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# Designing and Development of a DNA Vaccine Based On Structural Proteins of Hepatitis C Virus

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**ABSTRACT**

***Background*:** Hepatitis C virus (HCV) infection is one of the most prevalent infectious diseases responsible for high morbidity and mortality worldwide. Therefore, designing new and effective therapeutics is of great importance. The aim of the current study was to construct a DNA vaccine containing structural proteins of HCV and evaluation of its expression in an eukaryotic system.

***Methods*:** Structural proteins of HCV (core, E1, and E2) were isolated and amplified from *JFH* strain of HCV genotype 2a using PCR method. The PCR product was cloned into pCDNA3.1(+) vector and finally were confirmed by restriction enzyme analysis and sequencing methods. The eukaryotic expression of the vector was confirmed by RT-PCR.

***Results*:** A recombinant vector containing 2241bp fragment of HCV structural genes was constructed. The desired plasmid was sequenced and corresponded to 100% identity with the submitted sequences in GenBank. RT-PCR results indicated that the recombinant plasmid could be expressed efficiently in the eukaryotic expression system.

***Conclusion*:** Successful cloning of structural viral genes in pCDNA3.1(+) vector and their expression in an eukaryotic expression system facilitates the development of new DNA vaccines against HCV. A DNA vaccine encoding core-E1-E2 antigens was designed. The desired expression vector can be used for further attempts in the development of vaccines.



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## Introduction

Hepatitis C virus (HCV) is a small enveloped, positive-sense single-stranded RNA virus that belongs to Flaviviridae family (1). The HCV genome is 9600 nucleotide bases in length. The positive-sense RNA genome is translated into a single polyprotein then processed by cellular and viral proteases into three structural proteins (core, E1 and E2) and seven nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). Core protein is located at the N terminus

of the polyprotein and is followed by E1 and E2 proteins (2, 3).

It is estimated that more than 170 million people globally (3% of the world's population) are persistently infected with HCV infection (4). HCV causes severe liver disease that is a serious common health problem. It slowly affects the liver and causes few symptoms that eventually results in cirrhosis or liver cancer. HCV is a blood borne virus transmitted through direct blood-to-blood contacts between humans. Injecting drug users, recipients of blood products, healthcare staff, and patients undergoing haemodialisis are at an increased risk (5).

Administration of pegylated IFN-α and ribavirin are standard therapies for the treatment of HCV infection. However, IFN therapy has side effects in many patients such as headache, fever, severe depression, myalgia, arthralgia, and hemolytic anemia and in many infected people the only way to save a patient's life is liver transplantation that is very costly and risky. Therefore, development of a new treatment and vaccine is urgently needed.

HCV has 7 genotypes that differ in global prevalence and responses to treatment. Genotypes 1, 2 and 3 have a worldwide distribution. They differ by 31%–34% in their nucleotide sequence and by about 30% in their amino acid sequence. This diversity is mostly related to low fidelity of the viral RNA-dependent RNA polymerase. Currently an effective vaccine against HCV has not yet been developed. In order to control HCV, an ideal vaccine should be able to stimulate cellular and humoral immune responses (6, 7). Diversity of the HCV genome is a major obstacle in vaccine development and may be greater when chronic infection is occurred. E1–E2 complex is expressed on the surface of the HCV particle and once viral particles reach the liver, they attach to receptors on the surface of hepatocells. C or core, and envelope proteins E1 and E2, are three structural proteins with core protein being the most conserved one among various HCV genotypes. Core protein is a multifunctional protein with different properties (8-12). There is a relationship between the presence of neutralizing antibodies and the elimination of acute HCV infection. Therefore, induction of broadly neutralizing antibodies usually targeting the viral surface antigen could be a useful approach to minimize the infection (13).

The aim of the present study was to isolate core-E1-E2 genes from *JFH1* strain of HCV and construction of a fusion antigen DNA vaccine encoding these antigens. *JFH1* strain is a unique strain with propagating capability in Huh7.5 cell line. This strain has frequently been used for drug and vaccine studies (14, 15). In the current study, the genes encoding core-E1-E2 were isolated and inserted into the pcDNA3.1(+) plasmid and the constructed plasmid pcDNA3.1(+)/core -E1-E2 was transfected into Huh7.5 cells by calcium phosphate transfection method; this is a simple way to introduce extracellular DNA to eukaryotic cells. In this method, DNA reacts with calcium ions which then deposits on the cell surface. Finally extracellular DNA enters into the cell by endocytosis or pinocytosis. Benefits of this method can be routinely used for different types of cells and resistant to intracellular and serum nucleases (16, 17). Finally the HCV core -E1-E2 RNA production was detected by RT-PCR method.

