Cloning and Expression of a Secretory form of Truncated ORF2 (112 - 607 aa) from Hepatitis E Virus in the pVAX1 Vector

Behnam Azizolahi,1,2,3 Manoochehr Makvandi,1,2 and Reza Taherkhani3,4
1Health Research Institute, Infectious and Tropical Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran
2Department of Medical Virology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran
3Department of Microbiology and Parasitology, School of Medicine, Bushehr University of Medical Sciences, Bushehr, IR Iran
4Persian Gulf Tropical Medicine Research Center, Bushehr University of Medical Sciences, Bushehr, IR Iran

Abstract

Background: The hepatitis E virus (HEV) accounts for the hepatitis E infection with a high mortality rate in pregnant women. Therefore, the design of the novel and effective vaccines seems essential and the DNA vaccine approach could be useful to achieve this anticipated goal.

Objectives: The present study aimed at cloning the lone tPAsp-PADRE-truncated ORF2 (112 - 607 aa) from HEV into the eukaryotic expression pVAX1 vector and evaluating the expression of this recombinant protein in eukaryotic cells.

Methods: The truncated ORF2 gene (112 - 607 aa) was cloned in the pVAX plasmid, followed by digestion, and confirmed by digestion and sequencing. Then, the recombinant plasmid was transfected into eukaryotic cells to confirm expression. The expressed protein in the cell lysate and supernatant was evaluated by immunofluorescence assay (IFA) and Western blot assay.

Results: The cloning of the tPAsp-PADRE-truncated ORF2 gene (112 - 607 aa) to the pVAX eukaryotic expression path was set by colony PCR, restriction enzymes digestion, and DNA sequencing of the recombinant plasmid. The appearance of the truncated ORF2 protein in the eukaryotic cells was accepted by the Western blot assay, reverse transcriptase polymerase chain reaction (RT-PCR) method, and the immunofluorescence assay (IFA).

Conclusions: All outcomes of the present research showed that pVAX-tPAsp-PADRE-Truncated ORF2 (112 - 607 aa) recombinant plasmid was able to express truncated ORF2 from HEV as a potential candidate vaccine.

Keywords: HEV, pVAX1, Truncated ORF2, Vaccine

1. Background

The Hepatitis E virus (HEV) belongs to a species known as Orthohepevirus A, which is a part of the Orthohepevirus genus as well as the Hepeviridae family (1). HEV is transmitted through the fecal-oral path and accounts for clinical hepatitis, but with self-limiting infection. Clinical manifestations of HEV infection vary from moderate to severe hepatitis among all age groups (2). High mortality rate of 25% to 31% have been reported among pregnant women (3) and 30% to 70% in patients who have chronic liver disease (4, 5). In 2016, the world health organization (WHO) reported that approximately 20 million individuals are infected with HEV, of whom approximately 3.3 million have a severe form of HEV, and 56 600 annual mortality worldwide (6).

HEV has been classified into 5 genotypes although all the genotypes share 1 serotype. Genotypes 1 and 2 are for human infections, Genotypes 3 and 4 are zoonotic infections, while Genotype 5 is restricted to avian infection (7). According to the recent data, several Asian and African countries including Iran are classified in the high HEV endemicity (8). To date, no exclusive treatment has been available for severe hepatitis E, or a protective licensed vaccine against the HEV infection (9). Thus, based on the aforementioned data, an efficient vaccine against HEV infection should be developed because it is needed to prevent the infection. To date, no tissue culture system has existed for the cultivation of HEV. Therefore, molecular approaches are only alternative tools for the vaccine preparation (10).

HEV genome comprises 3 overlapping open reading frames (ORF) (11). ORF1 gene is responsible for enzymatic function and encodes the nonstructural proteins. ORF2 gene involves the synthesis of a viral capsid protein (72 kDa) (5, 8), and ORF3 gene encodes a small multifunctional protein (13.5 kDa), which may have a regulatory function (12). The capsid protein with 660 amino acids (aa) is highly immunodominant and a good candidate for the HEV vaccine development (6, 8). Antibodies against the capsid protein exhibit HEV protection and neutralization in vitro (8, 9). It has been shown that the ORF2 protein could be utilized in serological test kits to diagnose and determine
the seroepidemiological surveillance of the HEV infection (13). However, it was found that the full-length capsid protein expression is insoluble and less immunologically reactive. The construction of a truncated ORF2 protein (112 to 660 aa), which can be self-assembled into virus-like particles (VLPs), showed that it is able to induce systemic and mucosal immune responses in experimental animals (14). Therefore, most of the attempts have been made to use the truncated ORF2 proteins for the development of HEV vaccines (6). Thus, different truncated forms of the ORF2 protein (112 - 607 aa, 458 - 607 aa, 112 - 660 aa, and 368 - 606 aa) have been constructed and used in numerous DNA vaccines (15). Since the recombinant vaccines including DNA vaccines have been developed, several factors were studied to advance the vaccine potency such as codon optimization of the gene, coadministration of different adjuvants as well as using plasmids, encrypting a concealed form of the protein. The occurrence of the codon usage is diverse amongst the numerous organisms. Thus, codon optimization can steer towards an extreme expression of the target gene to mimic the host cell genes. Although mRNA secondary structures are considered as having negative effects on the translation efficiency, they can be removed by codon optimization (16).

