Original Article

Evaluation of full length E1 and E2 glycoproteins of HCV expressed in *P. pastoris* as a protein-based vaccine candidate

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ABSTRACT

Introduction: The development of an effective vaccine against Hepatitis C virus (HCV) is still a target of intensive vaccine research. The HCV envelope proteins E1 and E2 which can induce broadly neutralizing antibodies are the major candidate for this purpose. Different types of expression systems have been used to express these glycoproteins. In this study, an expression system using *Pichia pastoris* was used to express E1 and E2 in full length. **Methods:** E1 and E2 regions containing the restriction sites from HCV 1b were separately amplified and cloned into a pPICZAa vector. The km71h strain of *P. pastoris* was transfected with the confirmed vectors separately using electroporation. The recombinant E1 and E2 proteins were evaluated for their antigenicity in an ELISA test and the induction of humoral immunity in mice. **Results:** The expression of full length HCV glycoproteins E1 and E2 in *P. pastoris* strain km71h was successfully achieved and their specific antibody was detected in serum samples from HCV infected patients. Furthermore, the recombinant glycoproteins could elicit a significant humoral immunity in mice as a vaccine candidate. **Conclusion:** *P. pastoris* is one of the best eukaryotic expression systems for the production of HCV glycoproteins in full length and the expressed proteins could be used in diagnostic tests such as ELISA. The induction of humoral immune responses in mice should lead to further studies on these glycoproteins for designing an effective vaccine.

KEYWORDS: HCV E1 and E2, *Pichia pastoris*, Yeast expression system, Protein-based vaccine.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped virus in *Flaviviridae* family with a positive-stranded RNA genome of approximately 9.5kbp which encodes a single 3010 to 3033 amino acid polyprotein. This poly-protein is processed by cellular and viral proteases to produce the mature structural and nonstructural proteins, namely C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [1,2]. Among them, E1 and E2 are two structural proteins with several glycosylation sites which are enveloped membrane anchor glycoproteins with an ability to interact non-covalently with each other to form heterodimers [2-5]. The hydrophobic carboxyl-terminal (C-terminal) region of these proteins act as membrane anchors. Therefore, the deletion of this region is necessary for their secretion, especially when they are expressed in *Escherichia coli* [2-7].

The eukaryotic systems are known as the best expression systems, especially when a high yield production and post-translational modifications such as glycosylation, methylation and acylation are needed. Amongst them, the yeast systems have several advantages over other expression systems [8,9]. *Pichia pastoris* is considered as one of the better eukaryotic expression systems due to its ability for protein processing and folding, posttranslational modifications and its ease of manipulation, similar to *E. coli* or *Saccharomyces cerevisiae* [8-11]. Since it has been proposed that an antibody response against the envelope proteins is important for neutralization and clearance of the virus [15, 17], we tried to express these viral glycoproteins in a host which is able to perform post-transcriptional modifications on the recombinant proteins with a high yield production.

There are many studies on expression of viral proteins

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including different HCV proteins in yeast expression system such as *S. cerevisiae* and *P. Pastoris* [8, 10-17]. Herein, the HCV E1 and E2 glycoproteins from HCV genotype 1b were expressed in full length in *P. pastoris* for the first time. Moreover, we investigated the antigenicity of these proteins by detecting its specific antibody in HCV-infected patients and the induction of humoral response in the immunized mice.

MATERIALS and METHODS

PCR and cloning

HCV E1 and E2 (from base pairs 1490-2608 in genotype 1b (NCBI accession numbers KU879443 and DQ682381, respectively) were commercially synthesized by Biomatik (Canada) and were used as a PCR template along with primers designed by CLC workbench and Gene Runner software, containing *Eco*R1 and Nde1 for E1 primer pairs as well as *Eco*RI and *Xba*I restriction sites for E2 primer pairs, as shown below:

E1+*NdeI* Forward: 5'- **CATATG**GCTGCCCAGGTGAAGA -3' and **E1+*Eco*RI reverse:** 5'- **GAATTC**TTAGGTGCCCGCGTCCA -3'.

E2+*Eco*RI Forward: 5'- **GAA TTC** GGC ACC ACCACC GT-3' and **E2+*Xba*I reverse:** 5'- **TCT AGA** GAC CAA CTT CTC CAA TGC-3'.

