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Full Length Research Paper

A recombinant E_0 gene of bovine viral diarrhea virus protects against challenge with bovine viral diarrhea virus of sika deer

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Eukaryotic expression plasmid (PVAX 1/E_0) of E_0 gene was constructed from an isolated strain of bovine viral diarrhea virus (BVDV) from sika deer and transfected into the BHK-21 cells to detect E_0 gene transcription expression level and protein expression level. It was found that E_0 gene could express in BHK-21 cells by RT-PCR and ELESA results. The humoral and cellular immune response levels were detected in rabbits immunized with PAVX1/E_0 and inactivated vaccine by ELESA technology, which reached the peak after 42 days immunization and PVAX1/E_0 immune rabbits could produce specific higher cells immune responses level in comparison to inactivated vaccine group. This is the first report demonstrating and "immunogenicity" of BVDV E_0 gene on "sika deer", indicating a new vaccine target for BVDV.

Key words: Sika deer, bovine viral diarrhea virus, E_0 gene, eukaryotic expression, immune response.

INTRODUCTION

Bovine viral diarrhea virus (BVDV）is a kind of small, single-stranded enveloped RNA virus, a member of the family Flaviviridae (Francky et al., 1991; Pringle et al., 1999), BVDV is a globally distributed virus in many animals, such as deer, cattle, pigs, sheep, and horizontal transmission has been considered the possible mode of infection. BVDV has been established as a causative agent of substantial economic and genetic losses to the beef and dairy industries (Passler et al., 2007), and can cause fatal diarrhoea syndrome, respiratory problems and reproductive disease in cattle. There is evidence of manifestations with early embryonic death, abortion, cerebellar hypoplasia, infertility in reproductive form of the disease and congenital or persistent infections of the fetus (Grooms et al., 2004; Campen et al., 2001). Over the years, a number of reports that natural infection with BVDV may cause persistent infection in white-tailed deer (Chase et al., 2004; Passler, 2007). However, there was only limited information and research on sika deer (Cervus nippon, cervid endemic to mainland and insular Asia).

Different strategies are available to control the spread of BVDV in a herd: vaccination, including test-and-cull schemes. Among these strategies, test-and-cull schemes have been successfully applied in Scandinavian countries on the condition of combining with strict control of new animal introductions into herds (Viet et al., 2006). For sika deer, it is an economic loss to adopt the test-and-cull, therefore vaccine research is likely to be an attractive alternative to prevent BVDV throughout the animals. Since the immunogenicity of BVDV inactivated

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vaccine was not strong enough to develop formal domestic-made BVDV vaccine, the development of genetic engineering vaccine has become a. BVDV has an approximate 12.5 kb RNA genome and its genome possessing a single open reading frame. (Collett et al., 1988a). BVDV has four structural proteins: the nucleocapsid protein C and three envelope glycoproteins E_0 (gp48, also named E^{rns}), E_2 (gp53) and E_1 (gp25) (Donis and Dubovi, 1987a, b; Collett et al., 1988b; Thiel et al., 1991). Among these proteins, E0 and E_2 were identified as two major targets for the design of the (Weiland et al., 1990). E_2 is considered to be the major neutralizing antigen for BVDV infection (Greiser-Wilke et al., 1990; Rumenapf et al., 1993), while E_0 serving as a glycosylated protein has several functions, such as virus attachment and entry to target cells, production of neutralizing antibodies, and virulence.

BVDV is easily escaped from the neutralizing antibody could be due to the large sequence variation and higher rate of structural protein mutation of E_2 gene, however, E_0 gene have more conserved sequence. These suggest that the E_0 of BVDV is a candidate protein for a subunit vaccine. Obtaining E_0 gene is the key factor to develop gene engineering vaccine which could prevent bovine viral diarrhea (BVD). It is more to prevent the deer from BVD by employing deer BVDV separate strain E_0 gene. In this study, we carried out the eukaryotic expression of deer BVDV separate strain E_0 gene and evaluated the immunogenicity of it.

MATERIALS AND METHODS

Cells and viruses

Suspected deer BVDV was collected from deer abortion fetal livers Li et al., 2003. $C_{24}V$ standard strain of (Madin-Darby bovine kidney) MDBK cells was bought in Chinese Veterinary Drug Control Room. The suspected deer BVDV was propagated in Madin Darby bovine kidney (MDBK) cells. Cytopathic effect (CPE) Virus was harvested from cells by a single freeze-thaw method. CPE Virus was centrifuged at 5000r/min for 15 min at room temperature. The supernatants were centrifuged at 3000r/min for 45 min. The precipitation was subsequently dissolved to be virus concentrate by Serum-free 1640 Nutrient solution of 1% original virus solution.