Antigen processing and presentation in cells is an important key to activate both humoral and cell-mediated immunity (17). Moreover, due to the fact that vaccines for DNA can be constructed in precise cell compartments for antigenic processing, vaccination with recombinant DNA can usually activate both arms of the immune system. On the other hand, the existence of a secretary target gene to the signal sequence of human tissue plasminogen activator (tPA) are found highly unsusceptible for both the T as well as B cells than the plasmids without a concealed form (18). PADRE epitope, which is also known as a universal Pan HLA-DR (allelic DR) epitope peptide, is capable to bind to the various human major histocompatibility complex (MHC) class II molecules DR with high-affinity and is applicable in humans (19). The PADRE epitope showed a good potency to enhance Cytotoxic T lymphocyte (PADRE epitope), 22-amino-acid-sequence (MDAMKRGLCCVLLLCGAVFVSP) of the secretory peptide of human tissue-type plasminogen activator protein, (tPA signal peptide), and a complete kozak sequence, GCCGCCACCAGGG for increasing the efficiency of translation initiation. To retrieve tPAsp-PADRE-truncated ORF2 (112 - 607 aa) gene fragment, 2 restriction enzyme digestion sites for Scal were located at the position of 1609 and 1858 at the end of the cassette gene. Arrangement of fragment junctions is presented in Figure 1A.

2. Methods

2.1. Bacterial Hosts, Eukaryotic Cells, Plasmid and Culture Conditions

Escherichia coli strain DH5α (Novagen Inc., Madison, Wis., USA) is utilized for change, and the pVAX1 plasmid (Invitrogen®, Carls-bad, USA) for the replication of tPAsp-PADRE-truncated ORF2 (112 - 660aa). Purchased from the national Iranian cell bank (Pasteur Institute), HEK293 and CHO cell lines were used for protein expression. The Qiagen Company (Germany) prepared all needed kits. The New England Biolabs (New England Biolabs Inc., USA) is where the T4 DNA ligase and restriction endonucleases were all purchased. The chemicals reagents were obtained from Sigma-Aldrich Corporation (Germany) as well as Merck (Germany).

2.2. Construction of ORF2 (112 - 607 aa) from Designed, Optimized pVAX-tPAsp-PADRE-truncated ORF2 (112 - 660 aa) Cassette of Hepatitis E Virus

Based on the primary construction, the 112 - 660 amino acid arrangement of the ORF2 gene of the hepatitis E virus Genotype 1 (Ser55 strain) was developed from the records of the UniProtKB/Swiss-Prot (Pakistan/Ser-55 strain, accession number P33426), which was designed from the Vector NTI software (Vector NTI software version Advance® 11.5, Invitrogen), optimized using GenScript software, and inserted into the pVAX1 plasmid as mentioned in the previous study (21). The recombinant pVAX1 plasmid contains following sequences at the N-terminal of the ORF2 gene (112-660 aa). These sequences are 13-amino acid sequence (AKFVAAWTLKAAA) of universal epitope of T CD4+ lymphocyte (PADRE epitope), 22-amino-acid-sequence (MDAMKRGLCCVLLLCGAVFVSP) of the secretory peptide of human tissue-type plasminogen activator protein, (tPA signal peptide), and a complete kozak sequence, GCCGCCACCAGGG for increasing the efficiency of translation initiation. To retrieve tPAsp-PADRE-truncated ORF2 (112 - 607 aa) gene fragment, 2 restriction enzyme digestion sites for Scal were located at the position of 1609 and 1858 at the end of the cassette gene. Arrangement of fragment junctions is presented in Figure 1A.