To amplify the full length E1 and E2 separately, PCR was carried out in a 50µl reaction mixture consisting of 10x pfu amplification buffer (5µL; Thermo Scientific, Fermentas, Lithuania), 1µL of 10mM dNTPs (Thermo Scientific, Fermentas, Lithuania), 2µL of each forward and reverse primers (10pmol/µL), 2µL of each template DNA (13ng/µL), 1µL of 2.5u/µL Pfu DNA polymerase (Thermo Scientific, Fermentas, Lithuania) and 37µL double-distilled water. The thermo cycling program at first denaturation was 5min at 94°C, then 94°C for 45s, followed by 35 cycles of 95°C for 5s, 60°C for 45s, 72°C for 90s with a final extension at 72°C for 5min.

Another 25µL PCR was performed to prepare each previously mentioned PCR product for TA-cloning with the same reagents. The reaction mixture contained 12µL of each first PCR product, 1µL of Taq DNA polymerase, 0.5µL of dNTP, 2.5µL of 10x Taq amplification and 9µL double-distilled water. The thermo cycling profile was the same as the first PCR. Both PCR products underwent electrophoresis on a 1.5% agarose gel containing 0.5µg/ml ethidium bromide (CinaClone, Iran). The amplified products were ligated into a T-PTG19 vector using 10µL of the insert DNA (92ng/5µl) and 10µL of the T-vector DNA (PCR Cloning Vector PTG 19, 25ng/µl, Vivantechnologies) and 1µL of T4 DNA ligase (Lithuania-Thermo Scientific- Fermentas,) and 2µL T4 ligase buffer (Lithuania - Thermo Scientific- Fermentas, 10X). The reaction mixture was incubated at 4°C for 16h. Five µL of this ligation reaction was used to transform *E. coli* DH5a competent cells using the heat shock and CaCl2 method. The transformed cells were grown on low salt LB agar plates containing (50µg/ml ampicillin) and the plates were incubated at 37°C for overnight. T-vector DNA construct was isolated using QIAprep spin Miniprep Kit (Qiagen, Germany) and verified by PCR using (Master Mix Red- Taq 2x,1.5mM MgCl2, Ampliqon, Denmark) as well as restriction enzyme digestion. For E1 gene, 26µL of 65ng/5µl isolated DNA, 2µL of each *Nde*I and *Eco*RI restriction enzymes, 4µL of 10X orange buffer were incubated for 24h at 37°C. For E2 gene, in a 25µL reaction mixture, 20µL of 60ng/5µl of isolated DNA, 2.5µL of *Xba*I restriction enzyme and 2.5µL of Tango buffer (Thermo Scientific, Fermentas, Lithuania) were incubated for 16h at 37°C. For E2 gene second restriction enzyme reaction, 1µL of *Eco*RI and 2µL of each 10X Fast buffer and green buffer (Thermo Scientific- Fermentas , Lithuania) were mixed and incubated at 37°C for 1h. Samples were analyzed on 1% agaros gel. The bands of E1 and E2 genes were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

E. coli strain DH5a containing pPICZAa vector was grown on LB broth containing 25µg/ml zeocinTM (Invitrogen, USA) antibiotic in 37°C for 16h in shaking incubator (300rpm). pPICZAa vectors were isolated by using QIAprep spin MAXI prep Kit (Qiagen, Hilden, Germany) and verified by enzyme digestion reaction containing 1µl of each *Nde*I/*Eco*RI and *Eco*RI/*Xba*I for 30min at 37°C. The ligation reaction in upstream of the 6×histidine tag was performed in a reaction mixture containing 10µL of digested PCR product of E1 and E2 genes separately as described previously (with concentration of 65ng/5µl and 60ng/5µLf or E1 and E2 genes ,respectively), 5µL of pPICZAa vector with concentration of 90ng/5µl, 1µL of T4 DNA ligase (Thermo Scientific, Fermentas, Lithuania ,1 weiss u/ml, 200u) and 2µL of 10X ligase buffer (Thermo Scientific, Fermentas, Lithuania).The two mixtures were incubated in 4°C for 16h. Ten µL of each ligation reaction was used to transform *E. coli* DH5a competent cells by the heat shock method. The selection was done by plating the cells on LB agar containing (25µg/ml Zeocin: 100mg/ml), incubation at 37°C for 16-18h overnight and at dark. pPICZAa vectors were isolated from several colonies using QIAprep spin Miniprep Kit and were verified by enzyme digestion reaction (*Nde*I/*Eco*RI and *Xba*I/*Eco*RI for E1 and E2 genes, respectively) as well as PCR and sequencing using Master Mix Red- Taq 2x, 1.5mM MgCl2(Ampliqon, Denmark) AOX1 universal primers (Bio-Neer, South Korea). pPICZAa-E1 and pPICZAa-E2 constructs were linearized using the restriction enzyme *Sac*I (5000u, 40u/µL, Roche, Germany) for 16h at 37°C and were isolated by a gel extraction kit (YTA Plasmid DNA Extraction Mini Kit, Iran). The density of the gel-extracted products for E1 and E2 were 37.1µg and 35.2µg, respectively.

