

Recombinant CoreE1E2 Protein Expressed in *Pichia pastoris* Yeast a Candidate Vaccine for Hepatitis C Virus

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Abstract

Background: Hepatitis C virus (HCV) infection is a major health problem both in developed and developing countries and HCV infection is a global blood borne disease that affects almost 3% of the world's population with a morbidity and mortality rates. The efficacy of hepatitis C treatment is less than satisfactory and development of an effective vaccine may be essential in the control of HCV infection. The E1 and E2 proteins, two heavily glycosylated enveloped proteins, which can elicit neutralizing antibodies against HCV infection in the host and Core, E1 and E2 proteins are the major vaccine candidates for vaccination and ELISA is one of routine testes which has been used in clinical laboratories and different studies to detect the rate of antibody in sera against HCV infection.

Aim: Evolvement and gradual development of a useful vaccine can be the main point in the control and eradication of hepatitis C virus (HCV) infection. Recent studies have reported that HCV envelope glycoproteins can induce neutralizing antibodies against antigen domain of HCV. So HCV envelope proteins are considered as the main HCV vaccine candidate.

Methods: In this study, we used *Pichia pastoris* yeast expression system to express recombinant HCV CoreE1E2 protein, which consists of Core (269 nt-841nt) E1 (842 nt-1417nt) and E2 (1418 nt-2506nt). The *Pichia pastoris* can produce high level of recombinant HCV CoreE1E2 protein. The protein has glycosylation and also by codon optimization based on *pichia* expression system we could increase the rate of recombinant proteins. Moreover, the purified protein can efficiently induce anti-CoreE1E2 antibodies in rabbits, and also by developing homemade ELISA kit we can detect antibody of HCV Iranian patients with 1a genotype.

Results: Although little is known about the mechanism of hepatitis C virion assembly, in our study the virus like particle of rCoreE1E2 with 70 nm size, were shown by Electron microscopy and have proved the self-assembly in vitro in yeast expression system.

Conclusion: These findings indicate that the recombinant CoreE1E2 glycoprotein is effective in inducing neutralizing antibodies, and is an influential HCV vaccine candidate.

Keywords: Hepatitis C Virus; Virus like particles; *Pichia pastoris*; Vaccine

Introduction

Hepatitis C virus (HCV) infection is a major health problem both in developed and developing countries and HCV infection is a global blood borne disease that affects almost 3% of the world's population with a morbidity and mortality rate [1]. HCV is an enveloped virus with a single-stranded positive RNA and the viral particle consists of a nucleocapsid surrounded by an envelope derived from host membranes containing RNA genome with approximately 9500 nucleotides (nt). Hepatitis C virus (HCV) is a member of the Hepacivirus genus of the Flaviviridae family and the viral products (core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are processed from a 3000-amino acid (aa) polyprotein expressed from a single open reading frame [2,3].

More than 70% of HCV-infected patients develop chronic hepatitis and treatments for chronic HCV infection are generally effective in only 55% of cases. The efficacy of hepatitis C treatment is less than satisfactory and development of an effective vaccine may be essential in the control of HCV infection [4]. The E1 and E2 proteins, two heavily glycosylated enveloped proteins, which can elicit neutralizing antibodies against HCV infection in the host and Core, E1 and E2 proteins are the major vaccine candidates for vaccination and ELISA is one of routine testes which has been used in clinical laboratories and different studies to detect the rate of antibody in sera against HCV infection [5]. The combination of pegylated interferon α and ribavirin is useful to some of treated patients depending on the viral

or host factors [6]. Additionally, this method needs prolonged therapy, sometimes with different side effects and only some of those with chronic HCV infections meet the criteria for treatment [7]. Recently, production of virus-like particles as a model system for HCV receptor binding, cell entry and for vaccination studies is used [8]. Virus-like particles are the structural proteins of many viruses which produced and self-assembled in the absence of other viral materials and can be extracted and purified directly after expression in prokaryote and eukaryotic cells, or assembled in vitro from protein subunits purified from any recombinant hosts [9,10]. VLPs recognized in the average 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. In this study, the HCV virus-like particles (VLPs) obtained from *Pichia pastoris* cells were produced, in order to construct a recombinant

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HCV Core E1E2 glycoprotein (rCoreE1E2), hoping the rCoreE1E2 can induce neutralizing antibodies [10]. It has been shown that HCV antigens produced in *Pichia pastoris* induce strong immune responses in animals [8,11]. *Pichia pastoris* is a successful system for production of a wide variety of recombinant proteins with its promoter derived from the alcohol oxidase I (AOX1) gene of *P. pastoris* that is suited for controlled expression of foreign genes [12,13].

Post-translational modifications performed by higher eukaryotic cells such as proteolytic processing, folding, disulfide bond formation and glycosylation can be done by *P. pastoris*. This system is also faster, easier, and less expensive than expression systems derived from higher eukaryotes such as insect and mammalian tissue culture systems and usually gives higher expression levels [13,14]. With *P. pastoris*, heterologous proteins can either be expressed intracellular or secreted into the medium and because of self-assembly process which is present in recombinant proteins, the virus like particles produced and secreted in medium [15]. Secretion requires the presence of a signal sequence on the foreign protein to target it to the secretory pathway and the *S. cerevisiae* α factor has been used with the most success [16,17]. Expression of different genes in *Pichia pastoris* cells is a common practice in the biopharmaceutical industry. These expression systems can have raised error rates in translation due to codon bias, that is, preferential use of codons for the same amino acid. As codon bias differs between the host organism and the organism from which the gene was extracted, substituting synonymous codons can improve translational fidelity and in this study codon optimization has been used for increasing the protein expression efficacy [18,19].

