Journal of Virological Methods 212 (2015) 17–22

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Expression of *E2* gene of bovine viral diarrhea virus in *Pichia pastoris*: A candidate antigen for indirect Dot ELISA

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Article history:
Received 16 June 2014
Received in revised form 24 October 2014
Accepted 31 October 2014
Available online 13 November 2014

Keywords:
Bovine viral diarrhea virus
E2 gene
Immunogenicity
Indirect Dot-ELISA

A B S T R A C T

The *E2* gene containing *the Eco*R I and *Not* I sites of bovine viral diarrhea virus (BVDV) was amplified from the plasmid pMD-18T-E2 of the HB-bd isolated, and inserted into *Pichia pastoris* (*P. pastoris*) expression vector pPIC9K, and transfected into *Escherichia coli* DH5α. The recombinant plasmid pPIC9K-E2 was digested by the *Sal*I restriction enzyme and transformed into the *P. pastoris* strain GS115 by electroporation. High copy integrative transformants were obtained by G418 screening and induced for expression with methanol. The expressed products in the culture medium were identified by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the Western blotting and the antibody test for immunity. An indirect Dot-ELISA for the detection of antibody against BVDV was established by the recombinant E2 protein as the coating antigen. The reaction conditions of the indirect Dot-ELISA were optimized. The coating concentration of the E2 recombinant protein antigen, the dilution of serum sample, the optimal concentration of HRP labeled antibody, the optimal blocking reagent and blocking time were studied. 100 sera samples from cows in the field were tested for the antibody against BVDV by the Dot-ELISA and the IDEXX HerdChek BVDV antibody ELISA kit simultaneously to compare the specificity, sensitivity and accuracy.

The results showed that the expressed products in the culture medium resulted in single band of 44 kDa by SDS-PAGE and Western blotting. The results of the immunogenicity assay indicated that the protein E2 expressed in *P. pastoris* could induce the experimental animals of the rabbit to produce BVDV specific antibodies. The results of the indirect Dot-ELISA showed that the optimal coating concentration of the E2 recombinant protein was 2.0 μg/mL, the bovine serum dilution was 1:100, the optimal concentration of HRP-labeled rabbit anti-bovine antibody IgG was 1:500, and the optimal blocking reagent was 3% glutin-TBS and blocking for 45 min. The indirect Dot-ELISA showed 96.7%, 92.5% and 95% in the terms of specificity, sensitivity and accuracy compared to the IDEXX ELISA test kit. The indirect Dot-ELISA using the E2 recombinant protein can be used for the detection of antibody against the BVDV and could be considered in the surveillance programs.

1. Introduction

Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* within the family Flaviviridae. The virus has a positive stranded RNA (Pringle, 1999) with genome length of approximately 12.5 kb (Deng and Brock, 1992; Xu et al., 2006; Zhu et al., 2009; Li et al., 2010). The viral genome consists of a single open reading frame encoding both structural and non-structural proteins. The structural proteins comprise the nucleocapsid C protein and three envelope glycoproteins, Erns, E1 and E2. The Erns and E2 protein are required for virus attachment and/or cell invasion (Hulst and Moormann, 1997). The envelope glycoprotein E2 contains the major immunogenic epitopes of BVDV responsible for eliciting strong humoral immune responses, principally neutralizing antibodies (Bolin, 1993; Nobiron et al., 2001, 2003) which are protective against subsequent infections (Meyers et al., 1989; Harpin et al., 1999). Therefore, the E2 protein is suitable as a candidate antigen for developing a BVDV engineered vaccine and for immunodiagnosis and immunoprophylaxis tools (Bruschke et al., 1997; Reimann et al., 2010).

Pichia pastoris (*P. pastoris*) is an ideal eukaryotic expression system which has the dual characteristics of good maneuverability

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http://dx.doi.org/10.1016/j.jviromet.2014.10.017
0166-0934/

of prokaryocyte and posttranslational modification of eukaryocyte. The *P. pastoris* eukaryotic expression system which contains strong promoter AOX (alcohol oxidase) is tightly regulated and induced by methanol and it is used for the expression of the gene of interest. The yeast expression vector pPIC9K is a shuttle vector containing the kanamycin gene enabling to get transformants with high copies (Scorer et al., 1994) and substantial expressions of the target protein. The fusion proteins expressed in *P. pastoris* GS115/pPIC9k are secreted into the culture medium. With only low levels of the endogenous protein secreted to the media by the yeast itself and no added proteins to the media, a heterologous protein builds the majority of the total protein in the medium and facilitate following the protein purification steps (Harpin et al., 1997). Therefore, the present study was designed to express the *E2* gene of the isolated BVDV strain in *P. pastoris* and develop an indirect Dot-ELISA for detection of antibody against BVDV using the recombinant E2 protein as the coating antigen.

2. Materials and methods

2.1. Strain, plasmid and reagents

The P. pastoris GS115 strain and *P. pastoris* expression vectors pPIC9 K were purchased from Invitrogen (USA). The plasmid pMD-18T-E2 of HB-bd strain BVDV E2 gene was provided by the Health inspection and quarantine Laboratory of the College of Veterinary Medicine, Agricultural University of Hebei, China (Zhao et al., 2008). The cloning vector pMD18-T, Restriction endonuclease *Eco*R I, *Not* I, *Sal* I, *Taq* DNA polymerase, T4 DNA ligase were provided by TaKaRa (Dalian, China). The mini plasmid extraction kit and DNA gel Extraction kit were from Huashun (Shanghai, China). The IDEXX HerdChek BVDV antibody indirect enzyme-linked immunosorbent assay (iELISA) Kit was purchased from the IDEXX Corporation (USA). The BVDV standard positive and negative sera, Classical swine fever virus (CSFV) standard positive sera and Bovine rotavirus (BRV) standard positive sera were obtained from China Institute of Veterinary Drug Control (Beijing, China).