Transformation of competent *P. pastoris* strains

Control flow test: *P. pastoris* strain km71h strain was selected on minimal dextrose (MD) medium plate and confirmed on minimal methanol (MDH) medium plate and yeast extract peptone dextrose (YPD) medium plate containing 100µg/ml zeocin antibiotic. Moreover, the strain was grown separately on media plates without the antibiotic. All plates were incubated for 72h at 28-30°C in dark. The confirmation of km71h strain was done through not growing in a medium containing zeocin and growing in a non-zeocin medium. Then the strain was re-grown in YPD broth and was incubated at 28-30°C in a shaking incubator (300rpm) to reach OD 1.3-1.5 at 600nm. Then 40ml of 5X sorbitol (36.4g of sorbitol was dissolved in 20ml double-distilled water and filtered) was added to the medium and incubated in 20-30°C in shaking incubator with the speed range of 100-110rpm to prepare the competent km71h cells. pPICZAa- E1 and pPICZAa-E2, were linearized and km71h were transformed by electroporation method using Mini Gene-Pulser apparatus (Bio Rad, USA, Gen Pulser X Cell; 2000w, 25µf, 200Ω, 5ms). The transformed cells were selected on YPD, yeast extract peptone dextrose medium plate with different concentrations of 50, 100, 1000 and 1500µg/ml zeocin for 72h at 28-30°C in darkness. Both transformed strains were

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grown at 28-30°C for 2 days in 20ml of YPD broth containing 100µg/ml zeocin.

Genomic DNA Extraction of *P. pastoris*

Genomic DNA of the transformed cells were extracted using a mixture containing acid-washed glass beads with the size of 300 and 700 micron (0.2g of each size; SIGMA Glass Beads 710-1, 180 microns ,USA) with 200µL of phenol-chloroform prepared on ice and 100µL of isoamyl - alcohol incubated at 4°C in darkness. The micro tubes were centrifuged in 2000rpm for 5min. Then the supernatants were discarded and 250µL of TEN buffer (at first 0.12g of Tris–HCl 10mM, 0.04g of EDTA 1mM, 0.58g of NaCl 0.1M, 2ml of 2% Triton X-100 and 1g of 1% Sodium Lauryl Sulfate were dissolved in 50ml water to a final volume of 100ml with pH 8) were added to the pellet to resolve it. The mixture was then processed according to the phenol-chloroform DNA extraction protocol. Finally, to verify the recombinant yeast DNA, colony PCR was performed using Master Mix Red- Taq 2x, forward and reverse specific primers for E1 and E2 genes separately. Another colony PCR was also done using the AOX1 and AOX2 primers. PCR amplicons were analyzed by electrophoresis on 1% agarose gel. Nucleotide sequencing was also done for further confirmation.