Method

Study design

Construction of recombinant expression plasmid: The coding regions of Core (269 nt-841nt), E1 (842 nt-1417nt) and E2 (1418 nt-2506nt) were amplified by PCR from infected Iranian patient's blood with HCV (genotype 1a), using primers Core forward [5'-TTG AATTCGTCATGAGCAGCAATCCTAAACCTC-3'], and E2 reverse [5'-TTGCGGCCGCCGCCCTCCGCTTGGAATA-3'], (restriction sites for Xho I, NotI and the reverse stop codon were set in primers). In the forward primer for insertion of the CoreE1E2 gene in the pPICZaA vector it was not necessary to add the Kozak sequence because of the presence of the secretion factor in this vector and restriction sites added in the 5' end of primers for insertion into the pPICZaA vector and also the poly-histidine (6xHis) tag present in the vectors. The CoreE1E2 amplified product was ligated into pMD18-T vector (Takara) and then cloned into the XhoI/NotI sites of the pPICZaA vector (Invitrogen, Carlsbad, CA, USA) to produce pPICZaA-CoreE1E2 according to standard DNA manipulation methods. The clones in *E. coli* TOP 10 were obtained by transformation with CaCl_2 and selected on low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 2% agar) with ZeocinTM (Invitrogen) (due to the presence of the *Sh ble* gene in the vectors that confers resistance to this antibiotic). Recombinants were confirmed by PCR, restriction enzyme digestion and sequencing. Then, the recombinant plasmids were linearized and electro transformed into the competent *P. pichia* cells. Transformation, screening and recombinant protein expression were performed as described by the instruction manual of *Pichia* expression kit (Invitrogen), and the resulting in a recombinant plasmid nominated pPICZaA-CoreE1E2.

Expression and purification of rCoreE1E2: The rCoreE1E2 was expressed by *P. pastoris* yeast according to the manufacturer's manual

(Multi-Copy *Pichia* Expression Kit, Invitrogen). Briefly, pPICZaA-CoreE1E2 was linearized by Pme I and electroporated into *P. pastoris* strain GS115; GS115 (His⁻, Mut⁺) transformants were selected on Minimal Dextrose (MD) medium plate and confirmed on Minimal Methanol (MM) medium plate. Multiple inserted recombinants were isolated on Yeast Extract Peptone Dextrose (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, and 2% agar) medium plate containing ZeocinTM (Invitrogen) at final concentration of 2.0 mg/ml. *P. pastoris* GS115 was also transformed with empty vectors pPICZaA for negative control tests. Clones were detected by colony PCR using the conditions and primers provided in the EasySelectTM *P. pastoris* expression kit. The primers used for detection anneal in the flanking regions of the insert (AOX1 and α -factor). The control strains of the intracellular (GS115/ β -galactosidase) and extracellular (GS115/albumin) expression provided by the EasySelectTM *P. pastoris* expression kit. After choosing the most resistant colon, a single colony of multiple inserted His⁻, Mut⁺ GS115 recombinants was inoculated into 25 mL buffered glycerol complex medium (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base (YNB), 4×10^{-5} % biotin, 1% glycerol) and cultured at 250 rpm and 30°C until the culture medium reached an OD₆₀₀ of 2-6. The cells were harvested by centrifuging at 3,000 rpm for 5 min at room temperature. The cell pellet was re-suspended in buffered methanol complex medium (BMMY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5} biotin, and 0.5% methanol) to an OD₆₀₀ of 1.0 and cultured in a 250-mL flask at 250 rpm under 30°C. Inductive expression was carried out with the addition of methanol (0.5%, v/v) per 24 h at 30°C lasted for 3 days. For subsequent analysis of CoreE1E2 gene expression, samples were taken from cultures every 24 h, including aliquots of supernatants and pellets stored at -80°C. The samples were collected every 12 h and analyzed by SDS-PAGE and Western blot. For extraction and purification of rCoreE1E2, the cells were disrupted by glass beads in a TEN buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl) and then centrifuged. The rCoreE1E2 mainly existed in the deposit with cell fractions, and was not soluble in a TEN buffer. The deposit was resuspended in a TEN buffer with 8 M urea and centrifuged again. The supernatant was dialyzed against 5 mM Tris-HCl (pH 9.3) overnight at 4°C and then clarified by centrifugation at 12,000×g for 30 min. The supernatant was first loaded to a Q-Sepharose Fast Flow column with 20 mM Tris-HCl (pH 9.3). After that washing with 0.05 M NaCl and 20 mM Tris-HCl (pH 9.3), the rCoreE1E2 was eluted with 0.5 M NaCl and 20 mM Tris-HCl (pH 9.3) was performed. Then the eluted was loaded to a Phenyl Sepharose Fast Flow column with 20 mM Tris-HCl (pH 9.3) and 0.1 M NaCl. The rE1E2 was eluted with 20 mM Tris-HCl (pH 9.3). The rCoreE1E2 was further purified with Sephadex G150 column for removing low molecular weight fractions. The purified rCoreE1E2 was dialyzed against 5 mM Tris-HCl (pH 9.3) and sterilized with 0.45 μm filter, and then stored at 4°C.

Protein purification: The secreted *Pichia*-expressed protein in supernatant samples was spun at 15,000 rpm for 30 min. The rCoreE1E2 protein was purified from the soluble fraction. Briefly, the supernatant was loaded onto Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) in the presence of 0.5% Tween 80 and 40 mM imidazole. The column was washed extensively, and the protein was eluted with 1 M imidazole. rCoreE1E2 was purified from the insoluble fraction by using 0.5% N-lauryl sarcosine (Sarkosyl; Sigma Chemical Co., St. Louis, Mo). The protein was purified over Ni-NTA-super flow and the Sarkosyl was replaced by 0.1% Tween 80. The protein was eluted in the presence of 200 mM imidazole and 0.1% Tween 80 in phosphate buffer.

SDS-PAGE and western blotting: Supernatant of yeast culture harvested and the cells were harvested and washed twice in TEN buffers (50 mM Tris-HCL pH 8.0, 1 mM EDTA, 150 mM NaCl). Cell disruption was performed by vortex with glass beads in TEN. The lysate was clarified by centrifugation at 12 000 g for 20 min and the supernatant and pellet used. The rCoreE1E2 was mixed with sample buffer (New England Biolabs, Ipswich, MA, US). The sample was boiled for 10 min in the presence of DTT, and around 200 ng was loaded on a 4-12% Bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA, US). The primary antibodies against Core E1 E2 were (Santa Cruse); the secondary antibody was polyclonal rabbit anti-mouse horse radish peroxidase labeled. Staining was done and the molecular weight marker was a mix of proteins from Sigma: bovine serum albumin (A-7517), ovalbumin (A-7642), carbonic anhydrase (C-2273), beta lacto globulin (L-4756) and alpha-lacto albumin (L-6385) or the Precision and Precision plus Protein Standards (Bio-Rad, Hercules, CA, US, 161-0374 and 161-0361).