2.3. Subcloning of the Codon-Optimized tPAsp-PADRE-Truncated ORF2 Gene Cassette in pVAX1 Eukaryotic Expression Vector

Bacteria DH5α containing the pVAX1 eukaryotic expression vector with tPAsp-PADRE-truncated ORF2 (112 - 660 aa) linker in the previous study (21) was grown overnight in

Luria-Bertani broth containing kanamycin at 37°C. According to the kit's manufacturer's instructions, the pVAX1 plasmid was extracted using QIAquick® Gel Extraction Kit (Qiagen, Germany). The purified plasmid was digested using Scal restriction enzyme at 37°C for 4 hours. The digested plasmid was analyzed on 1% agarose gel, and the tPasp-PADRE-truncated ORF2 (112-607 aa) gene and the linearized pVAX1 plasmid were separated from 1% agarose gel using the QIAquick® Gel Extraction Kit (Qiagen, Germany), and were then ligated by the T4 DNA ligase (New England Biolabs Inc., USA) at 15°C overnight. The recombinant plasmid pVAX-tPasp-PADRE-truncated ORF2 (112-607 aa)-linker was assembled (Figure 1B) and converted into Escherichia coli DH5α proficient cells by 100 mM CaCl2, and chosen on Luria-Bertani agar containing kanamycin (22). Multiple colonies were analyzed by the colony PCR, and recombinant plasmid with the accurate limitation patterns were chosen by restriction digestion with Nhel and Xhol enzymes (New England BioLabs, USA) and then used for DNA sequencing with T7 forward and bovine growth hormone (BGH) reverse primers (Bioneer, Korea).

The gene fragment encoding tPasp-PADRE-truncated ORF2 (112-607 aa) with linker sequence (Figure 1B) was PCR amplified using the Q5 High-Fidelity DNA Polymerase (New England BioLabs, USA), the order of forward primer, which when including a Kozak consensus sequence was 5’-CCAAGCTGGCTAGCTGGAGCCGCCACCATGGATGCA-3’, and the order of the reverse primer was 5’-CCCGCTGAGTATCACGTACGTGAGCGCCACCATGGATGCA-3’, and then the order of the reverse primer was 5’. The cloning of the tPasp-PADRE-truncated ORF2 (112-607 aa) gene into the pVAX1 expression vector was confirmed by enzyme digestions, colony PCR by universal primers (T7, BGH) and sequencing. The verified construct was called pVAX-tPasp-PADRE-truncated ORF2 (112-607 aa) (Figure 1C).

2.4. Expression of the Recombinant pVAX-tPasp-PADRE-Truncated ORF2 (112-607 aa) in Mammalian Cells

According to the manufacturer's instructions, the recombinant plasmid pVAX-tPasp-PADRE-truncated ORF2 (112-607 aa) was extracted by the Plasmid Purification Midi Kit (Qiagen, Germany). To express the recombinant protein in the eukaryotic cells, CHO and HEK293 cells were transfected with the recombinant plasmid pVAX-tPasp-PADRE-truncated ORF2 (112-607 aa) by PolyFect® Transfection Reagent (Qiagen, Germany) in 6 well culture plates, according to the manufacturer’s guidelines (Nunc, Denmark). The cells were cultured in Opti-MEM medium (Gibco-Fisher Scientific, USA) without serum and antibiotics at 37°C and 5% CO2 to allow gene expression. To verify mRNA expression of truncated ORF2 gene (112-607 aa), RT-PCR was performed after 48 hours transfection. In short, the overall cellular RNA was removed via the RNaseasy Mini Kit (Qiagen, Germany), and RT-PCR was then executed by a QIA-GEN One Step RT-PCR Kit (Qiagen, Germany) according to the manufacturer’s protocol by specific primers (upstream primer: F29 (724) 5'-ATGAAAAGGGTTCTTGGC-3' and downstream primer: R329 (1053) 5'-ATGCCCATGACCGTCGCCAGC-3'). Then, the RT-PCR product was electrophoresed on an agarose gel of 2%. 

2.5. Confirmation of the Recombinant Protein Expression in Eukaryotic Cells

Indirect immunofluorescence assay (IFA) and Western blotting were used to evaluate the expression of the recombinant truncated ORF2 (112-607 aa) protein in the transfected CHO and HEK293 cells. Momentarily, the transfected cells were incubated with cold methanol-acetone (1:1) for 20 minutes and then washed and blocked with 2 mL of 0.15 M phosphate buffered saline (PBS), which contained 1% bovine serum albumin (BSA). The cells were incubated with anti-HEV ORF2 antigen antibody (abcam, USA) and then diluted 1:500 in PBS for 60 minutes at room temperature. The cells were washed once again and then tarnished with 10 µg/mL FITC-labeled anti-mouse IgG (Biolegend, USA). Then, 1 ng/mL of 4’, 6-diamidine-2’-phenylindole dihydrochloride (DAPI) (Roche, Germany) was used for tarnishing the nuclei of the cells. After washing with PBS, the cover slips were instantaneously seen under a fluorescence microscope (Nikon, USA).

