

Original Article

Construction and identification of Complex DNA vaccine of hepatitis B and *Toxoplasma gondii*

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Received March 23, 2015; Accepted June 3, 2015; Epub June 15, 2015; Published June 30, 2015

Abstract: Objective: To construct and identify multi-gene recombinant expression vector pcDNA3-HBsAg-p30-ROP2. Method: Primers were designed according to the gene sequences of restriction enzyme cutting site of recombinant pcDNA3-p30-ROP2 and hepatitis B surface antigen (HBsAg). The target fragment of HBsAg was amplified and cloned to expression vector pcDNA3-p30-ROP2 by restriction enzyme digestion and ligation. The recombinant expression vector pcDNA3-HBsAg-p30-ROP2 was identified by PCR detection, followed by enzyme restriction and sequencing. Results: The target fragment of HBsAg was successfully amplified, and the multi-gene eukaryotic expression vector pcDNA3-HBsAg-p30-ROP2 was established. PCR detection and restriction enzyme digestion showed that the length of the target fragment was consistent with the theoretical value. The recombinant expression vector contained the complete sequences of p30-ROP2 compound gene and HBsAg. Conclusion: Multi-gene recombinant expression vector pcDNA3-HBsAg-p30-ROP2 was successfully established. The constructed expression vector could be used to develop multi-gene nucleic acid vaccines.

Keywords: *Toxoplasma gondii*, surface antigen 1 (p30), Rhopty protein 2 (ROP2), HBsAg, genetic recombination

Introduction

Hepatitis B virus (HBV) is a circular, partially double-stranded DNA virus and also the smallest double-stranded DNA virus ever known to have pathogenicity in human [1]. Hepatitis B surface antigen (HBsAg) is a constituent of HBV envelope protein with good immunogenicity and used as a candidate molecule for preparing HBV vaccines. The genetically engineered vaccines against HBV are usually the products of eukaryotic expression of processed HBsAg gene [2]. Recent studies show that HBsAg can be also used as the carrier for genetically engineered vaccines [3-5]. Possessing the activity of adjuvant, HBsAg can greatly improve the immunogenicity of exogenous genes [1, 6, 7].

Toxoplasmosis which is globally epidemic zoonotic disease is prevalent among Chinese population and livestock [8], but there are no effective control and treatment measures against toxoplasmosis. Efforts have been made to prepare vaccines against toxoplasmosis, especially nucleic acid vaccines [9]. It is indicated by

literature that *Toxoplasma gondii* surface antigen (p30) and ROP2 have good immunogenicity, which can effectively trigger the immune response [10-12]. When used in combination, the two antigen genes can produce a greater immunoprotective effect than a single gene [13-15]. In the study, the p30, ROP2 and HBsAg genes were simultaneously cloned to the eukaryotic expression vector pcDNA3 to construct the multi-gene recombinant. The findings provided a basis for the preparation of multifunctional vaccines against both HBV and *Toxoplasma gondii* infection.

Materials and methods

Materials

E.coli DH5 α strain was preserved at our laboratory. Recombinant plasmid pcDNA3-p30-ROP2 was prepared by our research team in preliminary experiment [16]. Restriction endonucleases (Hind III, Xba I), HiFi Taq DNA polymerase and T₄ DNA ligase were purchased from TaKaRa

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Biotechnology (Dalian) Co., Ltd. DNA extraction kit and gel extraction kit were purchased from Tiangen Biotech (Beijing) Co., Ltd. All other reagents were analytically pure and were repacked from imported materials or domestically manufactured.

Primer design and synthesis

Since many common loci were already removed from the recombinant pcDNA3-p30-ROP2, Hind III restriction enzyme was used for cutting and cloning. Primers of HBsAg gene were designed by the principle of ensuring accurate expression of the target proteins and preventing reading frame shift. The upstream primer was P1: 5'-AT AAGCTT ATGGAGAACATCACATC-AGGATTC-3' (T_m 62°C) and the downstream primer was P2: 5'-AT AAGCTT tgTGCCCAAAGACAAAAGAAAATTGG-3' (T_m 60.3°C). The theoretical length of the amplified fragment was 672 bp. The primers were synthesized by Sangon (Shanghai) Biotech Co., Ltd.

HBV genomic DNA extraction

The cases were detected for HBsAg, HBeAg+ and anti-HBcAg by using HBsAg (surface antibody, e antigen, e antibody, core antibody) ELISA Kit (Yingke Xinchuang Company, Xiamen). Fluorescence quantitative PCR was performed to analyze 19 serum samples with HBV-DNA content > 1.0 × 10⁸ copies/ml. HBV genomic DNA was extracted using viral genomic DNA extraction kit (spin column, Beijing BioTeke Corporation).