Endoglycosidase digestion assay: Purified rCoreE1E2 was digested N-glycosidase F (PNGase F). The digested proteins were treated according to the manufacturer's instructions (New England Biolabs) and then analyzed by Western blot with mAb fore Core E1 E2.

Animals, immunization and ELISA: Approximately rCoreE1E2 (300 g) diluted in 2 ml sterilized 0.9% NaCl used for immunization. A New Zealand rabbits was provided from the Experimental Animals Center. The rabbit was subcutaneously immunized in multiple sites on the back with 0 g (negative control) or 300 g purified rCoreE1E2, respectively. Booster injections were given with the same doses at 2, 3, 4, 5 and 13 weeks later. Sera were obtained every week. The anti-rCoreE1E2 titers in sera were measured by ELISA. An indirect ELISA to measure anti- rCoreE1E2 antibodies in rabbit's serum was developed. In brief, recombinant protein (10 mg/ml) diluted in 50 mM carbonate buffer (pH 9.6) was used to coat microtiter plates (Costar, U.S.A.), overnight at 4°C. After blocking with 2% (w/v) skim milk powder (Oxoid Ltd., England) in PBS with 0.05% (v/v) Tween 20 (Sigma) (PBST), pH 7.2 for 1 h at room temperature. The serum samples were added in duplicate, either 1/20 in dilution buffer (PBST), containing 1% (w/v) skim milk powder, to test seroconversion or in a double serial starting at 1/50 dilution for titration. They were incubated at 37°C for 1 h. A horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Sigma) was added to 1/10,000 in the dilution buffer. After 1 h of incubation at room temperature (RT) and washing, tetramethylbenzidine substrate (Sigma) reactions were stopped with 50 ml of 2.5 M H₂SO₄. Absorbance was read at 492 nm in a SensIdent Scan (Merck, Germany). The cut-off value used to consider a positive antibody response was established as twice the mean of absorbance values of the preimmune sera. Antibody titers against rCoreE1E2 were detected as the maximum dilution that in ELISA gave for the immunized serum.

Enzyme-linked immunosorbent assays (ELISA) with human sera: HCV rCoreE1E2 particles were coated at 1 g/mL in carbonate buffer in microtiterplates and were incubated for 2 h at 37°C. The plates washed to remove the non-adsorbed particles and incubated with blocking buffer (0.5% casein in PBS). Sera of chronically HCV-infected people (Human sera were previously screened for the presence of anti-HCV) or sera of healthy persons were added in a dilution of 1:20. The plates were washed to remove the non-bound antibodies and the plates were incubated with horse radish peroxidase labeled rabbit anti-human immunoglobulins. The tetramethylbenzidine substrate (Sigma) reaction was stopped by addition of 2 N H₂SO₄ and the absorbance was measured at 450 nm. The cutoff value to consider a positive antibody

response was established as twice the mean OD 492 nm of the negative control sera.

Transcriptional analysis of the CoreE1E2 gene: Total RNA was extracted from recombinant clones by the total RNA extraction kit (Qiagen). The cDNA was synthesized using the IMPROM-IT™ Reverse Transcription System from Promega (USA). The presence of heterologous gene mRNA in *Pichia* was detected by RT-PCR and Real-time PCR with Forward primer 5'-TTGGGACATGATGATGAATTGG-3' and Reverse primer 5'-TGCCTGTGGGATTCTAAGC-3' and probe 5'-FAM ACAGCCGCATTTGGTTGTCGCC-BHQ1-3' that anneal in the internal sequence of the CoreE1E2 genes. Quantitative determination of the amplified products was done with the Rotor Gene 6000 (Corbett Research, Australia).

Electron microscopy: Our supernatant collected and recombinant CoreE1E2 was purified and fixed in glutaraldehyde and negatively stained with uranyl acetate prior to analysis by transmission electron microscopy.

Immunofluorescence: The *Pichia pastoris* cells after expression of rCoreE1E2, were applied to slides, air dried, and fixed with methanol. The slides were blocked with bovine serum albumin (BSA) diluted to 1% in PBS (PBS-BSA) for 30 min. Primary antibodies diluted in PBS-BSA were added to the wells, and the slides were incubated in a humidified chamber for 1 h at room temperature. The slides were washed with PBS, and fluorescein isothiocyanate-labeled goat anti-mouse antibody (Promega) was added and left for 30 min at room temperature. Slides were washed and mounted in Vectashield (Vector Labs, Burlingame, Calif.) and viewed on an Olympus microscope (Melville, N.Y.).

