV C protein expressed in *E. coli* have been studied and reported that 75 N-terminal residues of the C protein are necessary to assemble and generate nucleocapsid-like particles (NLPs) in vitro. This small protein fused to the endoplasmic reticulum (ER) anchoring domain also generated NLPs in yeast cells such as *P. pastoris* which had been shown that this yeast could be an appropriate host for the analysis of HCV structural polyprotein processing and nucleocapsid assembly. For HCV virus like other RNA viruses, assembly is started by the binding of the core protein to a unique encapsidation sequence within the viral genome, often a UTR with stem-loop structure. This interaction makes oligomerization of the core protein and virus particle. Some studies have been introduced new systems to assemble nucleocapsid-like (NCL) particles in vitro. More assembly studies using the Baculovirus expression system described that nucleic acid binding to HCVAg is necessary for in vitro virus like particle assembly process. Recently, have been illustrated that processed HCVAg (P21) produced in *P. pastoris* cells assembled into structured NLPs (Lorenzo et al., 2001; Acosta-Rivero et al., 2003; 2004; Mihailova et al., 2006).

The study of HCV assembly and morphogenesis have been done by HCV core protein expression with a Semliki Forest virus expression system and self-assembly into HCV-like particles (HCV-LP) at the endoplasmic

reticulum (ER) membrane has been reported. This system was used to show whether the processing of the HCV core protein by the signal peptide peptidase (SPP) is required for the HCV-LP assembly (Vidalin et al., 2000).

## Conclusions

Recent research about HCV structure, genome and virus lifecycle showed many target sites for intervention and help us to improve HCV treatment. Although the molecular characterization of the virus has been investigated, many fundamental questions still remain unanswered. Hepatitis C virus was the first pathogenic human virus which has been identified by molecular methods. Active disease is detected by viral RNA in serum regardless of antibody or ALT levels and the acute to chronic changing is detected by HCV RNA evaluation 6 months after exposure. Molecular diagnosis is used to monitor the antiviral response by measuring the presence and levels of virus (Cao et al., 1996). ELISAs have many advantages in the diagnosis such as cost-effectiveness, low variability and high sensitivity in screening and their false-positives results can be cleared by using RIBAs (Erensoy, 2001). INNO-LIPAs can be used to evaluate patient's responses after INF therapy and also detection of HCV RNA by reverse transcription or PCR can be used for virological response to INF. According to genetic, geographical and etiological variations of hepatitis C virus, genotyping is vital to design hepatitis-related vaccines and biotherapeutic agents (Kamili et al., 2012).

A successful HCV vaccine is require stimulating broad humoral, T helper, and CTL responses. The combination of multiple epitopes on the HCV-LP and HCV nonstructural proteins can stimulate CTL and interferon responses and also it is necessary that it contains epitopes which are restricted by diverse HLA alleles. According to genetic heterogeneity of HCV, the vaccine should be able to induce cross-protective immunity against various HCV genotypes by using epitopes with high conserve part for the 6 different genotypes to induce interferon production and CTL response. Synthesis of peptides (i.e. 15-20 amino acids), can help to find immunodominant T cell epitopes and elicit neutralizing antibodies, or cell-mediated responses and antigen-driven process to be enhanced by binding of the HCV envelope proteins to a cellular ligand such as CD81 (Harris et al., 1996; Nishimura et al., 1999; Uno-Furuta et al., 2003; Roohvand et al., 2007; Palomares-Jerez et al., 2012). HCV infection is global health problem which needs effective treatment. Unfortunately there is no effective vaccine available for prevention and therapeutic options are limited especially for genotype 1. For genotypes 2 and 3, pegylated interferon with Ribavirin, can make a sustained virological response (Clarysse et al., 1995; Comanor et al., 2003; Albertoni et al., 2010; Eskander et al., 2012).

The recombinant Baculovirus system is successful in recombinant proteins expression such as multi-subunit proteins and VLPs production in insect cells. This system is popular in protein glycosylation, folding, cleavage and secretion to enhance the yield of recombinant proteins produced by infected insect cells. R-BV is a tool

for studying antigen presentation and protein-protein interactions and applied for the display of foreign proteins and epitopes on the viral surface. This new gene delivery has number of advantages such as superior biosafety, inability of virus replication, absence of cytotoxicity and technical simplicity. Our knowledge about Baculovirus-cell interactions will lead future efforts to enhance vector design to increase transduction efficiency. The HCV-like particles synthesis by recombinant Baculoviruses in insect cells is an important tool for studying viral assembly to produce particles composed of fusion proteins carrying viral structural domains and foreign epitopes and make effective subunit vaccines which can mimic the virus particles structure without genetic material to develop HCV vaccine (Belyaev et al., 1995; Matsuo et al., 2006; Futatsumori-Sugai and Tsumoto, 2010; Palomares et al., 2012).

*P. pastoris* is a powerful and standard tools used for recombinant protein expression. This includes the AOX1 promoter, which is useful for regulating heterologous protein expression in large high density fermenter cultures. This methylotrophic yeast has many advantages such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems such as Baculovirus or mammalian tissue culture, and generally gives higher expression levels. As yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and it has the added advantage of 10-to100-fold higher heterologous protein expression levels (Acosta-Rivero et al., 2001; 2004).

Virus like particles are created tools for immune responses induction and their development as vaccines or vehicles for delivering small molecules have been made after increasing awareness of the structure and the mechanism of assembly and interaction with host cells. They are carrying many antigens by coupling with them and become vaccines tools for disease prevention and therapy. This novel class of subunit vaccine is able to induce efficient cellular and humoral immune responses for both viral and non-viral diseases. VLPs are safer than many live viruses because they are free of viral genetic material and more effective than many subunit vaccines because of their conformational properties. HCV-like particles synthesis without the nonstructural genes required for viral replication can be an excellent Candida for HCV vaccine production. VLPs structure characterized by X-ray crystallography and advanced EM techniques and focuses on the comparison of VLPs composed of different numbers and combinations of structural proteins (Lal et al., 1997; Falcón et al., 1999; Acosta-Rivero et al., 2003; Balamurugan et al., 2003; Lunsdorf et al., 2011; Patil and Khanna, 2012).

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