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Short Paper

Cloning and secretory expression of *VP2* gene of infectious bursal disease virus in eukaryotic cells

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Summary

VP2 gene coding region of a vaccinal strain (D78) of infectious bursal disease virus (IBDV) was cloned in a eukaryotic expression vector, pSec Tag2A. The gene was placed downstream of Ig κ chain leader sequence, under the control of human cytomegalovirus (hCMV) immediate early enhancer and promoter. The construct pSec Tag2A-*VP2* was transfected in COS-7 cell line and the expression and secretion of *VP2* was assessed by dot blotting and antigen capture ELISA. The antibody used in the immunological assays was a neutralizing monoclonal antibody (1A6) against *VP2*. Positive reaction with the antibody indicated the construct was functional with respect to expression and secretion of a native *VP2*.

Key words: Infectious bursal disease, IBDV, *VP2*, Eukaryotic expression, COS-7 cell line

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Introduction

Infectious bursal disease (IBD), also named as Gumboro, is an acute and highly contagious viral disease of young chickens. The disease has a worldwide distribution and adversely affects poultry health and production revenue in the poultry industry (Luckert and Saif, 1997). Infectious bursal disease is caused by infectious bursal disease virus (IBDV), a member of the Avibirnavirus genus in the family Birnaviridae (Leong *et al*., 2000).

The target organ for IBDV is the developing bursa of fabricius of young birds. Replicating IBDV causes a depletion of B cells by inducing apoptosis, resulting in severely immunodepressed chickens (Luckert and Saif, 1997).

The genome of IBDV consists of two segments, A and B, of double-stranded RNA, which are localized within a single shelled icosahedral capsid of 60 nm diameter (Dobos *et al*., 1979; Müller and Nitschke, 1987). The segment A (3.2 kb) contains two partially overlapping open reading frames (ORFs), A1 and A2. Open reading frame A2 encodes *VP5* (Mundt *et al*., 1995), recently shown to be non-essential for virus replication *in vitro* (Mundt *et al*., 1997). Open reading frame A1 encodes a 106 kDa polyprotein that is self-processed to give three mature proteins: *VP2*, *VP3* and *VP4*. *VP2* and *VP3* form the viral capsid, and *VP4* appears to be responsible for proteolytic cleavage of the polyprotein (Hudson *et al*., 1986). The segment B (2.9 kb) encodes *VP1*, which has RNA dependent RNA polymerase activity (Müller and Nitschke, 1987).

The protein *VP2*, placed on the outside of capsid, contains virus neutralizing epitopes that are responsible for eliciting neutralizing antibody (Azad *et al*., 1987; Becht *et al*., 1988). Neutralizing, *VP2*-specific antibodies protect susceptible chickens from IBDV infection (Fahey *et al*., 1989), and the protein is considered to be the

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major host-protective antigen. Therefore, attempts have been made to generate *VP2* subunit vaccines (Jagdish *et al*., 1988), and recombinant viruses (e.g., fowl pox virus) containing *VP2* gene of IBDV (Shaw and Davison, 2000).

In recent years, several groups have also tried the DNA vaccination, as an alternative approach to vaccinate and protect chickens against IBD (Fodor *et al*., 1999; Chang *et al*., 2001; Hilton *et al*., 2002; Mahmood *et al*., 2006). These studies have been pursued with some positive and promising results.

Recently, we described the cloning and expression of *VP2* gene in a eukaryotic expression vector (pCDNA4-*VP2*) (Hushiarian *et al*., 2007). The purpose of the present work was to construct a eukaryotic plasmid, expressing *VP2* in secretory form, for future application as DNA vaccine, compared to pCDNA4-*VP2*.

Materials and Methods

Virus and cell

Infectious bursal disease virus, vaccinal strain D78 (Intervet, Holland), was propagated in COS-7 cell culture (a kidney cell line of African green monkey, Pasteur Institute of Iran) and used for genomic extraction and as positive control in immunological assays. COS-7 cells were also used for plasmid transfections.

Construction of recombinant expression vector

Genomic dsRNA was extracted from D78 vaccine strain of IBDV by Tripure isolation reagent (Roche, Germany), as per the manufacturer instruction. The primers F (5′GCCGGGTACCCATGACAAACCTGC AAGAT-3′) and R (5′-GCCGGATATCTTACCTTATGCCCCGG A-3′) were designed, based on the *VP2* sequence of D78 strain (accession number: AF499929), and used for cDNA synthesis and PCR amplification of *VP2* coding region. PCR amplification was performed by pfu thermo stable DNA polymerase (Fermentas, Lithuania) to avoid introducing mutations. Amplified *VP2* was subsequently digested with *EcoRV* and *KpnI* restriction enzymes (Fermentas, Lithuania), through the sites created by the primers, electrophoresed in agarose gel, and purified by a gel extraction kit (Qiagen, Germany). Thereafter, the purified fragment was cloned into the eukaryotic secretory expression vector, pSec Tag2A (Invitrogen, USA), under the control of the CMV promoter and downstream of Ig κ-chain secretory leader sequence. Ligation of the insert and vector was performed by T4 DNA ligase (Fermentas, Lithuania). The recombinant plasmid (pSec Tag2A-*VP2*) was finally sequenced to verify that the gene was intact and in the correct orientation.