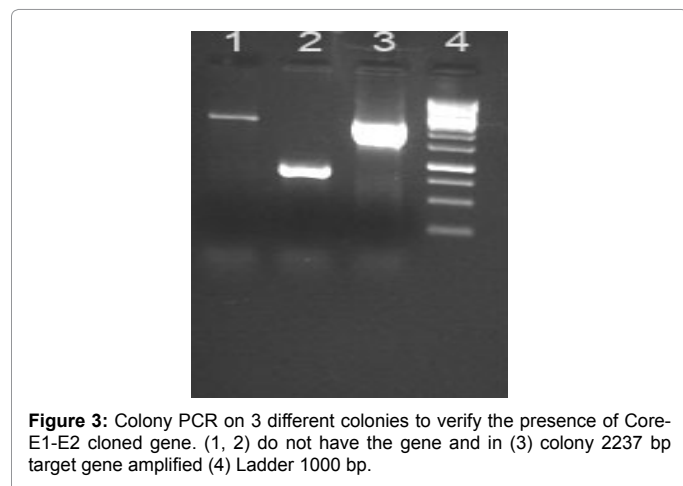
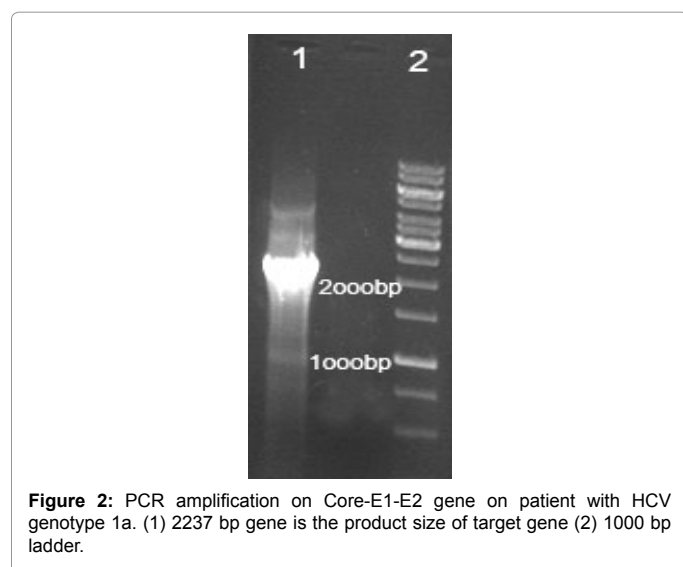
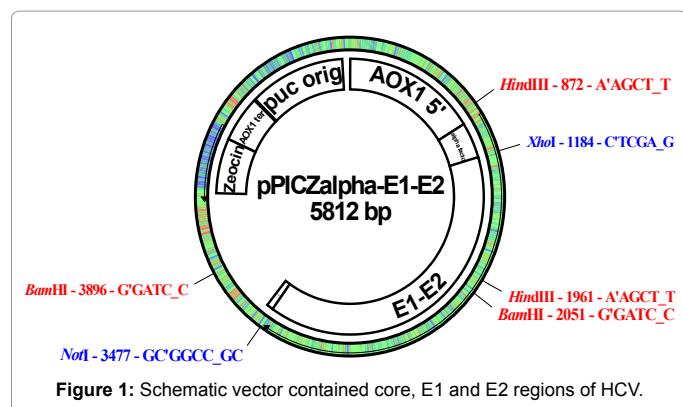
Codon optimization: The codon-optimized gene was designed based on the protein sequence of CoreE1E2 according to the codon bias of *P. pastoris* (<http://www.kazusa.or.jp/codon>). Codon optimization was performed by using the GenScript program. The entire CoreE1E2 gene with Xho I and Not I restriction sites at each end was designed and was in frame with α -factor of pPICZaA vector. The designed rCoreE1E2 was synthesized by GenScript (USA).

Sample size: We used one rabbit for Immunization study and 8 human patients for ELISA evaluation test.

Data analysis: Data was analyzed by SPSS software version 19.

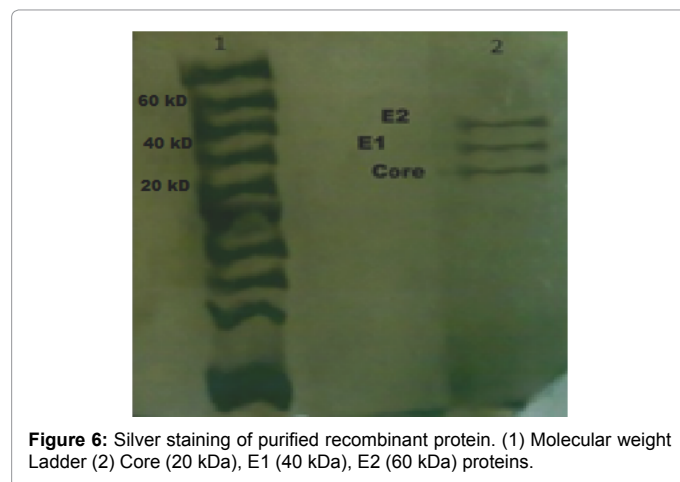
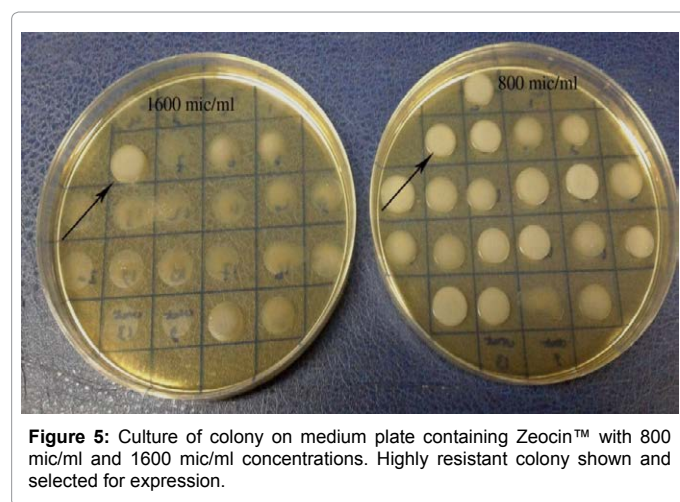
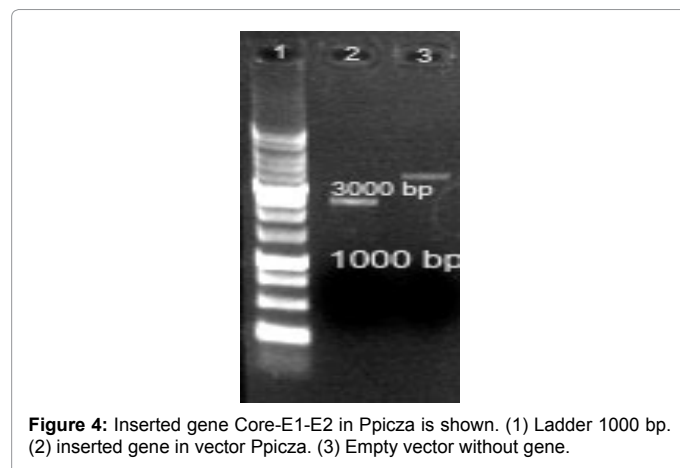
Results