2.2. Amplification of the E2 gene of BVDV

The HB-bd strain of BVDV was firstly isolated from the aborted cattle at a dairy farm in Hebei province, China. The E2 glycoprotein fragment containing the *Eco*R I and *Not* I sites was amplified by PCR utilizing the plasmid pMD-18T-E2 of BVDV HB-bd strain as a template using the forward primer (P1) and reverse primer (P2). The primer set (P1/P2) was designed by Primer 6.0 software based on the published *E2* sequence of HB strain of BVDV (Zhao et al., 2008) and multiple cloning sites (MCS) of the cloning vector pMD18-T and expression vectors pPIC9K. For cloning of the products in the expression vector pPIC9K, the primer set was designed to contain the restriction enzyme sites *Eco*RI and *Not*I in forward primer (P1) (5′-CGGAATTCATGGTGAAGGTGG-3′) and reverse primer (P2) (5′-GGCGGCCGCTTACACTATTTC-3′), respectively. The reaction mixture consisted of 10 × PCR buffer (4 μL), plasmid pMD-18T-E2 template (3 μL), dNTP Mixture (2 μL), forward primer p1 (1 μL, 2.5 pmol/μL), reverse primer p2 (1 μL, 2.5 pmol/μL), Taq DNA polymerase (0.3 μL, 5 U/μL) and DEPC-H_2O (up to 20 μL). PCR was initially denatured at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s and extension at 72 °C for 90 s. The reaction was subjected for final extension at 72 °C for 8 min. Products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under UV light. The purified *E2* gene fragments were cloned into the pMD18-T Vector and then transfected into *Escherichia coli* JM109. Positive clones were screened out based on white and blue colonies. The recombinant plasmid (pMD18-E2) was extracted by mini plasmid extraction kit and identified by the restriction *Eco*RI and *Eco*RI/*Not*I enzyme digestion.

2.3. Construction and identification of the recombinant expression vector pPIC9K-E2

The recombinant pMD18-E2 plasmids and pPIC9K vector were digested by the *Eco*R I and *Not* I, respectively. The digested products were extracted from the pMD18-E2 plasmids by DNA gel Extraction kit, ligated to pPIC9K vector by T4 DNA ligase at 14 °C for 12 h and transformed into competent *E. coli* DH5α. The transformed bacteria were plated on culture medium containing the ampicillin overnight at 37 °C. The recombinant expression plasmid pPIC9K-E2 was extracted by the alkaline lysis method and identified by the *Eco*R I and *Eco*R I/*Not* I enzyme digestion and PCR.

2.4. Transformation of GS115 yeast strain

The recombinant plasmid pPIC9K-E2 was linearized with the *Sal* I and transformed into *P. pastoris* strains GS115 by electroporation. The transformed products were spread on MD plates and incubated at 30 °C for 2–3 days (Barr et al., 1992) until colonies appeared. Selected His^+ single colonies were cultured on MM and MD medium plate at 30 °C for 3 days, yielding His^+Mut^+ recombinants. The high copy integrative transformants were screened by YPD plates that contained different concentrations of G418 at 30 °C for 3–4 days.

The single colonies of yeast transformants were picked up and identified by colony PCR utilizing yeast vector pPIC9K specific primers 5′α-factor/3′AOX1 (5′α-factor: 5′-TACTATTGCCAGCATTGCTGC-3′, AOX1: 5′-GCAAATGGCATTCTGACATCC-3′) and E2 primers P1/P2, respectively.

2.5. Induction of the E2 protein expression in P. pastoris

The single colonies of the yeast transformants were inoculated into 20 mL of BMGY medium and incubated at 25–28 °C with continuous shaking at 200 rpm for 16–24 h. At OD600 = 1.0, the cells were harvested by centrifugation at 2800 × *g* for 10 min. The cells were resuspended in 20 mL of BMMY medium. Expressions of the E2 fusion proteins were induced in recombinant *P. pastoris* strain GS115 by the addition of 1% methanol to the culture medium and incubation at 25–28 °C with continuous shaking at 200 rpm. The supernatant was obtained after centrifugation and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The expressed products were purified through Ni^{2+} affinity chromatographic column and the concentration of purified products was determined.

2.6. Immunogenicity of E2 expressed protein

Experimental animals were 2.0–2.5 kg, healthy and male rabbits, bought from Qingyuan Rabbit Breeder Farm in Hebei Province of China, provided with feed and water without any antibiotic. All animal studies complied with Guidelines for Ethical Conduct in the Care and Use of Experimental Animals, China.

The expressed products were emulsified in the complete Freund's adjuvant (CF, Sigma), and inoculated to 4 healthy male adult rabbits at a dose of 500 μg/rabbit by intramuscular injection. The animals received a booster dose in Freund's incomplete adjuvant after 21 days by the same route. In the control group, 4 healthy male adult rabbits were inoculated with physiological saline at dose of 500 μL/rabbit. One week later, the rabbits were bled and sera were collected, inactivated at 56 °C and used to detect anti-BVDV antibodies by indirect-ELISA using ELISA kit of DEXX.