E1 and E2 Expression, purification and verification of recombinant protein

One colony of each E1 and E2 gene transformed into the yeast was grown in 5ml YPD broth without antibiotic in 28-30°C for 1 day. Then it was centrifuged in 2000rpm for 5min and the pellet was re-suspended by adding 20ml of buffer complex medium containing glycerol (BMGY). Cells were re-grown in 28-30°C to reach to the optical density of 2-10 in 600nm. Two ml of un-induced sample was collected and the remaining 18ml was centrifuged. The pellet was resolved in 20ml yeast nitrogen base amino acids medium (YNB) containing 1% methanol. Cells were grown in 28-30°C in shaking incubator for 5 days. Samples were collected every 24h. Finally, Trichloroacetic acid (TCA) 100% was used to purify the secreted proteins. The C-terminal 6×His-tagged of each E1 and E2 recombinant protein was bound to Ni–NTA agarose beads and proteins were purified under native conditions with imidazole separately, according to the manufacturer's instructions (QIAGEN, Life Technologies, Germany) and subsequently, the eluted fractions were subjected to 12% polyacrylamide (v/v) SDS-PAGE gel electrophoresis. The purity and quantity of each protein was verified with Coomassie blue staining as compared to a known protein. Western blotting was carried out for each protein separately through electro-transfer of proteins from polyacrylamide gel to nitrocellulose membrane (Millipore, USA) at 20V for 1h. The membrane was treated with a PBS blocking buffer containing 2% non-fat dried milk (Sigma-Aldrich, USA) for 2h with shaking. After removing the blocking buffer, the membrane was incubated with Anti-His(C-term) antibody (diluted: 1/2000; Abcam Ms Mab and the secondary antibody (M-IgG-HRP diluted 1/10000; Abcam, Ms Mab) for 1h. After three rounds of washing, the bands corresponding to the appropriate proteins became visualized by adding 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic substrate (Roche Diagnostics GmbH, Germany). Then E1 and E2 were eluted with 20m MTris–HCl (pH 9.3) and further purified with Sephadex G150 column for removing low molecular weight fractions. Then they were dialyzed against 5mM Tris–HCl (pH 9.3) and sterilized with 0.45µm filter, and then stored at 4°C.

Concentration of the dialyzed proteins was measured by using the Bradford method [16].

Evaluation of recombinant E1 and rE2 specific antibody in human serum using ELISA

Plasma samples from 60 HCV chronic infected patients and from 20 healthy volunteers were used to evaluate the rE1 and rE2 specific antibodies. 100µl of 1µg ml-1 of each purified E1 and E2 in carbonate coating buffer (0.15M sodium carbonate, 0.435M sodium bicarbonate, 0.03M sodium azide, pH 9.6) was coated on the 96-well microplates separately. Microplates were stored at 4°C overnight. Washing solution containing PBS/0.5% Tween 20 was used to wash the plates for 6 times. Then, the plates were blocked with 100ml PBS/0.05% Tween 20/4% BSA per well at 37°C for 2h. The plates were then washed six times with washing solution. Each plasma samples was serially diluted twofold in PBS/0.05% Tween 20/2% BSA starting from 1:100 to 1:400. Hundred µl of each dilution was added in duplicate to each well of microplates and incubated at 37°C for 1h. Microplates were washed using the washing solution. Then, 100ml 1:2000-diluted horseradish peroxidase-conjugated (HRP) anti-human IgG isotyping monoclonal antibody (Sigma) was added per well and incubated at 37°C for 1h. Plates were washed with washing solution then, 100ml TMB substrate was added per well. Plates were incubated at room temperature in darkness for 10min. Hundred µl of 2M sulfuric acid was added to each well to stop the reaction. The absorbance of each well was read at 450nm. The mean absorbance value of those 20 healthy volunteers plus 2SD was used to determine the cut-off. A serum sample was considered positive when the absorbance was equal or above the cut-off.

Mice immunization

The female BALB/c mice aged 6-8 weeks were divided into 3 groups of six for each E1 and E2 separately. Each group was immunized 3 times subcutaneously at multiple sites in tail base at weeks 0, 3 and 6 with 20µg of each purified E1 or E2 alone diluted in sterilized PBS, E1 or E2 separately in combination with Freund's adjuvant (Sigma, USA) in 50µl of PBS, for the first immunization and incomplete adjuvant in 100µL of PBS was used as booster, (Sigma, USA).In each group of E1 and E2, 6 mice were injected with PBS and Freund's adjuvant alone as negative control. Mouse blood samples were collected from retro-orbital bleeding at weeks 0, 5, and 8 and the sera were stored at -70°C for further processing.