The continuous coding regions of Core (269 nt-841nt) E1 (842 nt-1417nt) and E2 (1418 nt-2506nt) were amplified by PCR from infected blood by HCV (genotype 1a) and the product (2237 bp) was cloned in vector (Figure 1). The colony PCR was done to verify the insertion of target gene in vector (Figure 2). After that target gene and Ppicza digested by XhoI and NotI enzymes and ligated to have one recombinant expression vector (Figure 3). The pPICZaA-CoreE1E2 was linearized by Pme I and electroporated into *P. pastoris* strain GS115; GS115 (His⁻, Mut⁺) and different colony in different concentration of Zeocin evaluated and the most resistant colons which tolerated 800 and 1600 mic/ml chosen for huge expression phase (Figure 4). The rCoreE1E2 protein was purified from the soluble fraction by Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) and the purified proteins were studied by SDS-PAGE and Western blot (Figures 5 and 6). Core protein with 20 kDa and E1 protein with 40 kDa and E2 with 60 kDa were shown. The optimization is one of best methods to increase the output of expression in *Pichia pastoris*. The codon-optimized gene was designed based on the protein sequence



of CoreE1E2 according to the codon bias of *P. pastoris* (<http://www.kazusa.or.jp/codon>). Possibility of high protein expression level is correlated to the value of Codon Adaptation Index (CAI). A CAI of 1.0 is considered to be ideal and perfect while a CAI of >0.80 is rated as good for expression in the desired *Pichia pastoris*. As Figure 7 illustrates the CAI perfect result for expression system is 1.00 (Figure 7A) and our codon optimization results is 0.85 (Figure 7B).

The ideal percentage range of GC content is between 30 to 70%. Any peaks outside of this range will adversely affect transcriptional and translational efficiency. In Figure 8 the average of GC content before optimization detected 40.49 (Figure 8A) but after optimization it became 45.20 (Figure 8B) and both were shown as GC curves. Figure 9 reported the percentage distribution of Codon Frequency Distribution (CFD) in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid



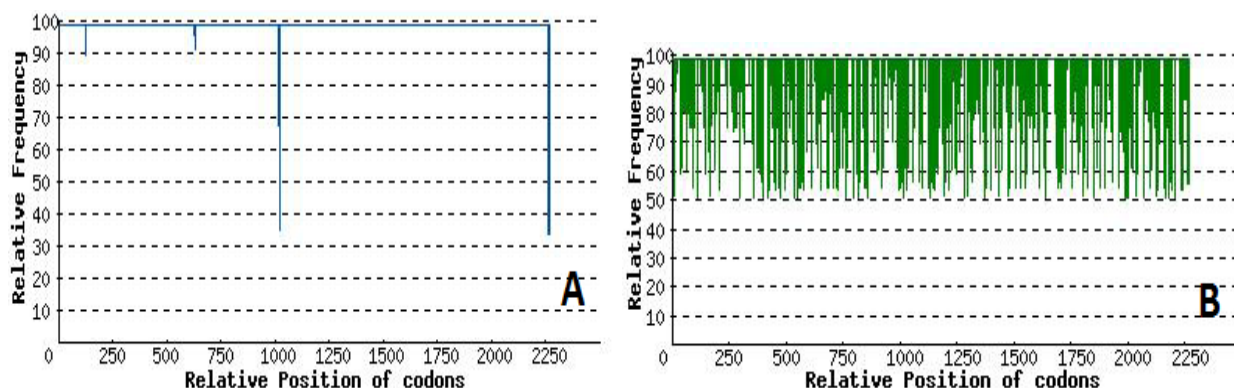


Figure 7: The distribution of codon usage frequency along the length of Core-E1-E2 to be expressed in *Pichia pastoris*. CAI of 1.0 is considered to be perfect in the desired expression organism (A) and our gene's CAI = 0.85 is regarded as very good (B) in terms of high gene expression level.

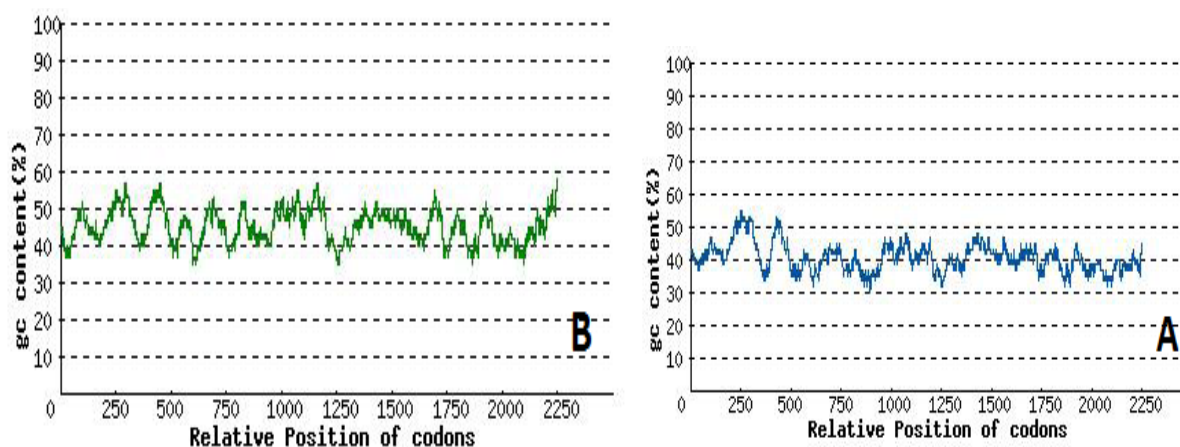


Figure 8: The distribution of GC content frequency along the length of Core-E1-E2 to be expressed in *Pichia pastoris*. (A)Before optimization it was 40.49 and (B) after codon optimization it became 45.20.

