Original Article

Cloning and expression of hepatitis E virus ORF2 as an immunogen protein in baculovirus expression system

Sadeghi SA[1], Shahanaghi M[2], Aghasadeghi MR[2], Motevalli F[2], Amiran MR[2], Mohammadi Pargoo S[2], Hamidi-Fard M[2*]

[1]Department of Research and Development of Hepatitis A vaccine, Pasteur Institute of Iran, Alborz, Iran.
[2]Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran.

ABSTRACT

Introduction: Hepatitis E virus (HEV) is a non-enveloped, single-stranded positive-sense RNA virus. It is one of the most important causes of liver failures and the mortality rate arising from HEV is more common in pregnant women. HEV is an enterically-transmitted virus and its outbreak is more common in the developing and poor-hygiene countries while vaccination against it can prevent its prevalence. The ORF2 is an immunogenic capsid protein of HEV with 660 amino acids that is being used in vaccine designs against HEV infection. ORF2 has been studied in a vast range of vectors and hosts, such as pRSET-C, pMAL and pSG vectors, as well as *Escherichia coli* BL21 and vaccinia virus hosts. A DNA vaccine expressing ORF2 has also been studied which has induced specific humoral and cellular immune responses in mice. This study was aimed to clone and express ORF2 as an immunogen protein in a eukaryotic host system. **Methods:** *orf2* gene corresponding to 660 amino acids of ORF2 protein was subcloned from a pET21avector into pFastBac. The protein expression was achieved by transforming Sf9 insect cells with a pFastBac-*orf2* construct. The over-expressed protein with ~72 kDa MW was assessed by SDS-PAGE. **Results:** The cloning was confirmed by PCR and restriction digestions. The expression of ORF2 with expected MW in Sf9 cells was confirmed by SDS-PAGE. **Conclusion:** ORF2 protein of HEV was successfully expressed in a baculovirus-based eukaryotic expression system as the first step for further studies on HEV vaccine designs, based on ORF2 protein.

KEYWORDS: Baculovirus, Hepatitis E virus, ORF2, Vaccine.

INTRODUCTION

HEV is a non-enveloped virus which belongs to *Hepeviridae* family and Orthohepevirus genus. It contains a polyadenylated, single-stranded, positive-sense RNA with 7.2 kbp length which encodes three overlapping Open Reading Frames (ORFs). HEV is responsible for a water-borne infection which causes liver inflammation [1]. Hepatitis that is caused by HEV can emerge either as an infection without signs or as a fulminant and fatal infection [2]. Capsid protein of HEV binds to the cell receptors and trigger pathogenesis [3]. HEV is the most common cause of acute hepatitis in adult persons in Asia and is the second cause of acute hepatitis in the Middle East after Hepatitis B Virus [4]. Diagnosis of HEV is performed by detection of Alanine Amino Transferase (ALT) as well as anti-HEV IgM and IgG in a patient's serum [5]. Real-time PCR is a sensitive assay for detection of HEV RNA in stool and blood samples [6]. HEV single-stranded RNA encompasses *orf1*, *orf2* and *orf3*. The biggest ORF in HEV genome is *orf1* which encodes non-structural proteins while *orf2* is responsible for encoding a structural protein with 660 amino acids in length and *orf3* encodes an ion channel [7, 8]. ORF2 is an immunogen protein which has been a target for vaccine design approaches [9].

***Corresponding Author:** Mojtaba Hamidi-Fard, Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran.
Email: mojtaba_hamidifard@yahoo.com
Tel/Fax: (+98) 2166969291

Acute hepatitis E in people with normal immune system is a self-limiting infection and usually doesn't need anti-viral therapy [10]. In patients with suppressed immune system, antiviral agents (such as Ribavirin) are needed. In China, p239 HEV vaccine (Hecolin®) based on ORF2 has been approved and is used to make protection in infection-prone persons, older than 16 while it has no approval license from the US Food and Drug Administration (FDA) and is not prescribed in countries other than China [11]. ORF2 expression was studied in a vast range of vectors and hosts, such as pRSET-C, pMAL and pSG as vectors and *Escherichia coli* BL21 and vaccine virus as hosts [12-15]. A recombinant Baculovirus has been designed to express orf2 which has been transduced to S10-3 human hepatoma cells and approximately 50% of the cells produced ORF2 protein with a MW of ~72kDa [16]. Baculovirus is a double-stranded DNA virus that infects insect cells and is also used for the protein expression. Because of its post-translational modification feature, it is an appropriate expression system for eukaryotic and viral proteins expressions [17]. SF9 is a cell line derived from *Spodoptera frugipedra's* larva cell and is common as the host for Baculovirus in researches [18]. BHK-21 cells are also used as the host for Semliki Forest Virus (SFV) replicon to produce ORF2 [19]. Insoluble ORF2 has been expressed on the cell surface of constructed recombinant *Lactococcus lactis* (*L.*