The presence of the recombinant truncated ORF2 (112-607 aa) protein inside the eukaryotic cells as well as in the supernatants (the secreted form) was also analyzed by Western blotting. Briefly, both of the supernatants and cell lysates of the transfected HEK293 and CHO cells were collected separately and were run on SDS-PAGE using 12% (w/v) polyacrylamide gel according to the method described previously (23). Following electrophoresis, the resolved proteins were blotted onto a 0.45 µm pore polyvinylidene fluoride (PVDF) membrane (Roche, Germany) using a semi-dry transfer cell (Bio-Rad, USA) at 15V for 1 hour.

After blocking the membrane with 2% (w/v) skimmed milk at 4°C overnight, the main antibody, rabbit polyclonal antibody against HEV ORF2 antigen (biorbyt, UK), diluted 1:100 in PBS, was then added and incubated at room temperature overnight. The secondary antibody, goat anti- rabbit IgG (heavy and light chains) conjugated with horseradish peroxidase (Nordic-MUBio, The Netherlands) diluted 1:5000 in PBS, was then added and incubated at 37°C for 2 hours. Washing with tris-buffered saline...
(TBS) (50 Mm tris pH = 7.5, 150 mM NaCl) having 0.1% between 20 was done in each interval 3 times. Finally, the response was identified using 3, 3’-Diaminobenzidine (DAB) reagents (Sigma-Alderich, USA).

3. Results

3.1. Construction of tPAsp-PADRE-truncated ORF2 (112 - 607 aa) Gene Fragment

To construct tPAsp-PADRE-truncated ORF2 (112 - 607 aa) gene fragment with linker sequence (Figure 1B), digestion with ScaI restriction enzyme was performed on pVAX-tPAsp-PADRE-truncated ORF2 (112 - 660 aa) cassette (Figure 1A), which had been designed and optimized in the previous study (21). This cassette fragment was 1948 bp in length and its length reduced to 1699 bp after digestion with ScaI (Figure 2). To remove the linker at the downstream of the cassette gene, PCR amplification was performed on tPAsp-PADRE-truncated ORF2 (112 - 607 aa) gene fragment with linker sequence and PCR product with 1624 bp in length; this fragment was named pVAX-tPAsp-PADRE-truncated ORF2 (112 - 607 aa) (Figure 1C). Figure 2 demonstrates agarose gel electrophoresis of pVAX-tPAsp-PADRE-truncated ORF2 (112 - 660 aa) digested with ScaI restriction enzyme.

3.2. Subcloning Results of Optimized tPAsp-PADRE-Truncated ORF2 (112 - 607 aa) Gene Cassette in the pVAX1 Eukaryotic Plasmid

Subcloning of pVAX 1 containing tPAsp-PADRE-truncated ORF2 (112 - 607 aa) gene cassette was approved by colony PCR and restriction enzyme digestion with NheI and XhoI enzymes (Figure 3). DNA sequencing with T7p as well as BGH primers confirmed the truncated ORF2.

3.3. Expression and Confirmation of the Recombinant tPAsp-PADRE-Truncated ORF2 (112-607 aa) in Mammalian Cells

The expression of the recombinant tPAsp-PADRE-truncated ORF2 (112 - 607 aa) was confirmed in eukaryotic cells, HEK293, and CHO cells by RT-PCR assay. The agarose gel electrophoresis analysis of RT-PCR product of truncated ORF2 mRNA in transfected CHO cells is exhibited in Figure 4. The PCR product was sized to be 329 bp. The expression of tPAsp-PADRE-truncated ORF2 (112 - 607 aa) protein in the both transfected HEK293 and CHO cells was confirmed by IFA using anti-HEV polyclonal antibody (Figure 5). Western blot analysis detected the expression of tPAsp-PADRE-truncated ORF2 (112 - 607 aa) protein in both the supernatant and lysates collected from the transfected HEK293 cells (Figure 6).

4. Discussion

Although most HEV infections are asymptomatic, some can cause acute hepatitis with several clinical manifestations including glomerulonephritis, thrombocytopenia, and neurological disorder (24). In addition, HEV infection can be severe in pregnant women, causing mortality and morbidity rates, especially in developing countries, leading to fulminant hepatic failure and superinfection. Chronic HEV infections may also occur in immunocompromised patients (25).

Due to the lack of proper treatments for HEV infections, the existence of active immunization remains only an alternative for the prevention of HEV infections. Several attempts have been made to develop HEV vaccine. As there is no efficient tissue culture system for cultivation of HEV, the recombinant DNA technology is the only approach to develop HEV vaccine (26). To achieve this, DNA vaccines have
been discovered to have several advantages including cost-effective, stability, and higher immunogenicity of a target antigen (27).