HBsAg gene amplification by HiFi PCR system

HBsAg gene amplification was performed using HiFi PrimeStar Taq polymerase (5 U/μl) with the extracted HBV genomic DNA as template. The 50 μl PCR reaction system consisted of the following: HBV genomic DNA 0.5 μl, upstream and downstream primers P1 (15 pmol/μl) and P2 (15 pmol/μl), 2 μl each, 5 × PrimeStar Taq buffer (containing MgCl₂) 10 μl, 2.5 mmol/L dNTP Mix 4 μl, PrimeStar Taq polymerase (5 U/μl) 0.8 μl, ddH₂O 30.7 μl. PCR reaction conditions: 94°C 5 min, 94°C 30 s, 55°C 30 s, 72°C 40 s, 30 cycles; finally, 72°C 10 min. The amplification products were analyzed by 1.0% agarose gel electrophoresis. PCR products were recovered by gel extraction kit.

Construction of recombinant pcDNA3-HBsAg-p30-ROP

Fresh recombinant plasmid pcDNA3-p30-ROP2 was extracted. Restriction enzyme digestion was performed using Hind III for the recombinant plasmid and the recovered HBsAg gene fragment, followed by recovery and purification for the second time. T₄ DNA ligase was used to clone HBsAg gene to plasmid pcDNA3-p30-ROP2 to construct recombinant pcDNA3-HBsAg-p30-ROP2. The 50 μl restriction enzyme digestion system consisted of the following: PCR product 30 μl, NE buffer 25 μl, Hind III 2 μl, ddH₂O 13 μl. The 30 μl pcDNA3-p30-ROP2 system consisted of pcDNA3-p30-ROP2 15 μl, NE buffer 23 μl, Hind III 1 μl and ddH₂O 11 μl (37°C, 2 h). The 20 μl DNA ligation system consisted of recovered products of pcDNA3-p30-ROP2/Hind III digestion 4 μl, recovered product of HBsAg/Hind III digestion 13 μl, T₄ DNA ligase 1 μl, buffer 2 μl (16°C, overnight).

PCR detection, restriction enzyme digestion and sequencing of recombinant pcDNA3-HBsAg-p30-ROP2

Some transformed clones were numbered, and a few clones were transferred to ddH₂O-containing PCR tube with the same number using toothpicks. Preliminary screening was performed. The 20 μl PCR reaction system consisted of the following: cloned colonies as template, upstream and downstream primer P1 (15 pmol/μl) and P2 (15 pmol/μl), 0.8 μl each, 10 × Taq DNA polymerase buffer (containing MgCl₂) 2.0 μl, 2.5 mmol/L dNTP Mix 1.6 μl, 5 U/μl Taq DNA polymerase 0.2 μl, ddH₂O 15.0 μl. The reaction conditions were the same as above. Some positive clones were inoculated to ampicillin-resistance LB medium. The plasmid was extracted and identified by restriction enzyme digestion. Since the recombinant pcDNA3-HBsAg-p30-ROP2 was constructed by using single restriction enzyme Hind III, there were two ways of insertion. Therefore, other enzymes were used to aid the identification. Restriction enzyme Xba I was chosen, and the 20 μl reaction system consisted of the following: NE Buffer 22 μl, pcDNA3-HBsAg-p30-ROP2 10 μl, ddH₂O 7.5 μl, Xba I 0.5 μl (37°C, 2 h). The positive clone pcDNA3-HBsAg-p30-ROP2 identified by restriction enzyme digestion was submitted for sequencing by BGI Tech. Sequence analysis was done by Blast program.

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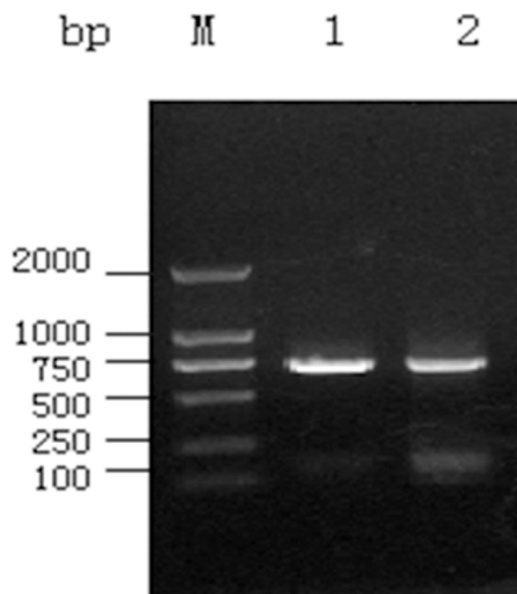


Figure 1. PCR amplification results of HBsAg gene. M: DNA Marker; 1-2: PCR products of HBsAg gene.