250 µl of the Virus concentrate was subjected to RNA extraction followed by TriPure Kit (Takara, China) the manufacturer's protocol and recommendations. The E_0 coding region was amplified by polymerase chain reaction (PCR) using a specific primer pair corresponding to E_0 sequence (the forward primer: 5'-CCGGATCCACCATGGAAAACATAACACAGTGG-3'; the reverse primer: 5'-GCCTCGAGTTAAGCGTATGCTCCAAACCACGT-3'). Amplification with a pre-heated thermocycler consisted of one cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 110 s and a final extension step of 10 min at 72°C. Plasmid pMD18-T/E_0 was constructed by inserting the fragment of E_0 gene into the *EcoRI* site of the vector pMD18-T (Takara, China). Cloning was performed as described by beer (1996).

Briefly, plasmid pMD18-T/E_0 was transferred into the expression vector strain JM109 (Promega, USA). All constructs were verified by restriction digestion and sequencing.

Construction, extraction and determination of eukaryotic expression vector PVAX 1/E_0

The pMD18-T/E_0 was excised from the by BmH I and Xho I digestion and ligated into the pVAX1 eukaryotic expression vector cut by the same enzymes, resulting in construction of the PVAX 1/E_0 plasmid. The recombinant plasmid PVAX 1/E^{rns} was transformed into the *E. coli* strain DH5a. We conducted the blue/white screening using X-gal and IPTG to select the white colonies (positive clones). Recombinant plasmid DNA from positive clones was extracted by the alkaline lysis method according to Birnboim and Doly (1979). The PCR analysis of recombinant plasmid DNA was performed as described above for E_0 gene amplification with the same primers as previously described.

Identification of PVAX/E_0 expression in eukaryotic cells *in vitro*

The baby hamster kidney (BHK)-21 cells were routinely cultured at 37°C in a 5% CO_2 atmosphere in 1640 nutrient medium containing 8% horse serum (HS) without antibiotics for 24 h. The recombinant plasmid PVAX 1/E_0 and PVAX 1 DNA transfection were performed with lipofectamine (Gibco, USA) as specified by the manufacturer After incubation at 37°C, two experiments were conducted as followed: first, total RNA was extracted and RT-PCR analysis of the E_0 gene was performed as previous described. Secondly, recombinant virus was recovered by three circles of freeze-thaw at -20. Supernatants were harvested and centrifuged at 10000 rpm for 5 min to remove the cell debris. 90 µl supernatants blocked with10 µl coating buffer (pH = 8.0) was added to the "microtiter" plate at 4°C overnight.

After removing the liquid, the medium was blocked with 10% horse serum at 37°C for 1 h, then washed three times with PBST, and incubated for 1 h with BVDV positive rabbit serum (or bovine serum) (1:20) at 37°C in a humidified box. The medium were then washed three times with PBST and incubated with goat anti-rabbit ELISA second antibody (or rabbit anti-bovine ELISA second antibody)(1:5000) for 1 h. Washed three times and the substrate was added for color reaction for 15min.

The division and immunity of laboratory animals

Healthy long-eared Japanese rabbits were randomly divided into ten groups (four rabbits per group). Group 1 was injected with normal sodium (1 ml); group 3 received deer BVDV inactivated oil vaccine (1 ml); group 4 $C_{24}V$ strain inactivated oil vaccine (1 ml); group 5 $C_{24}V$ strain inactivated water vaccine (1 ml); group 9 pVAX/E_0 (1.0 mg); group 10 PVAX1 (1.0 mg). The rabbits were five additional groups were injected according to the same regime. Animals in group 2 was injected with deer BVDV inactivated oil vaccine (1 ml); group 6 received with pVAX/E_0 (0.5 mg), group 7 was injected with pVAX/E_0 (1.0 mg) and group 8 with pVAX/E_0 (1.5 mg). The last five groups were immunized once at 0 day. Sera and venous blood (0.4 ml) were collected from rabbits' ear vein at 7, 14, 21, 28, 35, and 42 days after immunization.

Detection of rabbit serum antibody by ELISA

BVDV soluble whole virus antigen was diluted with coating buffer (pH = 8.0) and added into microtiter plate of 100 µl (containing viral protein of 100 µg) in each well. The microtiter plate was coated overnight at 4°C. After removing the liquid, the medium was washed three times with PBST, and blocked with 10% horse serum at 37°C for 2 h, then washed three times with PBST, and incubated for 1 h with 100 µl rabbit serum to be detected (1:200) at 37°C. Added the substrate for color reaction at 37 °C for 15 min and terminated the reaction by adding 2M H_2SO_4 (50µl/well).