[DOI: 10.29252/vacres.5.1.23]

lactis) strain NZ3900 that can be used as an oral vaccine for protection against HEV [20].
We used pET26b expression vector containing PelB sequence in our previous research to express ORF2 with a His-tag at the c-terminal [15]. PelB is responsible for secretion of the protein to the periplasmic space [21]. In this study, we used *Baulovirtus* expression system in SF9 cell line to express ORF2 protein to be used in future HEV vaccine studies.

MATERIALS and METHODS

Construction of vector containing *orf2*

Nucleotide sequence of HEV *orf2* was retrieved from GenBank (accession number AF444002.1). The *orf2* sequence was cloned into pET21a vector (containing a c-terminal His-tag) by Gene Transfer Pioneers Company. In order to confirm *orf2* cloning into pET21a vector, the construct was assessed by *Eco*RI and *Not*I restriction enzymes digestion. To sub-clone the gene of interest into pFastBac, both pFastBac vector and pET21a vector containing orf2 were double-digested by *Eco*RI and *Not*I restriction enzymes. The digested orf2 insert was ligated into the double-digested pFastBac expression vector (Fig. 1). PCR was conducted by forward and reverse primers (Forward: 5'-ATGATATCCATATGATCGCGCTGACCCTG and Reverse: 5'- TGTTAGCAGCCGGATCTCAGTGGTGG) to confirm the sub-cloning process.

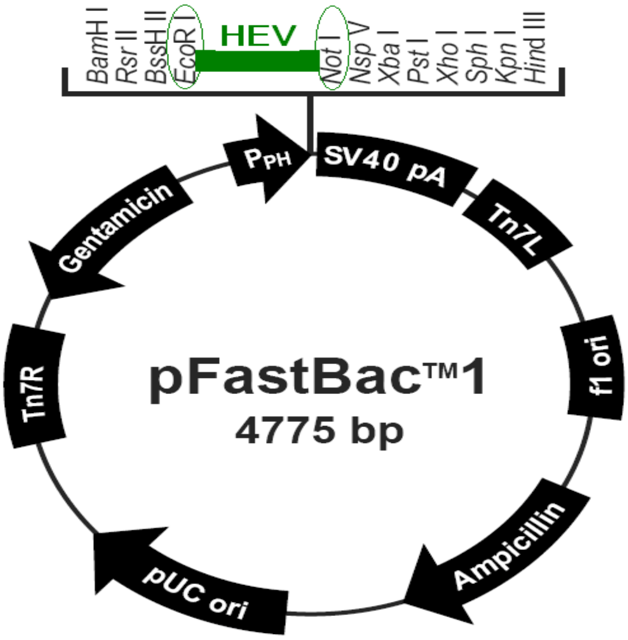

Fig.1. pFastBac containing *orf2* sequence.

Heat shock transformation of pFastBac-orf2

The cloning process was followed by transforming pFastBac-*orf2* into competent *E. coli* DH5α by adding 100 µl of the vector to 200 µl of competent *E.coli* DH5α and chilling on ice for 30 min. The microtube containing the vector and the bacteria was incubated at 42°C for 90 seconds and immediately transferred on ice for 5 min. LB media (1 ml) containing antibiotic (100 µg/ml ampicillin) was added to the prepared bacteria and incubated at 37°C for 60 min. The media containing transformed bacteria was centrifuged and the pellet was cultured on LB agar containing the antibiotic.

Colony selection

In order to select colonies containing pFastBac-*orf2*, plasmid extraction was performed (Yekta Tajhiz Azama plasmid extraction kit, Iran). The extracted plasmids were then digested by *Eco*RI restriction enzyme and run on agarose gel. A colon containing pFastBac-*orf2* was selected and cultured.

