Original Article

Cloning and Prokaryotic Expression of cDNAs from Hepatitis E Virus Structural Gene of the SW189 Strain

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Abstract

It is necessary to study the second open reading frame (open reading frame 2, ORF2) and antigenic peptides characteristic of genotype 4 of HEV, and create one kind of high sensitivity and specificity of anti-HEV IgM detection kit. The objective of this study was to obtain recombinant antigen for development of anti-HEV ELISA method and vaccine against hepatitis E virus infection. A 728 base cDNA was collected from 5'-terminus of open reading frame 2 (ORF2) among epidemic hepatitis E virus (HEV) isolated from Gansu, Western China. The fragment was digested with *Not* I and *Nco* I, and inserted into vector pET32a(+). The recombinant plasmid was transformed into *E. coli* Rosetta and the fusion protein expressed was confirmed by Western blot analysis. The recombinant plasmid was identified and confirmed with enzyme digestion, polymerase chain reaction (PCR) and sequencing, respectively. A protein band of about 45.3 kDa was demonstrated by SDS-PAGE. The result of Western blot analysis suggested that the fusion protein reacted with anti-HEV positive sera at a dilution of 1:1500. The recombinant protein pORF2 may be useful in developing anti-HEV ELISA kit and vaccine against hepatitis E virus infection.

Keywords: cloning, hepatitis E virus, prokaryotic expression, western blot

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บทคัดย่อ

การโคลนนิ่งและการแสดงออกของ cDNAs ในเซลล์โปรคาริโอต ของยีนโครงสร้าง เชื้อไวรัสตับอักเสบ อี สายพันธุ์ SW189

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การศึกษา ORF2 และคุณลักษณะของสายเปปไทด์แอนติเจนของจีโนไทป์ที่ 4 ของเชื้อไวรัสตับอักเสบ อี (HEV) มีความจำเป็นที่ต้องศึกษาเพื่อการพัฒนาชุดตรวจสอบสำเร็จรูปที่มีความไวและความจำเพาะต่อแอนติบอดี้ชนิด IgM ของ HEV วัตถุประสงค์ของการศึกษานี้เพื่อสร้างรีคอมบิแนนท์แอนติเจน เพื่อนำไปพัฒนาชุดตรวจอีไลซา และวัคซีนป้องกันโรคไวรัสตับอักเสบ อี โดยทำการสกัดสาย cDNA ขนาด 728 เบส จากส่วน 5' ของ ORF2 ของ HEV ที่แยกได้จากมณฑลกานซู ภาคตะวันตกของจีน นำชิ้นส่วนดีเอ็นเอมาย่อยด้วยเอนไซม์ *Not I* และ *NCO I* และนำไปใส่ในเวกเตอร์ pET32a(+) จากนั้นนำพลาสมิดที่ผ่านการตัดต่อใส่ใน *E. coli* Rosetta และทำการตรวจสอบโปรตีนลูกผสมด้วยวิธี Western blot จากนั้นนำพลาสมิดที่ผ่านการตัดต่อมาตรวจสอบโดยการสกัด แล้วทำการย่อยด้วยเอนไซม์ ตามด้วยปฏิกิริยาพีซีอาร์ และวิเคราะห์ลำดับคู่เบสตามลำดับ พบว่าการแยกโปรตีนด้วยไฟฟ้า ได้โปรตีนขนาด 45.3 kDa และเมื่อวิเคราะห์โดยวิธี Western blot โปรตีนดังกล่าวทำปฏิกิริยากับซีรั่มที่มีแอนติบอดี้ต่อ HEV ที่ความเข้มข้น 1:1500 ชี้ให้เห็นว่าโปรตีนรีคอมบิแนนท์ pORF2 สามารถนำมาใช้ในการพัฒนาชุดตรวจอีไลซา และวัคซีนป้องกันโรคไวรัสตับอักเสบ อี ต่อไปได้