Evaluation of anti-E1 and anti-E2 antibodies in immunized mice

Indirect ELISA was used to evaluate the anti-E1 and anti-E2 antibodies in immunized mice. Briefly, 5µg of each purified E1 and E2 protein was coated in ELISA plates overnight at 4°C. For evaluation of the total IgG and IgG isotyping serum dilution of 1:500 and 1:1000 of each mouse were prepared respectively. The diluted serums were added in triplicate to each well and incubated for 1h at 37°C, washed and further incubated with HRP-conjugated goat anti-mouse IgG (dilution1:10000) as secondary antibody. After adding the TMB, color development was measured at 450nm. IgG isotyping was done as described above using goat anti-mouse IgG1, IgG2a,and IgG2b antibodies in dilution of 1:2000 at room temperature (Sigma, USA) and HRP-labeled anti-goat IgG conjugate (dilution 1:10000 at room temperature).

Statistical analysis

Distribution of data was normalized by logarithmic transformation using GraphPad Prism 6. Statistical analyses for differences in antibody titer against HCV E1 and E2 protein between groups were then carried out using one-way ANOVA

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and Bonferroni's multiple comparisons tests. The results were considered statistically significant when P-values were equal to or less than 0.05.

RESULTS

The E1 and E2 regions were amplified separately by PCR using their specific primers. The PCR products were analyzed under gel electrophoresis and the bands with sizes of 576bp for E1 and 1100-bp for E2 were observed along with a band of 4176-bp for an undigested pPICZAa-E1 and a 4500-bp band for an undigested pPICZAa-E2 as well as a 3600-bp band for a semi-digested pPICZAa fragment using *Xba*I restriction enzyme. The pPICZAa-E1 and pPICZAa-E2 constructs were verified by restriction digestion reactions using *NdeI*/*EcoR*I and *EcoR*I/*Xba*I pairs, respectively. The results are depicted in Fig.1.

The recombinant E1 and E2 proteins were purified using Ni-NTA agarose and were verified using SDS-PAGE and Western blotting analyses. The recombinant proteins were then dialyzed overnight through a membrane with a cut-off of below 50kDa, against PBS 0.9% in room temperature and their concentrations were assayed by Bradford method which was estimated to be 6 mg/L and 4 mg/L for E1 and E2, respectively.

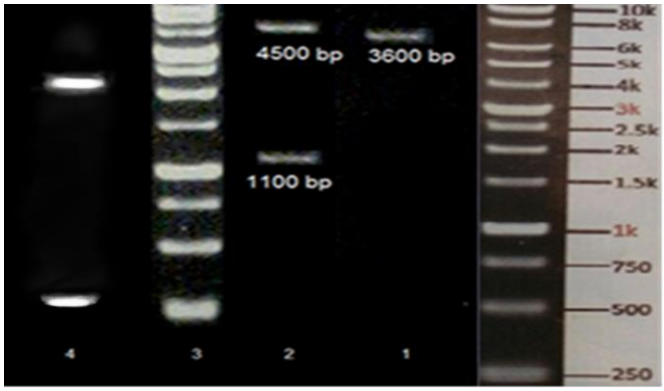

Fig. 1. Digestion reactions of pPICZAa-E1 and pPICZAa-E2 using *Nde*I/*EcoR*I and *EcoR*I/*Xba*I restriction enzymes, respectively.
Lane 1: a semi-digested non-recombinant pPICZAa (3600bp), Lane 2: upper band (4500bp); undigested pPICZAa-E2, lower band (1100bp); digested pPICZAa-E2 by using the *EcoR*I and *Xba*I. Lane 3: DNA ladder (1kb). Lane 4: upper band (4176bp); undigested pPICZAa-E1, lower band (576bp): digested pPICZAa- E1 by using *Nde*I/*EcoR*I restriction enzymes.
P. pastoris strain km71h was transfected by the linearized pPICZAa-E1 and pPICZAa-E2 separately. Different concentrations of zeocin were used to evaluate the resistance of the colonies of km71h strain and those resistant colonies in concentration of 100µg/ml of zeocin selected for the expression.