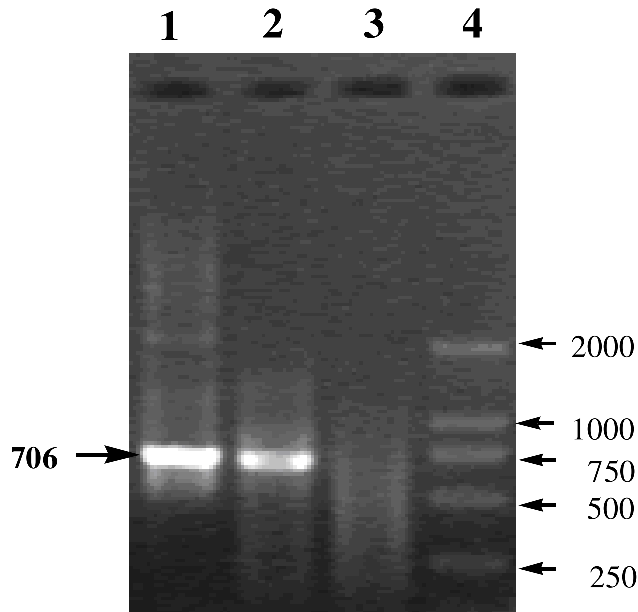

Figure 1. PCR identification of eukaryotic expression recombinant plasmid PVAX 1/E0: The band indicated by an arrow is a 706bp fragment. 1-2 PVAX1/E_0; 3.PVAX1; 4.DL 2000.

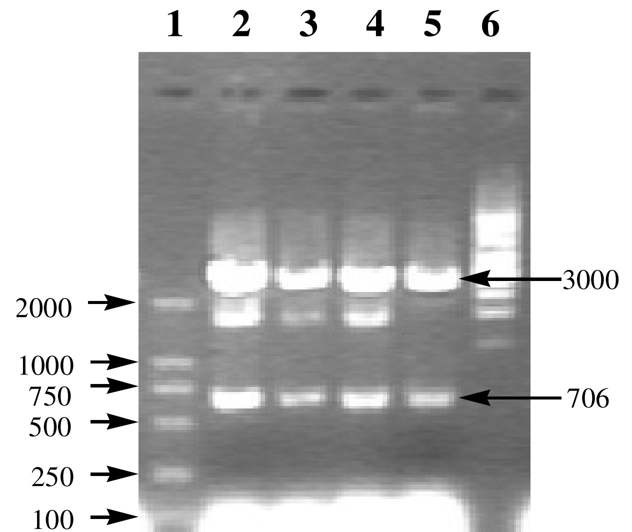

Figure 2. Identification of eukaryotic expression vector PVAX1/E_0 by digestion: 1. DL 2000; 2-5.product of PVAX1/E_0; 6.Ladder 15000.

Detection of cellular immune transformation of lymphocyte

2 ml cardiac blood was collected from each rabbit at 42 day after the first immunization using heparin as the anticoagulant. Lymphocyte was separated by stratification of lymphocyte and its survival rate was identified by 5% tyiophenol blue test. The survival rate> 95% was considered to be qualification. To obtain the cell concentration of 5×10^6 stain/ml, we added 10% calf serum 1640 cell culture medium containing double antibody to lymphocyte obtained. Cell suspension was added in the 96 microplate with each well containing at the same time, the negative control groups with only cell culture medium were set. Every group was set. After 40 to 48 h incubation at 37℃ in 5% CO_2 atmosphere, was added to each well and culture for 4 h. The supernatant were carefully 100 µl DMSO was added to each well, with pipettes blowing for several times.

The absorption value (A) was measured at 570 nm after micro shocks in high performance microplate spectrophotometer.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software (SPSS, USA) to determine the differences among groups.

RESULTS

PVAX/E_0 eukaryotic expression of recombinant plasmid PCR and enzyme digestion identification

E_0 gene was connected to the pVAX1 vector and was transformed into *E. coli* DH5a. After screening, recombinant plasmid (pVAX/E_0) was obtained. The use of E_0 gene-specific primers in PCR reaction carried out on RNA extracted from pVAX1/E_0 produced an amplified size of 706 bp of purpose band (Figure 1). The result was shown in Figure 2, PVAX1/ E_0 was digested with BamH I and Xho I two expected size of 3000 and 706 bp, respectively.