## Materials and Methods

### Polymerase chain amplification of core-E1-E2 genes

Primers for amplifying core-E1-E2 genes of *JFH1* strain were designed using Gene Runner (Version 3.05, Hastings Software Inc.) software. Two specific primers, 5′-ATCGTAAGCTTACCATGGGGAGCACAAATCCTAAACC-3′as a forward primer and 5′-AATGCGGATCCCTACTGGCCCAACAAGATGA-3′as a reverse primer were used for

PCR amplification of core-E1-E2 genes.

Forward primer included Kozak consensus sequence to enhance mammalian expression and *Hind*III restriction sites. Reverse primer had a stop codon and *Bam*HI restriction sites. Italic, bold and underlined letters indicate Kozak sequence, stop codon and restriction sites, respectively. PCR was performed for 40 cycles as follows: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min and was ended with the final extension of 10 min at 72 °C. PCR mixture consisted of 100 ng DNA, 5 pmol of each primer, 0.2 mM dNTPs, 1 unit *Taq* DNA polymerse (CinaGen, Iran), 1.5 mM $MgCl_2$, 2.5 µl of 10x PCR buffer, 17.3 µl double distilled water in total volume of 25 µl. PCR product was visualized on 1% agarose gel by Green viewer staining (ParsTous, Iran) and UV transilluminator.

**Cloning of core-E1-E2 genes into eukaryotic expression vector**

Amplified genes along with pcDNA 3.1(+) vector underwent double digestion with a similar pair of restriction enzymes *Bam*HI and *Hind*III (Fermentas, Germany). In separate sterile tubes 100 ng of core-E1-E2 PCR product and 100 ng of pcDNA3.1(+) were added into mixture containing 5 µl of 10x buffer, 2 IU *Hind*III enzyme, 2 U *Bam*HI enzyme and 16 µl double distilled water (DDW) were mixed gently. The reaction tube was incubated at 37 °C for 24 h. Digested products of core-E1-E2 gene and pCDNA 3.1(+) was loaded on 1% agarose gel. Specific bands were excided and extracted from gel by using DNA extraction kit in accordance with the manufacture's recommendations (Bioneer, South Korea). Core-E1-E2 fragment and pCDNA 3.1(+) gene were ligated at 16 °C overnight under the reaction mixture as follows: 2 IU T4 DNA ligase, 1.5 µl 10x buffer, 25 ng digested pCDNA 3.1(+) , 75 ng digested core-E1-E2 PCR product, 1 µl PEG. *E. coli* strain JM109 was transformed by the ligation product using heat shock procedure as descried previously (16). Transformed *E. coli* with chimeric pCDNA3.1(+) vector was able to grow in presence of ampicilin 100 mM/ml antibiotic in a selection procedure. Double digestion and sequencing were performed to confirm accurate cloning.

**Cell culture and transfection**

Huh7.5 cell line was used for the expression of structural HCV antigens. Huh7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS), 100U of penicillin per ml, 100µg of streptomycin per ml. The cells were grown at 37 ºC in a 5% $CO_2$ atmosphere. In order to transfect, first $10^3$ cells of Huh7.5 were seeded into 6 well tissue culture microplate and incubated at 37 °C, 5% $CO_2$ until cell confluency reached 85-90 . After that, they were transfected by the calcium phosphate method (18); briefly, 5-20 µg of the vector containing core-E1-E2 genes was sized 225 µl with DDW (19). Twenty five µl of $CaCl_2$ (2.5M) was added to the mixture until the final volume reached 250 µl. Finally, 250 µ1 of HBS buffer (NaCl 140 mM, $Na_2HPO_4$ 1.5 mM, HEPES 50 mM, pH=7) was added (16, 17). The solution was kept for 20-30 min at room temperature. The prepared mixture was added to Huh7.5 cell culture with 90% confluency. Four to six hours after transfection, cell supernatant medium was replaced with fresh medium. Seventy two hours after transfection, Huh7.5 cells were harvested for RNA isolation and RT-PCR assay procedures.

**RNA extraction**

RNA extraction procedure was performed on transfected Huh7.5 cells, using RNA X-plus solution (CinnaGen, Iran) according to the manufacturer's instructions. To remove

genomic DNA, extracted RNA was treated with DNase I (Fermentas, Germany) enzyme. Three µg of RNA was added to 5 units of DNaseI enzyme and 10x buffer in a total volume of 10 µl. The mixture was then incubated at 37 °C for 30 min. For inactivation of DNase I enzyme, the mixture was incubated at 65 °C for 10 min.