คำสำคัญ: โคลนนิ่ง ไวรัสตับอักเสบ อี การแสดงออกในเซลล์โปรคาริโอต western blot

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Introduction

Hepatitis E is caused by the hepatitis E virus (HEV), has two forms, epidemic and individual. Not only human can be infected with HEV, but also pig, boar, chicken and bird (Sun et al., 2004), rat (Makoto et al., 2003), cat (Choi et al., 2004; Okamoto et al., 2004), and deer. HEV genome consists of 5' and 3' non-genetic structure regions and three open reading frames (Engle et al., 2002; Emerson et al., 2003). It is approximately 7.5 kb in length. The ORF2 is 2 kb in length, encoding the viral capsid protein, which is the major structural gene coding region, where the nucleotide sequences is the most conservative. HEV has more antigenic epitopes. The structure is more complex, in addition to the N-terminal 25~38aa segment, the majority antigenic epitopes located in the C-terminal 2/3. Many experiments are based on recombinant ORF2 antigens expressed in *E. coli* (Li et al., 1997; Anderson et al., 1999), insect (Li et al., 2000; Sehgal et al., 2003), or animal (Jameel et al., 1996) cells. It is similar to other RNA viruses, RNA-dependent RNA polymerase of HEV does not copy the proofreading function, so it is prone to mutation and to produce the genetic and antigenic groups on the related virus, the existence is different HEV genotypes and subtypes. The study can lay the foundation for diagnostic antigens research with the protein of ORF2 of HEV, and provide the basis for the screening of the antigenic epitopes.

HEV is divided into four genotypes based on sequence homology in nucleotide, amino acid homology of the size and phylogenetic tree analysis. The four kinds of hepatitis E virus genotype are

genotypes 1 and 2 which are limited to humans, and genotypes 3 and 4 strains which are found in both humans and pigs (Meng, 2010). In recent years, genotype 4 of hepatitis E is mainly found in China (Hao et al., 2009; Li et al., 2009). A pandemic of HE involving 119 280 patients took place from 1986 to 1988 in Xinjiang, Western China, killing of 707 people. It is the largest and most serious hepatitis E epidemic in the world (Aye et al., 1992).

In recent years, the genotype 4 of ORF2 protein fragment of HEV has become a hot research topic (Li et al., 2009 ; Ran et al., 2010) because the viral proteins of ORF2 encoded can be used as a potential candidate of the hepatitis E virus vaccine. The objective of this study was to obtain recombinant antigen for development of anti-HEV ELISA method and vaccine against hepatitis E virus infection. Cloning and expression of ORF2 of swCH189 strains laid the foundation for studying the biological function of genes of HEV.

Materials and Methods

Virus strain: The swCH189 strains of HEV is positive by RT-PCR (Hao et al., 2011) and were preserved by laboratory of animal infectious diseases which belongs to Lanzhou Veterinary Research Institute of CAAS, PR China. The swCH189 strains of HEV belong to genotype IV. The GenBank ID number is FJ6101232.

Bacterias and vector: *E. coli* strains, pMD18-T carrier (Takara, Japan), Express bacterium Escherichia coli (E. coli) Rosetta and expression vector pET32a (+) were preserved by the laboratory of animal infectious diseases.

Primers: According to analysis of the translation product of ORF2 and the distribution of the antigen activity site, a pair of primer was designed. The primers were modified to contain restriction sites to facilitate cloning and ligating with expressed vector. The primers were synthesized (Takara, Japan). The nucleotide sequences of the primers were as follows:
U1:5'CATGCCATGGGTATTGCGCTAACCTTGTTTA ATCTTGCTGATA-3' (forward)
L1:5'ATTTGCGGCCGCTCAATACTCCCGGGTTTTA CCCACCTT-3' (reverse)
(*Note*: the underlined part of primers are enzymes digested sites, CCATGG is *Nco* I digested site, GCGGCCGC is *Not* I digested sites.)

RT-PCR: Viral RNAs were extracted from 200 µl of serum sample with RNA easy Mini Kit (Takara, Japan) according to the manufacturer's instructions, The main principles of the extraction are as follows: the main ingredient were guanidine thiocyanate and phenol in the TRIZOL. Guanidine thiocyanate could lysis cells, promote ribosome dissociation, and separate RNA and protein, then RNA was released into the solution. When adding chloroform, acidic phenol was extracted, which contributed to the RNA into the aqueous phase. The solution formed aqueous layer and organic layer after centrifugation, so RNA and the remaining organic phase proteins and DNA were separated. The extracted RNA was dissolved and precipitated with 60 µl no-RNase water. It was stored at -20^{0}C. The PCR amplification was carried out in a 20 µl reaction mix containing 8 µl of extracted RNA, 100 pmol of each of forward and reverse primers and 8 µl of 2xTakara OneStep RT-PCR Enzyme Mix. Water was added to obtain a final volume of 20 µl. The cycle conditions were 42^{0}C for 60 min and 70^{0}C for 15 min for RT, 94^{0}C for 2 min (for hot start) and then 35 cycles at 94^{0}C for 30 sec, 50^{0}C for 30sec and 72^{0}C for 1 min (for PCR amplification), and a final extension at 72^{0}C for 15 min. Finally, PCR products (2 µl) were electrophoresed in 1.0% agarose gels in a standard TAE buffer and visualised by UV light after staining with ethidium bromide.