Detection of PVAX/E_0 in eukaryotic cells expression and transcription

BHK-21 cells were transfected PVAX/E_0 recombinant eukaryotic expression plasmid after 72 h, total RNA of cells was extracted and subjected to RT-PCR amplification and obtained a DNA band of 706 bp (Figure 3), The OD_{490} values of all PVAX 1/E_0 group tested by indirect ELISA methods were significantly higher than those of pVAX1 empty vectors group, BHK cells group, respectively ($p<0.05$); and similar with that of viruses group (Table 1).

Value of recombinant eukaryotic expression plasmid of PVAX/E_0 and of inactivated vaccine antibodies of the immune rabbits is shown in Figure 4. Before immunization, each group showed no significant difference ($P< 0.05$), while after immunized rabbits for the first seven days, the serum antibody in vaccine immune group rabbit was significantly higher than that of saline and pVAX1 control group. Then antibody levels gradually increased with the trend rising quickly in inactivated vaccine immune group. The antibody levels of deer BVDV C_{24}V inactivated vaccine and inactivated vaccine immune group reached the peak after 35 day of immunization, while the antibody level of recombinant

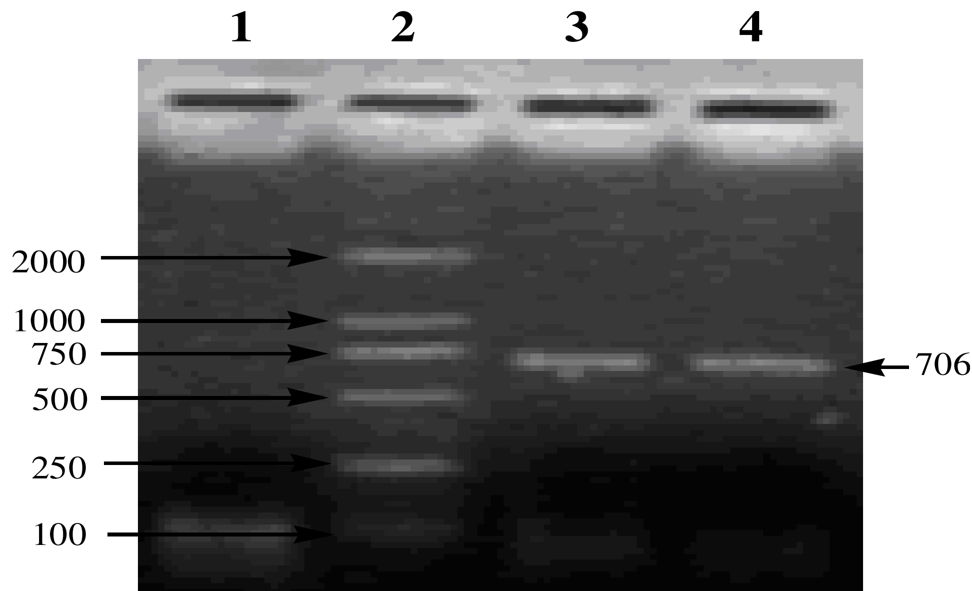

Figure 3. Detection of transcripts of E_0 genes in eukaryotic cells by RT-PCR: cell liquid of PVAX1 transfection BHK-21; 2 .DL2000; 3-4. Cell liquid of plasmid PVAX1/E_0 transfection BHK-21.

Table 1. PVAX1/E_0 expression in eukaryotic cells estimated by the ELISA tests, different letter represent the significant difference at $p<0.05$.

Groups	Cell	pVAX1/E_0	Virus	pVAX1
Rabbit anti-cattle	0.21 ±0.05a	0.37 ±0.07b	0.42 ±0.08b	0.21 ±0.03a
Goat anti-rabbit	0.24 ±0.05a	0.48 ±0.08b	0.47 ±0.08b	0.23 ±0.03a

eukaryotic expression plasmid of PVAX/E0 reached the peak after 42 day immunization. The antibody levels of gene vaccine group (Group 7, 8, and 9) were higher than those of the deer BVDV $C_{24}V$ inactivated vaccine and inactivated vaccine immune group, respectively.