Sf9 cell line preparation

Sf9 cell line was obtained from National cell Bank of Iran and cultured in Hink's TNM-FH insect Medium (Sigma-Aldrich chemie GmbH, Germany).

Electroporation

One microgram of the extracted pFastBac-*orf2* vector was added to the washed Sf9 pellet (~2.5×10^6 cells) and applied for electroporation procedure. Electroporation was performed by 750 V/cm and pulses with intervals of 2.8 ms.

Evaluation of expressed protein by SDS-PAGE

In order to evaluate the protein expression, SDS-PAGE was conducted on 12.5% resolving gel.

RESULTS

Gene cloning confirmation

The precise presence of *orf2* sequence in pET21a vector was confirmed by double-digestion using *Not*I and *Eco*RI restriction endonucleases. These enzymes were applied to pFastBac vector (4775 bp length) as well (data not shown). After, *orf2* was ligated with pFastBac. PCR was performed to select the right colon that contains pFastBac-orf2 vector. The PCR product was loaded on 1% agarose gel and a band with 1973 bp length demonstrated *orf2* sequence (data not shown). Following that, plasmid was extracted and then digested with *Eco*RI and electrophoresed on 1% agarose gel in which the band with the length of ~6700 bp confirmed pFastBac containing *orf2* (Fig. 2).

Fig.2. Agarose gel electrophoresis of extracted pFastBac-*orf2*. Lane1: digested vector. Lane 2: supercoiled plasmid. M: DNA ladder.

Following ligation of *orf2* insert into pFastBac vector, the Sf9 cells were transfected with plasmid by electroporation method, performed by 750 V/cm and intervals of 2.8 ms using a (Bio-Rad, USA) electroporator (Fig. 3).

[DOI: 10.29252/vacres.5.1.23]

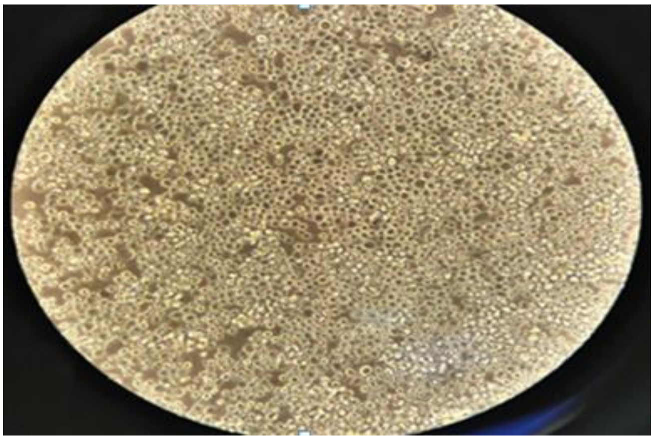

Fig.3. Electroporated Sf9 cells.

Protein expression assesment

In order to confirm and assess ORF2 expression, the transfected Sf9 cells were incubated at 27°C overnight and then were heated in boiling water for 5 min and applied to SDS-PAGE. An overexpressed band with MW of ~72kDa, confirmed the accurate expression of ORF2 protein (Fig. 4).

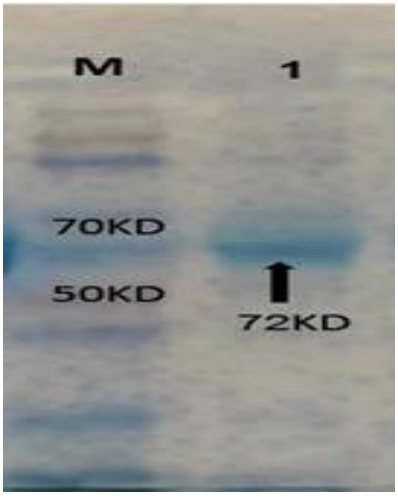

Fig.3. SDS-PAGE of the over-expressed protein. A band with MW of approximately 72 kDa demonstrates the recombinant ORF2. M: Protein marker. Lane 1: over-expressed ORF2 protein.