Digestion and plasmid construction: The 728 bp of ORF2 cDNA was digested with *Not* I and *Nco* I, and the pET32a(+) vector was digested with *Not* I and *Nco* I. The purpose fragments were recycled with MiniBEST Agarose Gel DNA Extraction Kit (Takara, Japan) according to the manufacturer's instructions. The restrict fragment was inserted to the pET32a(+) of prokaryotic expression vector and formed the recombinant plasmid pET32a(+)-CP239. The recombinant was then transformed into *E. coli* Rosetta and plated with agar containing ampicillin. For confirmation of target gene, the plasmid DNA was extracted by the alkaline lysis procedure, then examined for inserts of the expected sizes by enzyme digestion (*Not* I and *Nco* I), PCR and sequencing. The nucleotide sequences of 728bp from ORF2 cDNA were identified (Takara, Japan) (dideoxynucleotide method using a commercial kit).

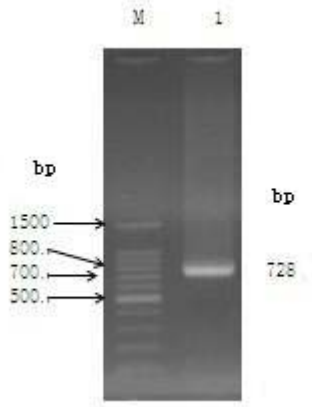
Production of pORF2 fusion proteins: The recombinant product was transformed into *E. coli* Rosetta. The transformants of pET32a(+)-CP239 was incubated into L-broth medium containing 100 µg/ml of ampicillin for 4 hours at 37^{0}C. The cultures were then induced with 0.3 mmol isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 37^{0}C with constant shaking. The supernatant and precipitate were separated through centrifugation after the bacterial pellet was ultrasonically broken (300V, 3x5 sec). Then the fusion proteins were separated on 12% SDS-PAGE gels. Bacilli were harvested by centrifugation at 3000 rpm for 10 min at 4^{0}C and kept frozen at -70^{0}C. The pellet was then resuspended in a 1/50 volume of phosphate-buffered saline (PBS) and subjected to three cycles of freeze-thawing in liquid nitrogen and cold (4-10^{0}C) water. The bacilli suspension was sonicated by five bursts of 30 sec each at 60% maximal power and centrifuged at 13,000 rpm for 15 min at 4^{0}C. 20% Triton X-100 lysis buffer was added to the postsonic pellet. The suspension was incubated for 30 min at room temperature, and centrifuged at 12, 000 rpm for 10 min. The pellet containing most of the fusion proteins was washed with PBS, and resuspended in 500 µl of 10 mmol Tris (pH 7.5) and stored in aliquots at 4^{0}C.

SDS-PAGE and western blot: The fusion protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) gel, and transferred to nitrocellulose membrane for Western blot with anti-HEV positive sera confirmed by a commercial ELISA kit (WanTai,

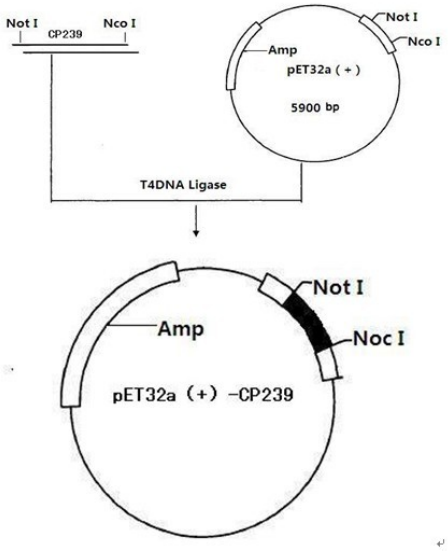
China). The membrane was blocked with 2% BSA/PBS for 2 hours at 37^{0}C. After a rinse with wash buffer (PBS containing 0.05% Tween 20, PBS-T20), strips were cut out and incubated with anti-HEV positive sera at 1:1500 dilution in PBS. After a 2 hours incubation with shaking at 37^{0}C, the strips were rinsed three times for 5 min each time with wash buffer. This was followed by incubation with a 1:1500 dilution (in wash buffer) of anti- sheep IgG horseradish peroxidase conjugate for 1 hour at 37^{0}C. After a rinse with wash buffer as above, color development was carried out with 3, 3'-diaminobenzidine as a substrate and photographed.

Results

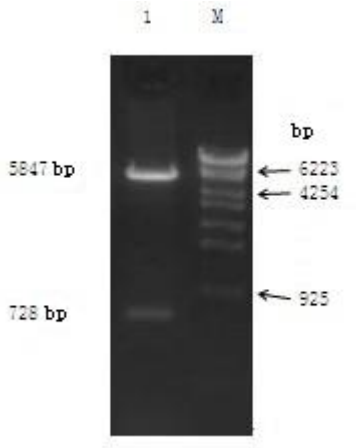
RT-PCR: The detection of HEV RNA positive was obtained from the serum of swine in Gansu Slaughter plant. A 728 basepair of ORF2 cDNA was collected by RT-PCR (Fig 1).