**cDNA synthesis and RT-PCR**

In order to prepare cDNA, 5 µg of total RNA, 1 µl oligo-dT primer and 3 µl DEPC-treated water were mixed together and the mixture was incubated at 65 °C for 5 min and was chilled on ice and the reagents were added as follows: 5x RT buffer, 2 u of RNase inhibitor, 1 mM dNTPs, 2u Thermo-resistance RT enzyme (ParsTous, Iran). Synthesis procedure was performed by Applied Biosystems thermo cycler using pre-set program (25 °C, 10 min; 47 °C, 60 min and 70 °C, 10 min). Synthetic cDNA was further used in the PCR method. PCR mixture contained 1 ng recombinant plasmid, 5 pmol Forward and reverse primers (specific for core region and partially of E1) each of them 1 µl, 0.5 µl of 0.2 mM dNTPs, 0.2 µl of *Taq* DNA polymerase (CinaGen, Iran), 1.5 µl of 1.5 mM $Mgcl_2$, 2.5 µl of 10 x buffer, 17.3 µl DDW in a total volume of 25 µl.

## Results

Primers were designed based upon published sequence of *JFH1* in Genbank and were used to amplify the fragment of the genes corresponding to 2241bp core-E1-E1 fragment (Figure1). The accuracy of the constructed plasmid was confirmed by restriction enzyme digestion (Figure 2) and sequencing of the insert. Sequencing data was analyzed with DNAMAN (Lynnon Biosoft version 5.2) and BLAST (www.blast.ncbi.nlm.nih.gov) softwares and no inconsistency was observed. Multiplicity and transcription of chimeric plasmid was confirmed in vitro by using RT-PCR (Figure 3). Using calcium phosphate method, core-E1-E2 antigens were successfully expressed in Huh7.5 cell line.

## Discussion

During the last decade, multiple vaccines

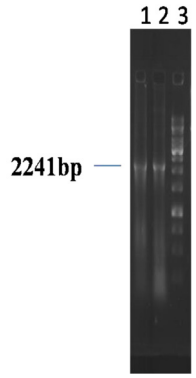


**Fig. 1**
Agarose gel electrophoresis of core-E1-E2 PCR product. Lane 1, 2: a 2241 bp PCR product; Lane 3: 1kb DNA size marker (Fermentas, Germany)

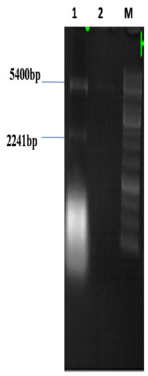


**Fig. 2**
Double digestion of recombinant vector by *Bam*HI and *Hind*III restriction enzymes that lead to excision of core-E1-E2 fusion gene. Lane 1: digested vector and core-E1-E2 fusion fragment; Lane 2: recombinant vector linearized by *Bam*HI; Lane M: 1kb DNA size marker (Fermentas, Germany)

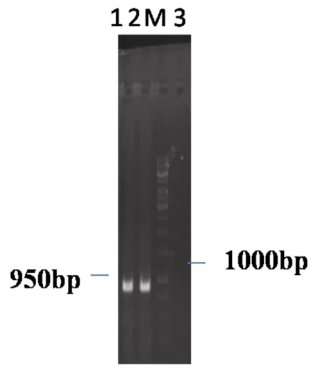


**Fig. 3**
Detection of core-E1-E2 mRNA in transfected and non-transfected Huh-7.5 cells by RT-PCR analysis. RT-PCR analysis using specific primers designated for N-terminal region of fragment showed negative results in non- transfected cells (lane3) and a band with a size of approximately 950bp in transfected cells with recombinant vector (lanes 1,2). Lane M: 1kb DNA size marker

against HCV have been evaluated on animal models but only some of them reached the clinical trial stages. Lack of an appropriate animal model and an efficient cell culture system in the laboratory are the major obstacles in vaccine development against HCV. Humans and chimpanzee are the only species susceptible to HCV infection. However, not to mention ethical issues, studies performed using them are very costly. As a result, these obstacles have prevented the development of an effective vaccine against HCV (20-22).

Approximately 20% of people with acute infection will spontaneously clear the infection. It seems that multiple components of the immune system, both innate and adaptive, play a key role in this process. Host immune system is naturally able to combat HCV virus and designing an effective vaccine that induces and motivates similar responses is a realistic goal (23-26). Production of more specific and mount of strong neutralizing antibodies responses are the hallmark in viral controlling at early stage of the HCV acute infection and can enhance viral clearance by cellular immune responses. Early antibody responses mainly target structural antigens (core, E2, E1). Thus, development of an effective vaccine containing these antigens could be of usage. In recent studies, core, E1 and E2 antigens have been regarded as an attractive candidate for vaccine development. These envelope proteins are variable across HCV genotypes. There are conserved neutralizing epitopes in E1 and E2 (27, 28).