Detection of E1 and E2 specific antibody in patients with chronic HCV infection

The evaluations of specific anti-E1 and anti-E2 antibodies were done using an in-house ELISA test. The mean absorbance values of 20 healthy volunteers using 2SD calculations were used to determine the cut-off. A serum sample was considered positive when the absorbance was equal or above the cut-off which was calculated by the following formula:
Cut-off = Mean + 2sd + 0.05
= 0.1 + 0.14 + 0.05
Cut-off = 0.29

The identification of recombinant HCV E1 and E2 expression in cell lysates of *P. pastoris* km71h strain using HRP-conjugated anti-His polyclonal antibody. On the blotted membrane, 2 bands for E1 (Lane 1) and E2 (Lane 3) with molecular weights of approximately 25kDa and 85kDa were detected, respectively. Lane 2: Pre-stained protein molecular weight marker (20-120kDa).

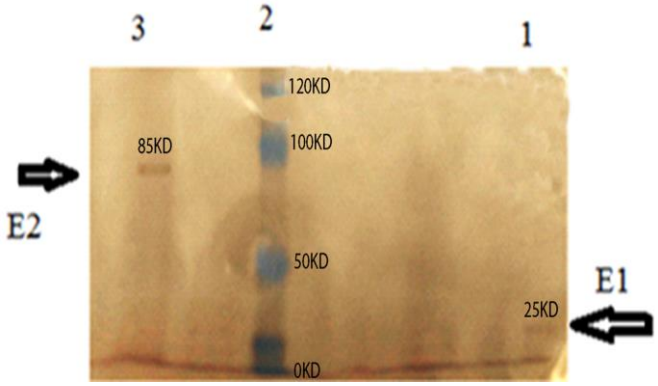

Fig. 2. Western blotting of recombinant HCV E1 and E2 glycoproteins expressed in *P. pastoris*.
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Cut-off = Mean + 2sd + 0.05
= 0.1 + 0.14 + 0.05
Cut-off = 0.29

There was a significant difference in anti-E1 and anti-E2 antibodies titers in the serum samples with dilution of 1:400 between the healthy volunteers and HCV chronic infected patients .The high antibody titer against both E1 and E2 was detected in patients with HCV chronic infection with the above cut-off value.

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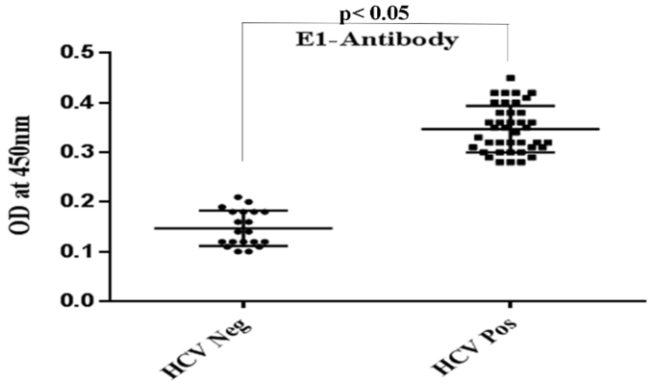

Fig. 3. ELISA assay for detection of anti -E1 specific antibody in 20 healthy volunteers and 60 HCV chronic infected patients using purified recombinant E1 as the antigen.

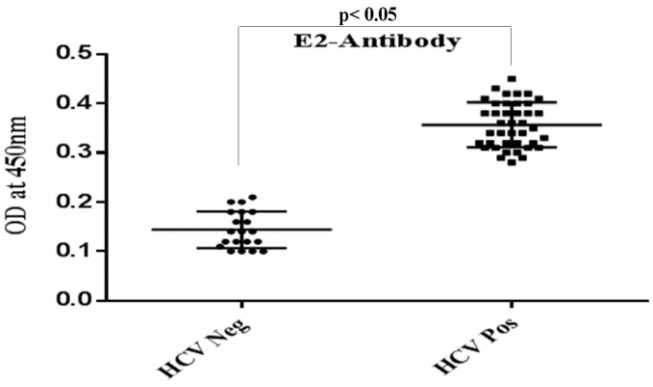

Fig. 4. ELISA assay for detection of anti-E2 in 20 healthy volunteers and 60 HCV chronic infected patients using purified recombinant E2 as the antigen.