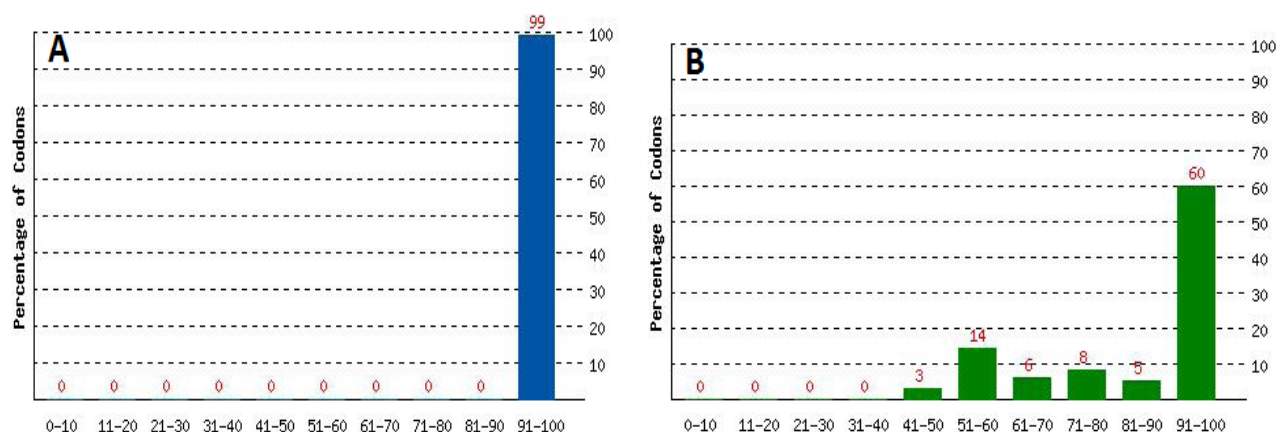


Figure 9: The distribution of Codon Frequency Distribution (CFD) frequency along the length of Core-E1-E2 to be expressed in *Pichia pastoris*. (A)The value of 100 is set for the codon with the highest usage frequency for a given amino acid in *Pichia pastoris* and (B)after codon optimization. All the cordon's distribution are upper than 50%.

in *Pichia pastoris* (Figure 9A). Codons with values lower than 30 are likely to hamper the expression efficiency and as it shows after codon optimization the value of Frequency of Optimal Codons (FOP) in our

gene is acceptable (Figure 9B) and all cordon's distribution are upper than 50%. Interestingly, 60 percent of Codon Frequency Distribution is 91-100%, 5 percent is 81-90%, 8 percent is 71-80%, 6 percent is 61-

70%, 14 percent is 51-60% and 3 percent is 41-50% and no distribution lower than 40% was detected. As in Figure 10 is clearly shown, the thickness of bands in optimized sample comparing with not-optimizes sample, shows that most codons are expressed better in *pichia pastoris*. To determine that the mRNA of recombinant vector are expressed, total RNA was extracted from recombinant clones by the total RNA extraction and the presence of target mRNA in *Pichia* was detected by RT-PCR and Real-time PCR to detect Core-E1-E2 and housekeeping genes (Figure 11). After 30 cycles the housekeeping genes and Core-E1-E2 gene were detected and analyzed by software. To digest and delete carbohydrate residues from recombinant Core-E1-E2, PNGase F was used to remove unnecessary N-glycan bands (Figure 12). As it is clear, before digestion the presence of smear in lane 1 is present but after digestion in lane 2 and also in positive control all bands are sharp and Core with 20 kDa and E1 with 40 kDa and E2 with 60 kDa is illustrated. Electron microscopy is one of reliable tests to ensure us that virus like particle is present in our supernatant. As in Figure 13

reported, the EM negative staining image shows the particles with 70 nm size in our sample. Immunofluorescence test by using monoclonal Antibodies against Core-E1-E2 was used to show the expression and being of recombinant protein in *Pichia pastoris* cells. In Figure 14, two images of positive expression recombinant proteins and normal cell without expression are illustrated. For evaluation of efficiency of HCV rCoreE1E2 particles against patient sera, we coated them in ELISA wells and the sera of some patients were evaluated with international QuickTiter™ HCV Core Antigen ELISA Kit (Catalog Numbers VPK-151) and homemade kit (Figure 15). From those patients, 8 samples were shown and interestingly in case number 4,5,6,7 the result of our kit is better than standard kit. The comparison between tests was carried on 3 times and after analysis by SPSS software the P value<0.001 was reported.

Number 7 has the highest titer of antibody and in our kit it is 118 ng/ml but in QuickTiter™ kit it is 90 ng/ml and in case 4 the lowest titer

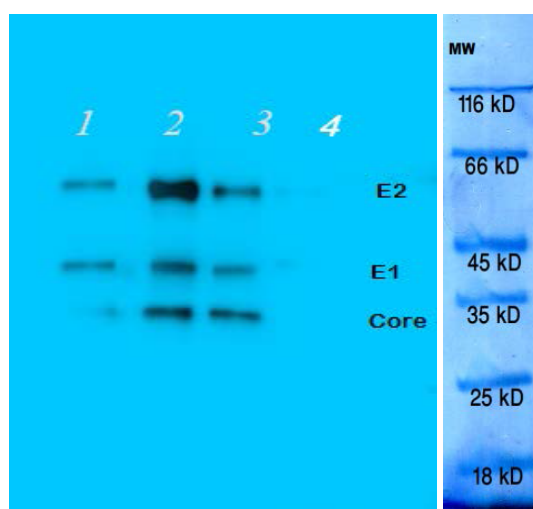


Figure 10: Western blot of recombinant Core (20 kDa), E1(40 kDa), E2 (60 kDa). (1) Recombinant protein before optimization. (2) Recombinant protein after optimization. (3) HCV proteins as Positive control. (4) Negative control. MW is molecular weight markers.