DISCUSSION

HEV is one of the most prevalent causes of sporadic hepatitis across the world. It is an acute self-limiting viral hepatitis with fecal-oral transmit in route which its genome was characterized in 1991 [1, 22].People in the developing countries and poor regions of the world as well as patients with organ transplantations or hepatic failures background are at high risk for viral hepatitis caused by HEV[1]. Among the 3 ORFs of HEV, ORF2 is a capsid protein that is essential for binding of HEV to the host cells and leads to the immune system stimulation [23]. The antibody against HEV capsid protein leads to viral neutralisation *in vitro* and makes protection in primates against HEV. Hence, ORF2 has been considered as a vaccine candidate for viral hepatitis caused by HEV[24]. Recombinant ORF2 proteins have been expressed in a wide range of hosts such as vaccinia virus[23], tobacco [25] and Huh-7 cells [26].

Hecolin® is a HEV vaccine that has been produced and approved for use in China in 2012 in which the HEV 239 protein is used as an immunogen. HEV 239 protein is the ORF2 protein selected from HEV genotype 1 that has been expressed in *E.coli* BL21in the form of inclusion bodies [27]. Although bacterial expression systems are commonly used in researches due to their relatively simpler gene manipulatin, low cost protein expression procedures and high quantity of the expressed proteins, they lack post-translational modification on the expressed protein. Therefore, attempt were made to express ORF2 by eukaryotic systems [21]. For instance, transgenic tomato has been designed to express ORF2 in its fruits and leaves. Such expressed ORF2 had the potential to stimulate the immune system while it could be used as a promising oral vaccine [28].

In the present study, we expressed ORF2 in a eukaryotic system instead of *E.coli*. We used eukaryotic Sf9 cells and baculovirus expression system to express domestic ORF2 protein of HEV genotype1 in which ORF2 was expressed, appropriatelly. The pathological indications in Sf9 cells indicated a high titer of infectious virus and high yield of the electroporation. In comparison with the expression in prokaryotic systems, the expression of ORF2 in baculovirus-based expression system has the advantage of post-translational modification which is closer to its native structure and with a reasonable efficiency that can potentially be developed as an immunogen for future HEV vaccine researches.

ACKNOWLEDGEMENT

The authors would like to acknowledge Department of Hepatitis and AIDS Pasteur Institute of Iran.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Purcell R, Emerson S. Hepatitis E: an emerging awareness of an old disease. Journal of hepatology. 2008;48(3):494-503.
2. Betts RF, Penn RL, Chapman SW. Reese and Betts'a practical approach to infectious diseases. Lippincott Williams & Wilkins; 2003.
3. Surjit M, Jameel S, Lal SK. Cytoplasmic localization of the ORF2 protein of hepatitis E virus is dependent on its ability to undergo retrotranslocation from the endoplasmic reticulum. Journal of virology. 2007;81(7):3339-45.
4. Anderson D. hepatitis E virus: in: Gerald L, Mandell JE, Bennet RD, editors. Mandell, Douglas, Bennet's principles and practice of infectious disease Volume2, 7th ed Philadelphia: Natasha andjelkovic. 2010:2411-20.
5. Bendall R, Ellis V, Ijaz S, Ali R, Dalton H. A comparison of two commercially available anti- HEV IgG kits and a re- evaluation of anti-HEV IgG seroprevalence data in developed countries. Journal of medical virology. 2010;82(5):799-805.
6. Jothikumar N, Cromeans TL, Robertson BH, Meng X, Hill VR. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. Journal of virological methods. 2006;131(1):65-71.
7. Ding Q, Heller B, Capuccino JMV, Song B, Nimgaonkar I, Hrebikova G et al. Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles. Proceedings of the National Academy of Sciences. 2017;114(5):1147. doi:10.1073/pnas.1614955114.
8. Chandra V, Taneja S, Kalia M, Jameel S. Molecular biology and pathogenesis of hepatitis E virus. Journal of biosciences. 2008;33(4):451-64.