Transfection of cells

COS-7 cells were transfected by pSec Tag2A-*VP2* to verify protein expression. For this purpose, the cells cultured in 25 cm^2 flasks were transfected with 20 µg plasmid DNA, using polyfect Transfection Reagent (Qiagen, Germany), based on the manufacturer protocol.

Cell culture supernatant was harvested 48 h post transfection and tested for the presence of *VP2* by dot blotting and antigen capture ELISA.

Dot blotting

Nitrocellulose membranes were dotted with the supernatant of transfected cells and blocked with 5% skim milk in PBS. The membranes were then probed with 1/200 dilution of monoclonal antibody 1A6 (provided by Dr Herman Müller, Leipzig Faculty of Veterinary Medicine, Germany), specific for a conformation dependent neutralizing antibody epitope on *VP2*. Thereafter, the membranes were incubated with 1/300 dilution of a goat anti-mouse IgG peroxidase conjugate (Sigma, USA) and developed by DAB (Biogen, Iran) peroxidase substrate. Supernatant of COS-7 cells, transfected with pSec Tag2A, or infected by IBDV strain D78 were treated in the same manner and used as negative and positive controls, respectively.

Antigen capture ELISA

In this assay, ELISA plate was first coated with 1/400 dilution of Mab 1A6 in carbonate coating buffer, overnight at 4°C and blocked with 5% skim milk. Cell culture supernatants were added (100 µl/well) and incubated for 1 h at room temperature.

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Captured *VP2* was then detected with a polyclonal chicken anti-IBDV serum (1:500 dilution), a goat anti-chicken IgG peroxidase conjugate (KPL, USA) and ABTs (Roche, Germany) peroxidase substrate. The plate was finally read at 450 nm by an ELISA spectrophotometer. As in dot blotting, supernatant of COS-7 cells, transfected with pSec Tag2A, or infected by IBDV strain D78 were respectively used as negative and positive controls.

Results

Plasmid construction

The 1356 bp, *VP2* coding region of segment A, was successfully amplified in RT-PCR (Fig. 1). The PCR product was subsequently cloned into the vector pSec Tag2A, (Fig. 2). Sequencing of recombinant plasmid with T7 and BGH primers indicated the correct sequence and orientation of *VP2* gene, so the plasmid containing *VP2* gene (designated pSec Tag2A-*VP2*) was correctly constructed.

Secretory expression of *VP2* from the recombinants plasmid

Secretion of *VP2* in supernatant of transfected COS-7 cells was assessed by dot blotting and antigen capture ELISA. As demonstrated in Fig. 3, supernatant of cells transfected with pSec Tag2A-*VP2*, dotted onto nitrocellulose membrane, compared to that of cells transfected with pSec Tag2A reacted positively with the Mab 1A6. The results of antigen capture ELISA are presented in Table 1. Supernatant of cells transfected with pSec Tag2A-*VP2* caused a higher optical density, compared to supernatant of cells transfected with pSec Tag2A. This indicated again the cells transfected with pSec Tag2A-*VP2*, secreted *VP2* in supernatant.

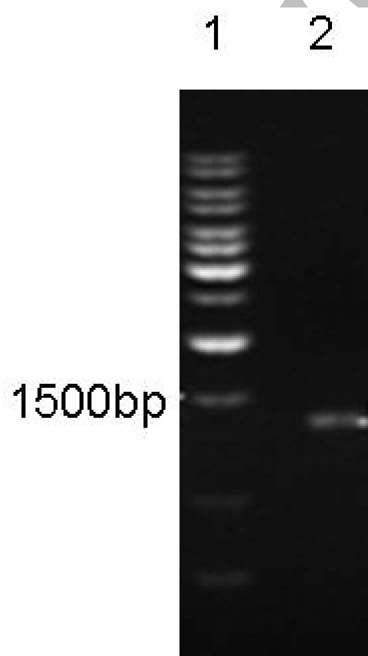

Fig. 1: PCR amplification of *VP2* coding region. Lanes 1 and 2 indicate 1 kb ladder and *VP2* PCR product, respectively