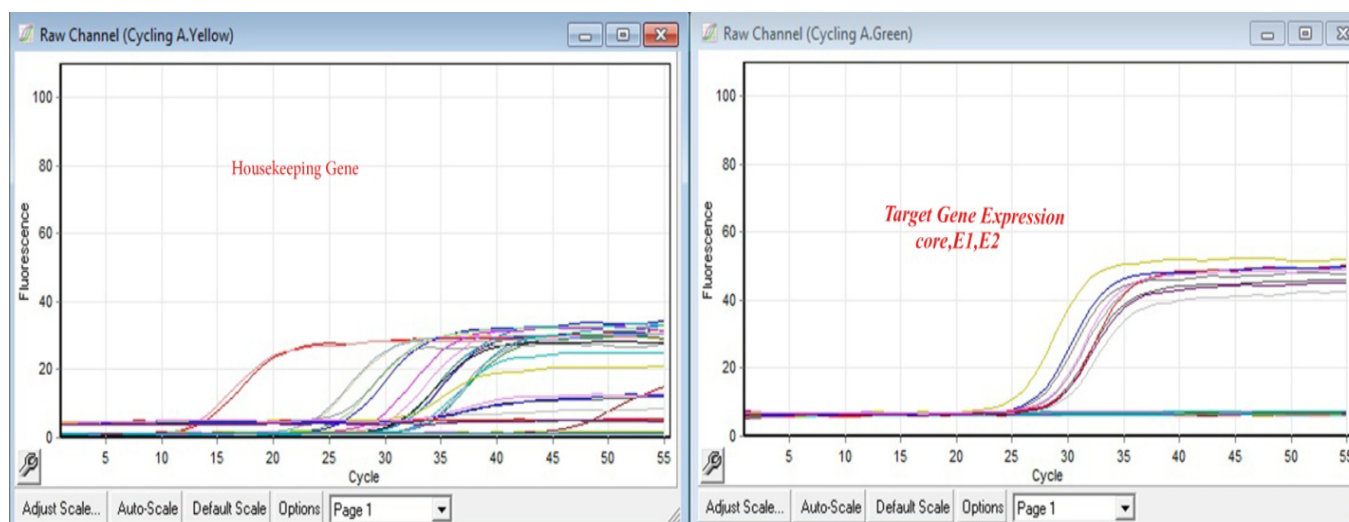


Figure 11: Real-time PCR of Housekeeping gene on left picture and presence of housekeeping gene detected and Target gene of Core-E1-E2 on right picture, after 30 cycles the result are positive.

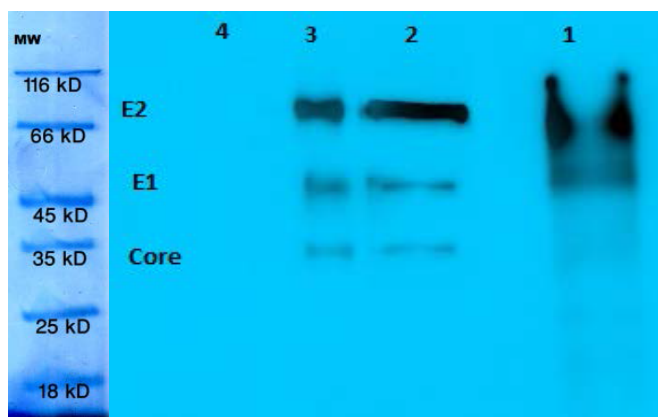


Figure 12: Western blot of recombinant Core (20 kDa), E1 (40 kDa), E2 (60 kDa). (1) Recombinant protein before PNGaseF and it is smear like and heavy with N-glycan bands. (2) Recombinant protein after PNGaseF without extra N-glycan linkages and sharp bands. (3) HCV proteins as Positive control. (4) Negative control. MW is molecular weight markers.

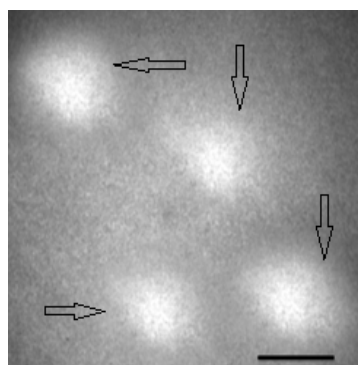


Figure 13: Virus like particle are shown with the size of 70 nm in negative staining EM as a golden standard method to verify the presence of recombinant particles. Bar=100nm.

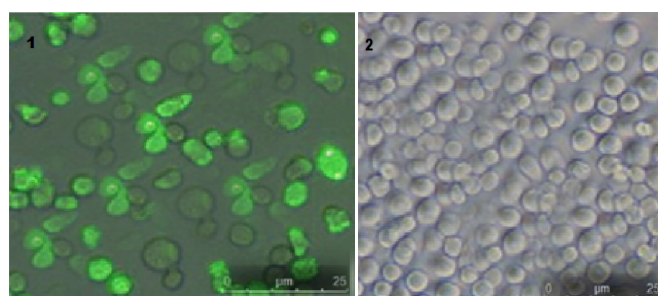


Figure 14: Immunofluorescence test on *Pichia pastoris* cells to show that expressed proteins are present in cells. (1) Positive cells in expression of recombinant Core E1 E2 proteins. (2) Control of *Pichia pastoris* cells without expression vector.

detected and our kit 38 ng/ml and QuickTiter™ kit 22 ng/ml antibody level reported. The rCoreE1E2 diluted and used for immunization of New Zealand rabbit to show the immunization potential *in vivo*. The rabbit was subcutaneously immunized in multiple sites and after last booster injection the titer of antibody checked (Figure 16). The first serum collected before immunization and no anti HCV antibody

detected by ELISA and this sample was kept as zero in data chart. After last injection, every week sample shows the raise of antibody for example in week 5 the rate of antibody is 49 ng/ml in week 8 is 100 ng/ml and surprisingly in the week of 10 the antibody titer is 120 ng/ml.

Discussion

Hepatitis C virus (HCV) infection is a main health problem both in developed and developing countries and Iran is a developing country which is located in Middle East [20]. One of the aims of HCV research is to develop an effective vaccine to produce acceptable immunity in human sera against HCV [21]. Last studies have reported that the HCV glycoproteins can induce neutralizing antibodies, making them as a candidate for HCV vaccine [22]. In our current study, the sample of HCV virus (genotype 1a) diagnosed and used as a template for amplification and expression. Due to the sample of Iranian HCV patient, we proposed an efficient vaccine candidate for more research. Although some research showed that it was difficult to express HCV envelope proteins by *P. pastoris* yeast, we could express HCV rCoreE1E2 by *Pichia pastoris* yeast expression system which was used in our study is pPICZaA vector (Invitrogen, Carlsbad, CA, USA) and this vector has α -factor to help us to express and secrete recombinant protein in high volume both in intra and extracellular [13,23]. Therefore, Western blotting using anti-Core/E1/E2 mAbs demonstrated a different bands for Core 20 kDa, E1 40 kDa, E2 60 kDa in yeast-expressed system [24]. The glycans of HCV envelope proteins are very critical for structures, functions and antigenicity. Using eukaryotic expression system for HCV envelope glycoproteins can help us to make native shape and function

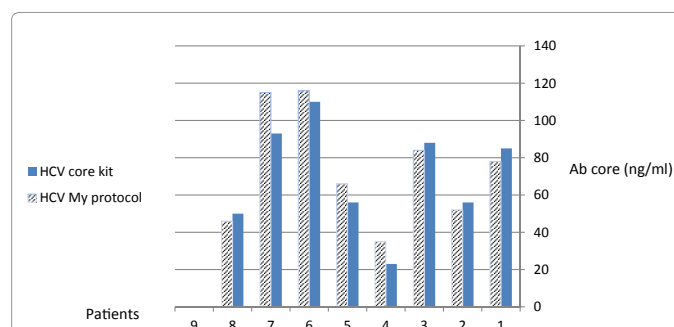


Figure 15: Our ELISA kit with recombinant Core-E1-E2 is compared with standard international kit. The blue bar is the result of antibody of patients Antigen ELISA Kit QuickTiter™ HCV Core and another bar is antibody titers of patients with our ELISA kit. In this chart 8 samples are shown and in number 4,5,6,7 the result of homemade kit is better than QuickTiter™ kit. Data were repeated 3 times and results were the same.