9. Schofield DJ, Purcell RH, Nguyen HT, Emerson SU. Monoclonal antibodies that neutralize HEV recognize an antigenic site at the carboxyterminus of an ORF2 protein vaccine. Vaccine. 2003;22(2):257-67.
10. Khuroo M, Kamili S, Khuroo M. Clinical course and duration of viremia in vertically transmitted hepatitis E virus (HEV) infection in babies born to HEV- infected mothers. Journal of viral hepatitis. 2009;16(7):519-23.
11. Proffitt A. First HEV vaccine approved. Nature Publishing Group; 2012.300 (30) 12. Haqshenas G, Huang F, Fenaux M, Guenette D, Pierson F, Larsen C et al. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. Journal of General Virology. 2002;83(9):2201-9.
13. Ropp S, Tam A, Beames B, Purdy M, Frey T. Expression of the hepatitis E virus ORF1. Archives of virology. 2000;145(7):1321-37.
14. Sehgal D, Malik PS, Jameel S. Purification and diagnostic utility of a recombinant hepatitis E virus capsid protein expressed in insect larvae. Protein expression and purification. 2003;27(1):27-34.
15. Sadeghi S, Aghasadeghi M, Amiran M, Bahramali G, Rahimi P, Owlia P et al. Cloning and expression of hepatitis E virus ORF2 as a vaccine candidate. Vaccine Research. 2017;4(3):81-4. doi:10.29252/vacres.4.3.4.81.
16. Parvez MK, Purcell RH, Emerson SU. Hepatitis E virus ORF2 protein over-expressed by baculovirus in hepatoma cells, efficiently encapsidates and transmits the viral RNA to naïve cells. Virology journal. 2011;8(1):159.
17. Kost TA, Condreay JP. Recombinant baculoviruses as expression vectors for insect and mammalian cells. Current opinion in biotechnology. 1999;10(5):428-33.
18. Jarvis DL, Kawar ZS, Hollister JR. Engineering N-glycosylation pathways in the baculovirus-insect cell system. Current opinion in biotechnology. 1998;9(5):528-33.
19. Torresi J, Meanger J, Lambert P, Li F, Locarnini SA, Anderson DA. High level expression of the capsid protein of hepatitis E virus in diverse eukaryotic cells using the Semliki Forest virus replicon. Journal of virological methods. 1997;69(1-2):81-91.
20. Gao S, Li D, Liu Y, Zha E, Zhou T, Yue X. Oral immunization with recombinant hepatitis E virus antigen displayed on the Lactococcus lactis surface enhances ORF2-specific mucosal and systemic immune responses in mice. International immunopharmacology. 2015;24(1):140-5.
21. Nakamura M, Saeki K, Takahashi Y. Hyperproduction of recombinant ferredoxins in Escherichia coli by coexpression of the ORF1-ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster. The Journal of Biochemistry. 1999;126(1):10-8.
22. Aggarwal R. Hepatitis E: epidemiology and natural history. Journal of clinical and experimental hepatology. 2013;3(2):125-33.
23. de Oya NJ, Escribano-Romero E, Blázquez A-B, Lorenzo M, Martín-Acebes MA, Blasco R et al. CHARACTERIZATION OF HEPATITIS E VIRUS (HEV) RECOMBINANT ORF2 PROTEINS EXPRESSED BY VACCINIA VIRUSES. Journal of virology. 2012:JVI. 00610-12.
24. Zhou Y-H, Purcell RH, Emerson SU. A truncated ORF2 protein contains the most immunogenic site on ORF2: antibody responses to non-vaccine sequences following challenge of vaccinated and non-vaccinated macaques with hepatitis E virus. Vaccine. 2005;23(24):3157-65.
25. Zhou Y-X, Lee MY-T, Ng JM-H, Chye M-L, Yip W-K, Zee S-Y et al. A truncated hepatitis E virus ORF2 protein expressed in tobacco plastids is immunogenic in mice. World Journal of Gastroenterology: WJG. 2006;12(2):306.
26. Graff J, Torian U, Nguyen H, Emerson SU. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. Journal of virology. 2006;80(12):5919-26.
27. Wu T, Li S-W, Zhang J, Ng M-H, Xia N-S, Zhao Q. Hepatitis E vaccine development: a 14 year odyssey. Human vaccines & immunotherapeutics. 2012;8(6):823-7.
28. Ma Y, Lin S-Q, Gao Y, Li M, Luo W-X, Zhang J et al. Expression of ORF2 partial gene of hepatitis E virus in tomatoes and immunoactivity of expression products. World journal of gastroenterology. 2003;9(10):2211.

[DOI: 10.29252/vacres.5.1.23]