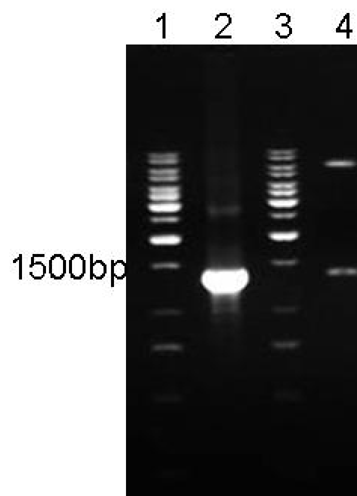

Fig. 2: Cloning of *VP2* gene in plasmid pSec Tag2A. Lanes 1 and 3 indicate 1 kb ladder, Lane 2: PCR amplification of *VP2* from the recombinant construct pSec Tag2A-*VP2* and Lane 4: restriction endonuclease digestion of pSec Tag2A-*VP2*

Fig. 3: Results of dot blotting. Dots 1 and 2 indicate supernatants of cells transfected with constructs pSec Tag2A and pSec Tag2A-*VP2*, respectively and Dot 3 indicates supernatant of cells infected with IBDV

Table 1: Results of antigen capture ELISA

Samples	Supernatant of cells transfected or infected with	OD_{450}
1	pSec Tag2A	0.341
2	pSec Tag2A-VP2	0.719
3	IBDV	1.033

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Discussion

IBDV is highly infectious and resistant to environmental exposure (Benton *et al*., 1967). Vaccination is therefore, essential to protect chickens from outbreaks of infection and is especially important during the first weeks after hatching. However, the chickens vaccinated with conventional IBDV vaccines are not fully protected, especially against virulent strains of IBDV. In general, live vaccines possess unstable antigenic and pathogenic characters, and induce moderate bursal atrophy (Tsukamoto *et al*., 1995; Tsukamoto *et al*., 2000).

Due to disadvantages of conventional IBDV vaccines, attempts have been made to generate subunit (Jagdish *et al*., 1988) and recombinant virus (e.g., fowl pox virus) vaccines (Shaw and Davison, 2000), based on *VP2*. Recently, several studies have shown that DNA vaccination can induce protective immune response against IBDV (Fodor *et al*., 1999; Chang *et al*., 2001; Hilton *et al*., 2002; Mahmood *et al*., 2006)

The concept of DNA as a vaccine was initially described in 1990 in a study by Wolff *et al*. (1990) demonstrating that direct intramuscular injection of purified bacterial plasmid DNA resulted in the expression of an encoded reporter gene. DNA vaccines employ genes encoding viral proteins in the absence of a live replicating virus; thus, problems with revertant virulence, divergent mutants, and the possibility of environmental contamination can be minimized.

Since the initial report on this novel vaccine technology, DNA vaccines have been successfully used to immunize a number of different animal species against a multitude of infectious agents (Fynan *et al*., 1993; Robinson *et al*., 1993; Corr *et al*., 1996; Sakaguchi *et al*., 1996). The potential advantages of DNA vaccines, such as overcoming maternal immunity, in ovo delivery and absence of requirement for a cold-chain, combined with immunological efficacy make this new vaccine technology very attractive for the poultry industry.

Enhancement of DNA vaccine efficacy in chickens is an area that requires more research. Application of CpG-ODN as DNA vaccine adjuvant (Mahmood *et al*., 2006), use of bacteria for efficient delivery of DNA vaccine (Li *et al*., 2006; Mahmood *et al*., 2007) and boosting the immune system by a killed vaccine, after using the DNA vaccine (Hsieh *et al*., 2007), all have been shown to enhance the efficacy of IBD DNA vaccines.

DNA vaccine efficacy in chickens may also depend to other features, like amount of the expressed protein and duration of *in vivo* expression. In order to get maximal protein production from a plasmid transfected into an animal cell, the regulatory elements of the plasmid must be optimized for the cell type. There have been a few studies done to determine the effect of promoter, transcription and termination signals (Kodihalli *et al*., 1997; Suarez and Schultz-Cherry, 2000) and plasmid conformation (Sakaguchi *et al*., 1996) on DNA vaccination in chickens.

The present study was aimed to prepare a construct for secretory expression of *VP2*, for future evaluation as DNA vaccine, compared to a non-secretory construct. The plasmid pSec Tag2A contains the CMV promoter, ampicillin resistance gene for selection in *E. coli*, zeocin resistance gene for selection in eukaryotic cell lines and a downstream histidin tag for protein purification. Based on the results of dot immunoblotting and antigen capture ELISA, cells transfected by pSec Tag2A-*VP2*, expresses the *VP2* of IBDV in supernatant efficiently. Monoclonal antibody 1A6, specific against a conformational neutralizing epitope on *VP2*, was able to recognize the expressed protein, suggesting that the expressed *VP2* has been processed to adopt a similar conformation and maintain the conformational epitope for 1A6 neutralizing antibody. Preliminary studies in our laboratory have indicated that mice immunized intramuscularly with 50 µg of the construct pSec Tag2A-*VP2* became seropositive against IBDV, two weeks post-immunization. Therefore, the construct pSec Tag2A-*VP2* is functional with respect to expression of *VP2* at the level sufficient to stimulate the immune response and can be used in studies to investigate *in vivo* expression and immunogenecity of *VP2* in chickens.

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