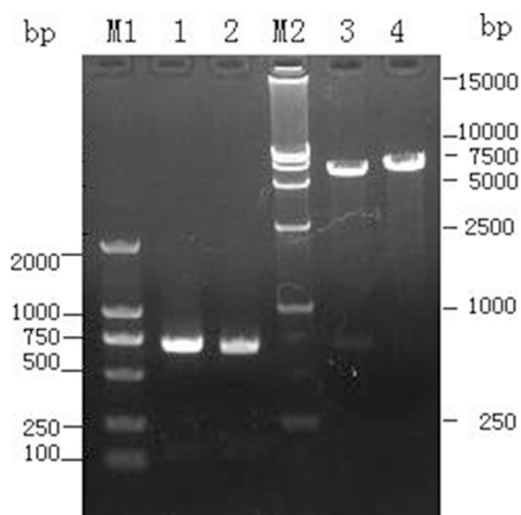


Figure 2. Enzyme products of HBsAg gene and vector pcDNA3-p30-ROP2. M1: DNA Marker (DL2000); 1-2: HBsAg gene segment digested with *Hind* III; M2: DNA Marker (DL15000); 3: pcDNA3-p30-ROP2 plasmid digested with *Hind* III; 4: Recombinant plasmid pcDNA3-p30-ROP2.

Positive single colony was picked for shaking cultivation and then cryopreserved.

Results

Results of PCR amplification of HBsAg gene

The products amplified by using HBV genomic DNA as template were identified by 1.0% aga-

rose gel electrophoresis. A band with the length of 670 bp was obtained (**Figure 1**), which conformed to the theoretical length.

Results of restriction enzyme digestion of HBsAg gene and recombinant pcDNA3-p30-ROP2

The amplified HBsAg gene fragment and recombinant pcDNA3-p30-ROP2 were digested by restriction enzyme *Hind* III. The electrophoresis results are shown in **Figure 2**.

Construction of eukaryotic expression vector pcDNA3-HBsAg-p30-ROP2

Eighteen positive cloned colonies identified by ampicillin-resistance LB medium were picked for PCR amplification. A clear band was found at about 670 bp, which conformed to the theoretical length of HBsAg gene fragment (i.e., 672 bp). Most colonies were positive clones, for example No. 1-5 and 7-18 colonies. However, the amount of amplified products of No. 6 colony was small, so No. 6 colony was considered as non-positive clone and eliminated one (**Figure 3**). Recombinant plasmid pcDNA3-HBsAg-p30-ROP2 was digested by restriction enzyme *Xba* I. Electrophoresis showed 1 clear band near 1200, 1300 and 5400 bp, respectively. They agreed with the theoretical length of the 3 fragments (1212 bp for ROP2 fragment, 1356 bp for HBsAg-p30 fragment, and 5400 bp for pcDNA3 fragment). See **Figure 4**. No. 1-3 and No. 5 colonies (channel 4 in **Figure 4**) were all positive clones as confirmed by PCR detection and restriction enzyme digestion.

Gene sequencing of recombinant pcDNA3-HBsAg-p30-ROP2

Gene sequencing was performed for recombinant pcDNA3-HBsAg-p30-ROP2, which was proved to contain the complete sequences of HBsAg gene and p30-ROP2 compound gene with a total of 2568 bases (672 + 1896). If the protective bases and the recognition bases were counted, the fragment was 2600 bp in length containing 856 amino acids. The rate of accordance with the sequences in genebank was 99.96% (2599/2600). However, there was one base mutation (A to G) at site 978 (site 288 of p30 sequence), which was identified as same sense mutation. That is, the amino acid corresponding to this position was not substituted, since both "ACG" and "ACA" are the codon for threonine (Thr).

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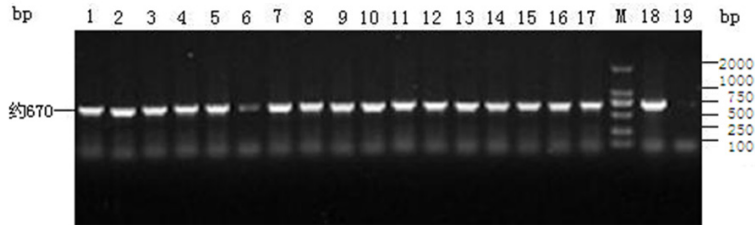


Figure 3. PCR identification of pcDNA3-HBsAg-p30-ROP2 clonal colony. M: DNA marker; 1-18: Results of pcDNA3-HBsAg-p30-ROP2 cloning PCR amplification of HBsAg gene fragments; 19: Negative control.