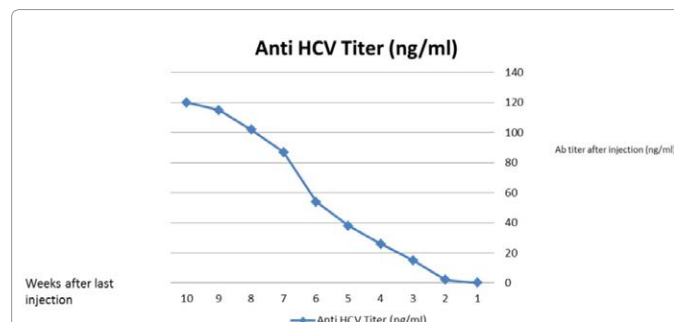


Figure 16: Antibody titers in rabbit after last injection of recombinant Core-E1-E2 protein is shown and 10 weeks after last injection the titer of 120 ng/ml was reported.

for our recombinant proteins [25]. *P. pastoris* yeast can synthesize and process rCoreE1E2 carrying glycans which could be digested by PNGase F and this glycosylation is similar to some of original HCV envelope glycoproteins. The truth is that glycans of rCoreE1E2 should be different to native CoreE1E2 because of the difference between yeast and mammalian cells [26]. The removes all three types of amino-linked glycans, high mannose- type glycans, hybrid-type glycans, and complex- type glycans. In *P. pastoris* cells, the N-glycosylation pathway is similar to the pathway in human cells except that *P. pastoris* cells have just high mannose structures [27]. Enzymatic deglycosylation with PNGase F resulted that the glycosylated smeared band disappear and sharp band with less molecular weight which corresponds to nonglycosylated proteins remain, indicating that the multiple bands arise by different degrees of N-glycosylation. The yeast expressed rCoreE1E2 has all potential N-glycosylation sites occupied [28].

We reported that codon optimization leads to increase expression of recombinant Core-E1-E2 in *P. pastoris* and also we evaluated the utility of codon optimization to improve the expression of Core-E1-E2 in *P. pastoris*. We designed the Core-E1-E2 gene by choosing the most preferred codons, while avoiding the formation of stable secondary structures in the corresponding mRNA sequence. Therefore, codon optimization moderately increased the expression level and our data was similar with other optimization studies which show increase of efficacy by codon optimization. The translational hypothesis related to translation initiation and elongation rates has been well accepted for explaining the codon usage bias in eukaryotes. Although the mRNA levels were similar between the native and the optimized constructs, suggesting that the increased expression is attributable to the enhancement of posttranscriptional processing (data not shown). As the, we expect that the increased expression by codon optimization should be mainly due to the enhanced translation elongation instead of translation initiation. It seems that other factors like protein folding within the endoplasmic reticulum and secretion signal processing may be important in secretion ability [29]. Moreover, in our study the native gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. We changed the codon usage bias in *Pichia pastoris* by to 0.85. GC content and unfavorable peaks have been optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were broken [30]. In addition, our optimization process has screened and successfully modified those negative cis-acting sites. In other past researches different parts of HCV glycoproteins were used for immunization in mice, goat, sheep and raising antibody detected but whole CoreE1E2 which is more immunogenic was not shown. In this study, the strategy of inducing broadly neutralizing antibodies is probably successful to produce anti HCV glycoprotein antibody as it succeeds in rabbit and our rCoreE1E2 can induce high humeral immune response and it can be one step forward for evaluation of HCV vaccine for in vivo research. The immune reactivity of rCoreE1E2 particles was tested by international ELISA Kit QuickTiter™ HCV and homemade ELISA kit, using sera from chronically HCV-infected persons. Indicating the epitopes presented by our particle's conformation is very analogous with the original HCV particle. The evaluation of human sera, showing anti-HCV positive sera against rCoreE1E2 proteins demonstrated that anti-HCV positive sera recognized our recombinant peptide by ELISA and even in some cases our results were better than international kit (P.value<0.001). Based on conformation, we have shown that recombinant Core-E1-E2 proteins can be expressed from a yeast system and purified to homogeneity [31,32]. Moreover our data shows that

human sera is anti-recombinant protein and can neutralize our protein in ELISA system which is better result than other researches and also because we produced whole CoreE1E2 protein of HCV virus, all the immunogenic sites are in our recombinant protein which has not been in other studies. These recombinant proteins efficiently self-assemble in vitro into virus like particles with a regular, spherical morphology. These particles have a modal diameter centered about 70 nm and were shown with negative staining by Electron microscopy and also because CoreE1E2 assembled together the size of particle increased which is similar to other studies base on HCV particles in vivo and in vitro [21,23,33]. In conclusion, the expression of the HCV structural proteins in *P. pastoris* would be useful for studying the mechanisms of HCV processing, morphogenesis, immunity and assembly. Natural HCV structural proteins are not useful for developing vaccines or specific anti-sera because the virus concentrations in the infectious materials are very low. Therefore, recombinant HCV structural proteins are useful as immunogens. For the development of preventive vaccines and therapeutic treatments against HCV, the rCoreE1E2 protein might be a crucial element and the results obtained in this work may therefore contribute to this effort. These recombinant proteins may be useful targets for HCV vaccine candidates. Moreover, *P. pastoris* yeast expression system is an efficient eukaryotic expression system and we believe that the *P. pastoris* yeast-expressed rCoreE1E2 is a promising HCV vaccine candidate for industrial purpose.

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