Evaluation of specific anti-E1 and anti-E2 total IgG and its subclasses in immunized mice

The antibody response in mice that were immunized separately with either E1 plus complete Freund's adjuvant and E2 plus complete Freund's adjuvant were significantly higher in comparison with mice that were immunized with each of E1 or E2 recombinant proteins alone. Also, the antibody response showed a significant raise in IgG titer after 8 weeks of immunization compared to the 5th week and before the immunization, respectively. IgG1 was the predominant isotype of detected IgG. Other identified IgG subclasses were IgG2a and IgG2b. However, the difference between the titer of E1-IgG1 and E2-IgG1 with the other subclasses of E1-IgG and E2-IgG was not statistically significant.

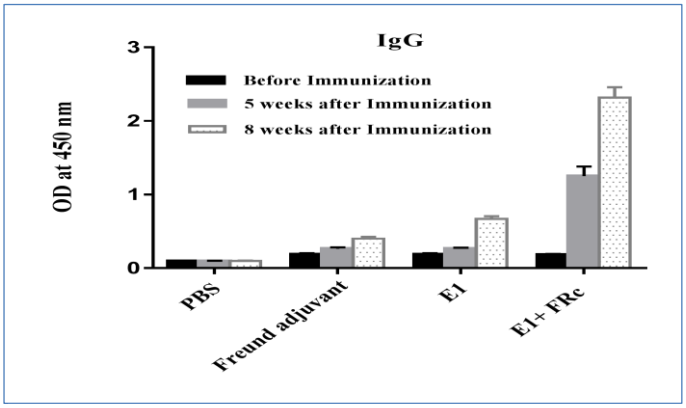

Fig. 5. ELISA analysis of humoral responses (Total IgG) in mice immunized with PBS (negative control), complete Freund's adjuvant, E1 and E1 plus FRc.

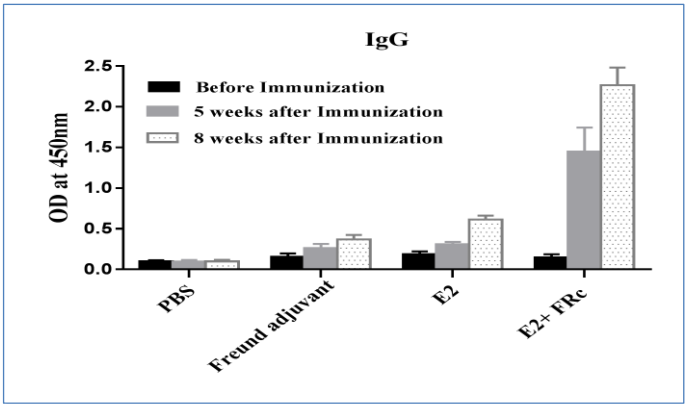

Fig. 6. ELISA analysis of humoral responses (total IgG) in mice immunized with PBS (negative control), complete Freund's adjuvant, E2 antigen and E2 antigen plus complete Freund adjuvant (FRc). IgG total antibody titer was measured in mice pooled serum in separate ELISA tests.

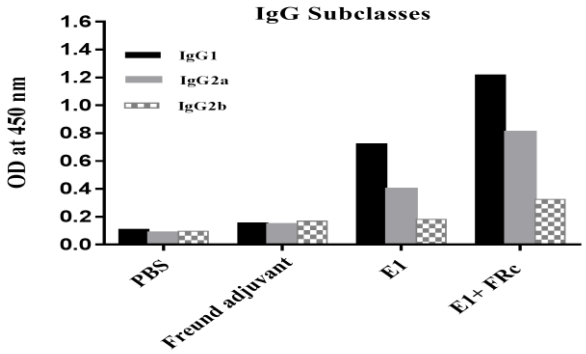

Fig. 7. Analysis of sub-classes of IgG antibody responses in mice pooled serum by ELISA test. Measurement of IgG sub-classes against E1 Ag.

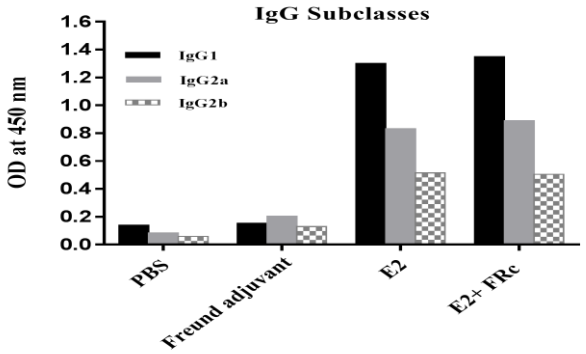

Fig. 8. Analysis of sub-classes of IgG antibody responses in mice pooled serum by ELISA test. Measurement of IgG sub-classes against E2 Ag.