Numerous reports revealed that the effectiveness of immune responses have been stimulated by DNA vaccines (28). DNA-based vaccine was found to stimulate the CD4+ T helper cell immune responses, following the activated CD4+ T helper cells outcome in the production of CD8+ T cell immune responses as well as the memory T cell responses (29).

Some studies have found that the HEV ORF2-induced antibody in animals as well as humans is long-lived, can neutralize HEV in vitro, and is also cross-reactive among diverse HEV genotypes. Due to its effective immunogenicity, the HEV ORF2 protein has been used as an antigen for all vaccine research studies until now (30). In DNA vaccine studies, it has been revealed that the codon optimization could enhance the expression of recombinant proteins (31). The present study reveals that the codon optimization for expressing the recombinant truncated ORF2 gene is effective in eukaryotic cells, and this is consistent with the previous report.

Several studies demonstrate that the expression of different target genes can be highly and efficiently achieved by pVAX vector. Using pVAXi to design and construct the target gene has also several advantages such as stability and cost-effectiveness, especially for developing countries (32). To enhance the protein yield, adding Kozak sequence to the upstream of the initiation codon can increase the protein expression level. As a result, in the present study, the Kozak sequence was adjoined to the N-terminal sequence of the truncated ORF2 (112 - 607 aa) gene exact before the start codon of the gene. The study conducted by Farshadpour et al. showed the proper expression of truncated ORF2 (112 - 607 aa) in HEK293 and CHO cells using the Kozak sequence (21). The presence of the signal sequence of tPA in a target gene leads to constructing a secretary form of the protein, which makes it better to present to both T and B cells (18). In other words, the secretion of a protein will boost the antigen presentation via MHC class II, which results in enhancing the activation of antigen-specific CD4+ T-cells. Thus, in the present study the secretary tissue plasminogen activator (tPA) sequence was used in DNA vaccine construction. In addition, it was found that the existence of PADRE sequence improves the presentation of a target peptide via MHC class II for CTL responses.
Figure 5. Expression Analysis of HEV Truncated ORF2 Protein (112 - 607 aa) in HEK293 and CHO Cells by Immunofluorescence Assay (IFA)

Figure 6. Expression Analysis of HEV Truncated ORF2 Protein (112 - 607 aa) in Transfected HEK293 Cells by Western Blotting

Lane M, pre-stained protein ladder; Lane 1, HEK293 cells transfected with pVAX (as negative control); Lane 2, detection of truncated ORF2 protein (112 - 607 aa) in the supernatant of HEK293 cells transfected with pVAX-tPAsp-PADRE-truncated ORF2 (112 - 607 aa) recombinant plasmid; Lane 3, detection of truncated ORF2 protein (112 - 607 aa) in cell lysate of HEK293 cells transfected with pVAX-tPAsp-PADRE-truncated ORF2 (112 - 607 aa) recombinant plasmid. A protein band about 56 kDa corresponding to tPAsp-PADRE-truncated ORF2 (112 - 607 aa) recombinant protein is detected.

Some studies revealed that PADRE peptide can fasten the alternates of human MHC class II molecules DR as well as provoke helper-facilitated resistance in humans and enhance the potency of vaccines in the preclinical models (33, 34). Therefore, in the present study, the sequence of tPA and PADRE were, respectively, added to the N-terminal sequence of truncated ORF2 (112 - 607 aa) gene after the Kozak sequence.

In conclusion, this study revealed that the encoded truncated form of HEV capsid protein (ORF2) (112 - 607 aa) was efficaciously communicated in eukaryotic cells. However, the immunogenicity as well as potency of pVAX containing truncated ORF2 (112 - 607 aa) requires assessing in vivo immunologically and cell mediated immune response.

Acknowledgments

The writers would like to recognize the financial help of the Infectious and tropical diseases research center of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (grant No. 93132).

Footnotes

Authors’ Contribution: Behnam Azizolahi, Reza Taherkhani and Manoochehr Makvandi developed and contributed in the performance of the study. Behnam Azizolahi and Reza Taherkhani contributed in the laboratory evaluation as well as data analysis. Reza Taherkhani designed the original idea. Manoochehr Makvandi acquired the accountability for the precision of the data and was
also the guarantor. The final manuscript has been read and approved by all authors.

Financial Disclosure: No conflict of interest related to the subject matter or materials discussed in this article was declared by the authors.

Funding/Support: Financial support was provided by the Infectious and tropical diseases research center of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran (grant No. 93132).

References