Our results indicated that the rabbits immunized with recombinant eukaryotic expression plasmid of PVAX/E_0 produced humoral immune responses. in the reaction of PHA or LPS induced lymphocyte proliferation of rabbits, there was a significant improvement on the lymphocyte proliferation of rabbits stimulated by recombinant eukaryotic expression plasmid of PVAX/E_0 and inactivated vaccine, with the inactivated vaccine exerting more effect (Table 2). High-dose group of recombinant eukaryotic expression plasmid of PVAX/E_0 presented stronger effect than that of low-dose group. Immunization times had no relationship with the lymphocyte proliferation of rabbits (Table 2). These results indicated that deer BVDV recombinant eukaryotic expression plasmid of PVAX/E_0 could stimulate cellular immune response in rabbits.

DISCUSSION

Over the years, bovine viral diarrhea disease control, despite of the quarantine, isolation and test-and-cull measures, they have not been able to fundamentally resolve this problem. Recently, an upward trend of BVD is present throughout the world, while the root of the BVDV prevalence is mainly related to the absence of vaccines and special medicines. So investigation on the new, safe, efficient bovine viral diarrhea disease vaccine has become the hotspot in controlling the popularity of the BVD. BVDV is capable of infecting pigs which has aroused people's concern. For the classical swine fever "immune failure", apart from the common agents such as the of immunization dose, unreasonable immunization procedures, and the possible variation of the virus antigen. BVDV infection may be one of the important

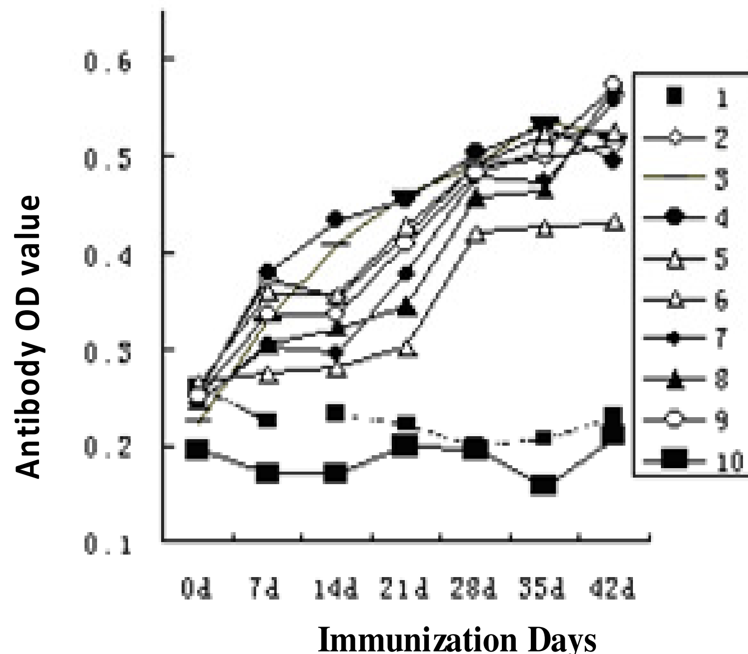

Figure 4. Humoral immunity of rabbits with PVAX1/E_0.

Table 2. Effect of PVAX1/E_0 gene vaccinate and kill vaccinate of lymphadenosis of rabbits, the same letters indicated significant difference (P<0.05); different letters indicated no significantly difference (P<0.05).

Groups	PHA stimulation OD value	LPS stimulation OD value
1	0.186±0.011a	0.168±0.020a
2	0.414±0.044c	0.289±0.025c
3	0.405±0.055c	0.304±0.026c
4	0.394±0.036c	0.313±0.037c
5	0.407±0.047c	0.308±0.043c
6	0.219±0.019a	0.242±0.033b
7	0.271±0.022b	0.256±0.026b
8	0.284±0.026b	0.331±0.031c
9	0.278±0.029b	0.260±0.028b
10	0.184±0.013a	0.162±0.017a

reasons to create the disease.

Recent epidemic atypical of classical swine fever could have the relationship with BVDV. Deer BVDV shared 78.3 to 79.9% homology of amino acid sequences with that of E_0 protein in all HCV strains. Further research is needed to determine whether it is effective when the vaccine strains are applied in preventing the atypical of classical swine fever. No surveys concerning the deer BVDV E_0 gene eukaryotic expression and immunogenicity of domestic and wild have been reported yet. The breakthrough of this study was to construct deer BVDV E_0 gene eukaryotic expression plasmid PVAX 1/E_0. It was found that E_0 target gene could be expressed in BHK-21 cells; otherwise, PVAX1/E_0 immune rabbits could produce specific humoral immune and cells immune responses as well. Therefore, made deer BVDV genetic engineering subunit vaccine and DNA vaccine become possible in further research.

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