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DISCUSSION

HCV infections with approximately 200 million chronic cases worldwide remain a serious global health burden. Only 30% of the infected patients can spontaneously resolve the infection and in some patients the infection may evolve into chronic hepatitis, cirrhosis, liver failure or hepatocellular carcinoma [1-4]. Previous studies have shown that CD8+ T cells are the key component for resolving HCV infection. However, neutralizing antibodies are also considered to be important in protecting people against the infection [1, 2]. Despite improved new therapeutic options, an effective and preventative vaccine against HCV is not yet available [2, 3]. E1 and E2 are type I membrane glycoproteins and are generally believed to constitute the components of the virion membrane involved in the receptor binding and membrane fusion which promote HCV entry into the target cells and as a result, E1 and E2 are two major targets in HCV vaccine research. Several studies have demonstrated that E1 and E2 structural proteins induce the production of neutralizing antibodies; hence, they might be considered as future vaccine candidates [2-10].

Yeasts have the advantages of molecular and genetic manipulations and have the added advantage of 10 to 100-fold higher heterologous protein expression levels. As a eukaryote, *P. pastoris* has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding and posttranslational modification. This system is faster, easier and less expensive to use than other eukaryotic expression protocols such as baculovirus or mammalian tissue culture while it generally gives higher expression levels. These features make Pichia a very useful protein expression system. *P. pastoris* is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. Two genes in *P. pastoris* code for alcohol oxidase, namely AOX1 and AOX2. The AOX1 gene product accounts for the majority of alcohol oxidase activity in the cell. The AOX1 gene has been isolated and a plasmid-borne version of the AOX1 promoter is used to drive expression of the gene of interest encoding the desired heterologous protein. The expression of the AOX1 gene is tightly regulated and is induced by methanol to very high levels [12-15, 17-19]. Here, the pPICZαA expression vector containing the α-factor was used to express and secrete recombinant proteins in high volume. Therefore, we could produce both E1 and E2 glycoproteins with concentrations of 6 mg/L and 4 mg/L, respectively. The expression of these proteins is difficult because of their transmembrane domains which are highly hydrophobic [9, 14, 17-19]. Cai *et al.* expressed these viral glycoproteins known as rE1E2 in truncated form in *P. pastoris* with a yield of 35mg/L [15]. However unlike rE1E2, we were able to express these 2 glycoproteins in their full lengths including their hydrophobic domains for the first time.

To evaluate the potential antigenicity of these proteins, they were used separately as the antigen in ELISA tests. The results showed that these yeast-expressed glycoproteins could be used in diagnostic methods to discriminate specific antibodies against E1 and E2 in HCV patients.

Our results were in agreement in terms of antigenicity with previous studies which had used a yeast expression system for producing E1 and E2 glycoproteins. However, they used it to express them in a truncated form as were the cases for rE1E2 or rCoreE1E2 [21-23]. Herein, we also investigated the immune reactivity of these 2 proteins in induction of neutralizing antibodies. Compared with the control groups of mice which were injected with complete Freund's adjuvant and each of E1 and E2 proteins alone, the injection with either E1 or E2 plus complete Freund`s adjuvant could induce a higher titer of neutralizing antibody that were statistically significant. These results were consistent with previous studies which had evaluated these HCV glycoproteins in induction of humoral immune responses in animal models [21-23].

In this study, we could express two HCV glycoproteins in full length for the first time and the antigenicity of these recombinant proteins were evaluated by an ELISA test using the serum samples of HCV infected patients in comparison with healthy volunteers as well as the induction of neutralizing antibody in mice. In conclusion, our study exhibited the potential application of recombinant E1 and E2 HCV glycoproteins expressed in *P. pastoris* either as a vaccine candidate or as a diagnostic tool such as ELISA. However, further studies with incorporating adjuvants that could be used in humans are remained to be performed on this subject.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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