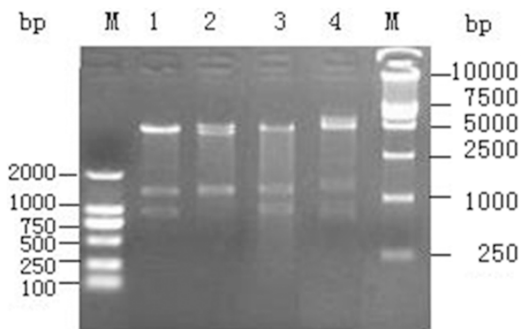


Figure 4. Endonuclease digestion identification of pcDNA3-HBsAg-p30-ROP2 clones. M: DNA marker; 1-4: Recombination vector pcDNA3-HBsAg-p30-ROP2 digested with Xba I (recombination vector after complete enzyme digestion should be divide into 1212 bp, 1356 bp and 5400 bp bands).

Discussion

Hepatitis B is an infectious disease that has a hazardous impact on human health. About 2 billion people worldwide are once infected by HBV [17]. HBV vaccination is considered as the most effective measure to control hepatitis B by triggering strong cellular immunity and humoral immunity [18]. HBsAg is commonly used in the preparation of HBV vaccines because of its strong immunogenicity. Commercial HBV vaccines are usually prepared by transferring the cloned HBsAg gene into the vector where the gene expression is induced. So far HBsAg is an ideal candidate molecule for vaccine preparation and provides the basis for manufacturing multifunctional vaccines against other diseases [2-5].

As shown by many studies on eukaryotic expression vectors, pcDNA3 contains high-efficiency promoter/enhancer sequences of human cytomegalovirus (CMV), Bostaurus growth hormone (BGH), polyA and ampicillinum resis-

tance gene. Expression vector pcDNA3 can be used for high-efficiency expression of exogenous genes in mammals [19, 20].

Surface antigens (SAG) of *Toxoplasma gondii* include microneme protein (MIC), ROP and dense granule antigen (GRA). ROP2 and p30 are generally recognized as the most potential antigens.

Our research team has carried out in-depth study on SAG of *Toxoplasma gondii* [21-24]. P30 is an important surface membrane protein related to the adhesion and invasion of *Toxoplasma gondii* [25, 26]. Zhang et al. [27] performed immunization with p30 protein to increase the resistance against *Toxoplasma gondii* infection in mice. Secreted by *Toxoplasma gondii* during host invasion and expressed in tachyzoites, bradyzoites and sporozoites [28], ROP2 plays the key role in host invasion by *Toxoplasma gondii* [29, 30]. Expression vector pcDNA3-HBsAg-ROP2 was established in one of our previous experiments, and the effect was satisfactory [31]. However, ROP2 and p30 alone failed to provide full protection despite the high titer, especially during the acute stage of *Toxoplasma gondii* infection [24]. In the present study, the SAG of *Toxoplasma gondii* was cloned along with HBsAg and ROP2-p30 compound gene into the expression vector pcDNA3. Sequencing showed that the multi-gene recombinant vector was successfully constructed and the full sequences of HBsAg, ROP2 and p30 genes were contained. This multi-gene recombinant expression vector can be used for in vitro expression experiment and immunogenicity analysis.

Though proved to be a good eukaryotic expression vector, pcDNA3 is expressed in small quantity and unstably. The reason may be related to the high transcriptional level of enhancer and promoter sequences of CMVIE gene and the exogenous genes along with the inherent characteristics of the host cells [20]. Since pcDNA3 vector does not contain fluorescent protein reporter gene, it is impossible to observe the cell transfection directly. The procedures of transfection and target gene expression will be greatly simplified if the eukaryotic

expression vector containing fluorescent protein reporter gene can be constructed.

Acknowledgements

This work was Supported by the Natural Science Fund of Shandong Province (NO. 2009ZRC-03083) Promotive Research Fund for Excellent Young and Middle-aged Scientists of Shandong Province (Grant code: BS2013SW015), and Grant from Shandong province medicine and health technology development program (NO. 2014WS0330).

Disclosure of conflict of interest

None.

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