Among different proteins derived from HCV, core protein is the most conserved antigen across various HCV genotypes and trigger immune responses to a broad range of virus variants (29, 30). Core protein has main role in protecting of HCV genomic RNA and recent researches highlighted modulatory aspects of it. Core antigen of HCV has potent immunomodulatory functions. Core antigen binds to gC1qR on T cells which leads to deregulation and suppression of CD8+ T cells so anti-core antibody can nullify its negative effects (31). HCV core protein has played experimentally a role in nucleocapsid formation modulating host-cell gene transcription, cell proliferation, cell death and cell signaling, lipid metabolism, and host immune responses. The core protein is highly conserved between various genotypes of HCV and is a target for designing DNA vaccine. However, mice immunized with the HCV core DNA vaccine alone showed no significant antibody responses (32).

E1 and E2 are viral envelope glycoproteins embedded in the lipid envelope and serves as a target for neutralizing antibodies. These antigens are variable among HCV genotypes and are able to induce the production of neutralizing antibodies. Neutralizing antibodies to one HCV strain typically are not effective to other strains but new studies identified conserved neutralizing epitopes in E1 and E2 (33, 34). E2 is a key protein in invading the liver cells because E2 binds to the CD81 receptor on the liver cells (35, 36). Binding site of E2 is conserved among different strains of HCV. These findings can pave the way for designing new HCV vaccines which will enhance the production of neutralizing antibodies effective against a wide range of viral strains. In order to enhance the humoral immune responses against this nonsecreted viral protein some cytokine genes such as GM-CSF, IL-2, or IL-4 genes was co-administered along with the HCV core DNA (37, 38). The first DNA vaccine was introduced in 2001. DNA vaccine is a plasmid containing gene of interest that could replicate in the host cells and results in protein expression *in-vivo* and subsequently induce and trigger host immune responses. Compared with conventional vaccines, DNA vaccine have some advantages including easy preparation, motivation wide range of immune responses such as CTL, T helper cell, and antibody responses, stability and no need for cold chain etc. (39, 40). CIGB-230A, a DNA vaccine expressing HCV structural antigens (core/E1/E2) is the first DNA-

based vaccine against HCV that reached to Phase I clinical trial. Patients with HCV genotype-1infection that failed PEG-IFN/ribavirin therapy received this vaccine. Nearly 73% of vaccinated subjects exhibited specific T cell proliferative and IFN-gamma secretory response (41). In a Phase I study effectiveness of recombinant E1/E2 adjuvanted with MF59C adjuvant was evaluated. In all healthy vaccinated subjects development of anti-envelope antibodies and T-cell lymphocyte proliferation responses to E1/E2 was seen (42). In a study, 20 healthy and 34 chronically infected individual received recombinant HCV-E1 protein in alum adjuvant. In both groups HCV-specific antibody and T-cell responses was detected. Despite the lack of change in the amount of HCV RNA levels improvements in liver histology and reduction of liver inflammation were seen (38, 43, 44). In previous studies recombinant E1/E2 antigens derived from genotype 1a, were administered to guinea pigs and chimpanzees. In vaccinated animals induction protective immune responses and reduction rate of chronicity were observed. These findings indicate that E1/E2 proteins are protective antigens. In phase I clinical trial safety and immunogenicity of this vaccine in healthy volunteers was examined and production of neutralizing antibodies against all seven genotype were seen but cross neutralization activity of this vaccine was especially against genotypes 1a and 2a and efficiencies of it differed among all genotypes. Direct injection of DNA opened new way to introduce various antigens to immune system. This discovery has been successfully used to generate protective immunity against some life-threating disease such as influenza virus, rabies virus, bovine herpes virus 1.DNA vaccine is able to stimulate CTL responses and antibody production (45).

The DNA vaccines expressing structural antigens of HCV provide a basis for future experimental studies. Currently structural antigens of some virus such as HPV, HBV and HCV have been used in making virus like particles (VLPs) that is a new strategy in vaccine. DNA vaccine and other strategies in the field of vaccine development need more research that is underway (46, 47).

In the present study, core -E1-E2 genes were isolated from *JFH1* strain of genotype 2a. This plasmid has an eukaryotic cytomegalovirus (CMV) promoter that efficiently regulate the expression of interested genes. Protective and therapeutic properties of this DNA-based vaccine remain to be determined in further studies.

## Conclusions

HCV disease is a serious public health problem. Despite many efforts, a reliable vaccine against it has not yet been developed.Based on the result of this study; this construction can be used to further develop a DNA vaccine against HCV infection. Immunological responses can be measured in animal model in future study.

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## Conflict of interest

None of the authors have any conflict of interest to disclose.

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