Construction of recombinant plasmids: The 728 basepair of ORF2 cDNA was inserted to the pET32a(+) of prokaryotic expression vector and formed the recombinant plasmid pET32a(+)-CP239 (Fig 2).

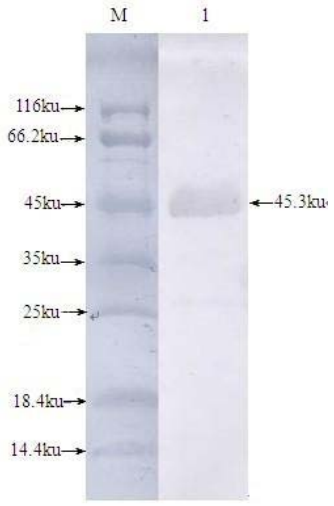


The recombinant was identified with enzyme digestion (Fig 3).



A nucleotide segment of about 728 bp was demonstrated and confirmed by sequencing. HEV nucleotide of recombinant was 100% homologous with that of the swCH189 strains of HEV.

Expression of HEV structural protein: The recombinant protein which contained 728 bp cDNA of ORF2 was expressed in *E. coli* Rosetta to produce the protein fused with GST (normal molecular mass, about 20 kDa). A clear protein band of about 45.3 kDa (Fig 4), consistent with the prediction of the molecular mass of the translation of hepatitis E virus sequences, was demonstrated by SDS-PAGE. This protein was designated GST-pORF2. The result of Western blot analysis showed that pORF2 protein was reactive with anti-HEV-positive serum at a dilution of 1:1000.



Discussion

The ORF2 is the major structural gene coding region. Kaur et al. (1992) positioned three antigen activity regions of pORF2 : 25-38, 341-354, 517-530. Khudyakov et al. (1992) also positioned four antigen activity regions of pORF2 : 319-340, 631-648, 641-660. Li et al. (1997) found that the expression of ORF2 full-length protein C-terminal peptide chain folding was good because of gene epitopes and cut the N-terminal peptide chain ORF2 antigen.

In this study, because GC content of ORF2 gene region was higher, we added the GC x buffer to improve efficiency in the cloning process. In addition, annealing temperature is one important factor in this experiment. because the undeserved annealing temperature can cause non specific amplification. If we improve the annealing temperature, it may affect the efficiency of the amplification, resulting in products decreased. The last annealing temperature was optimized at 50^0C.

In this cloning process, the RT-PCR method was used because it has higher sensitivity, quick and efficient than other methods. The index amplification of RT-PCR is a kind of very sensitive technology, it can detect very low RNA copy. RT-PCR amplification technique is widely used in the diagnosis of genetic diseases, and it may be used to quantitative monitoring the content of some RNA. The target fragment was successfully cloned, but its specificity was low, also some cloning errors occurred, We applied TaKaRa Taq™ Polymerase (Takara, Japan) to prevent its occurrence because it did not have 5'-3' exonuclease activity, but had the 3'-5' exonuclease activity. It could adjust the generated error of PCR amplification process, so the base mismatch rate of product was extremely low.

The recombinant protein pORF2 from 728 bp fragment of HEV ORF2 was expressed in *E. coli* Rosetta and confirmed by protein analysis. The molecular of the protein was 45.3 kDa, which are liable to form inclusion bodies (Kuang et al., 2009), it may be related to higher expression temperature (37^0C) and higher expression levels. It was reported that the expression protein of human HEV produced polymerization phenomenon (Meng et al., 2001; Zhang et al., 2001), but the expression product is not seen more emergence of the strip in this experiment

The recombinant protein was expressed as fusion protein that facilitates the purification process. The pET-32a (+) expression vector is a commonly used as prokaryotic expression vector. It is a higher level expression system. In this system the fusion protein is the most abundant cellular protein and is easily purified as a soluble protein. We used the method of gel purified and ultimately obtained the target protein. The western blot results showed that the fusion protein could be identified by positive serum of pig HEV and it had the main antigen epitope of natural strains of HEV. This showed that the pORF2 protein could be used in a Western blot format to detect anti-HEV in animal sera.

The next step of the research work will mainly focus on observation of whether immunization can be improved by adding or duplicating some certain antigenic epitopes and whether anti-pORF2 titers decline significantly over time. This part of the viral proteins can be used as a potential candidate of the hepatitis E virus vaccine, if we could demonstrate that it is immunologically reactive with a wide variety of human sera, not only in outbreak settings but also in sporadic cases.

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