

**Full Article**

# **Crude extract of bacterially expressed, infectious bursal disease virus VPX/VP2 proteins induces protective immune response to the virus in chickens**

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## **ABSTRACT**

Infectious bursal disease virus (IBDV) infects young chickens and causes serious loss to the poultry industry, worldwide. Previous attempts using purified bacterially expressed IBDV VP2 failed to elicit a protective immune response to the virus. This study was designed to investigate if the initial expressed protein contained neutralizing epitopes but became nonfunctional during purification steps. The full length IBDV VP2 and its precursor VPX genes from the highly virulent IBDV (hvIBDV) SDH1 isolate were cloned and expressed in BL21 bacterial cells. Specific pathogen-free (SPF) chickens were inoculated subcutaneously with 0.3 ml crude extract with/without adjuvant at week 0, 2, and 4. At week 2 post-immunization, IBDV antibody was detected in the vaccinated chickens and increased to its highest titre at week 4. Virus challenge using the SDH1 isolate did not result in IBDV specific clinical signs when chickens were vaccinated with adjuvant-extract preparation (100% protection) while mock-inoculated chickens died 3-4 days post-inoculation. The above results, taken together, demonstrated that bacterially expressed IBDV VP2 harbors neutralizing epitopes required for induction of protective response, and suggest less detrimental purification procedures for vaccine preparation.

**Keywords:** Immunization, Infectious bursal disease virus, VP2, VPX

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## **INTRODUCTION**

Infectious bursal disease (IBD, Gumboro) is a chicken highly contagious viral infection that causes high mortality in broiler chicken flocks worldwide. The disease is recognized by inflammation and atrophied bursa of Fabricius in acute and progressed cases, respectively. In addition to the bursal lesions, inflammation of kidneys (nephritis) and pin-point

haemorrhagic muscles are often observed. Despite significant progress towards preventing the disease, IBD remains a considerable threat to the poultry industry (Lasher & Shane 1994). IBD virus (IBDV), a member of the Birnaviridae family, is the causative agent of IBD. The virus contains two genomic double-stranded genomic segments namely segment A and B. The larger segment (segment A) with 3400 bp length contains two partially overlapping open reading frames (ORFs). The first one encodes for the 17 kDa viral

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protein 5 (VP5) which is partially overlapping at the 5' end of the large ORF with 435 nucleotides (Bayliss *et al* 1990, Mundt *et al* 1995b). The second ORF encodes a 110 kDa precursor protein which is cleaved by the cis-acting viral protease VP4 into three proteins named VPX (48 kDa), VP4 (24 kDa) and VP3 (32 kDa), respectively (Maraver *et al* 2003). The VPX is further cleaved into VP2 (38 kDa) during maturation of the viral particle (Kibenge *et al* 1997, Maraver *et al* 2003). The smaller segment (segment B) with an approximate size of 2800 bp contains a single ORF encoding VP1 that has RNA-dependent RNA polymerase (RdRp) activity (Müller & Nitschke 1987). Currently, lack of an efficient vaccine to control the hvIBDV strains causes serious financial loss to poultry industry and the development of an efficient vaccine is an eminent need. The live intermediate plus vaccines are recommended for farms where highly virulent strains are found. However, the commercial vaccines fail to induce a complete protection against the hvIBDV strains (Rautenschlein *et al* 2005). Recent attempts to develop a protective vaccine using purified bacterially expressed IBDV VP2 have failed to elicit a protective immunity. This study has been designed to investigate if bacterial expression system is a suitable system but the succeeding purification steps have detrimental effects on the protein.

## MATERIALS AND METHODS

**Constructs and cells.** Construction of the pRSET-B-VPX constructs containing the entire hvIBDV VPX gene has been described previously (Hosseini *et al* 2007). Adopting the same strategy as described for VPX, the IBDV VP2 gene was amplified and cloned into the pRSET-B vector (Invitrogen, USA). The final construct was sequenced and named pRSET-B-VP2. The VPX and VP2 constructs were transformed into competent BL21(DE3)pLysS cells (Invitrogen, USA) as described (Hosseini *et al* 2007).

**Western blot analysis.** Expression of VPX and VP2 was induced as described previously. Briefly, *E. coli* strain BL21(DE3)pLysS harboring the recombinant

plasmids were grown in lauryl broth (LB) containing ampicillin and chloramphenicol. Isopropylthio- $\beta$ -D-galactose (IPTG) was added to a final concentration of 1mM and the cultures were incubated for additional 7 hours. Total proteins were resolved onto 12.5% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gels were stained with Coomassie brilliant blue. Western blot analysis was performed as described (Hosseini *et al* 2007). Briefly, total proteins were resolved using a 12.5% of SDS-PAGE gel and transferred onto the PVDF membrane (Immun-Blot, BIO-RAD). To detect the viral proteins containing the 6-His epitope, the membranes were incubated with monoclonal anti-His antibody (Invitrogen, USA) at final concentration of 1: 5000 in Tris-buffered saline (TBS). The same dilution of anti-VP2 monoclonal antibody (a gift from Dr. Egbert Mundt, Institute of Molecular Biology, Insel Riems, Germany) to detect VP2. Antigen-antibody complexes were detected using phosphatase conjugated goat anti-mouse antibody (KPL, USA) combined with colorimetric detection system according to the manufacturer's instructions (KPL, USA).

**Preparation of VPX and VP2 for vaccination trials.** Following identification the time point post-induction at which the maximum yield was achieved, large scale expression for vaccination trials was performed. The bacterial cells were pelleted and resuspended in phosphate buffered saline (PBS). The suspension was sonicated on ice using 10-second bursts at high intensity with a 10-second cooling period between each burst. The suspension was clarified by centrifugation at 10 x g for 15 min to pellet cellular debris. The suspension was stored at -80 °C.

**Vaccination and virus challenge trials.** A total of 45 one-week-old SPF chicks were randomly allocated to five groups. Groups A and B acted as mock-inoculated controls. Groups C, D, and E were inoculated intramuscularly with 0.3 ml of the recombinant *E. coli*-VPX protein, recombinant *E. coli*-VPX+VP2 and recombinant *E. coli*-VPX+VP2 with TSA-71 adjuvant (50/50 v/v), respectively. Three

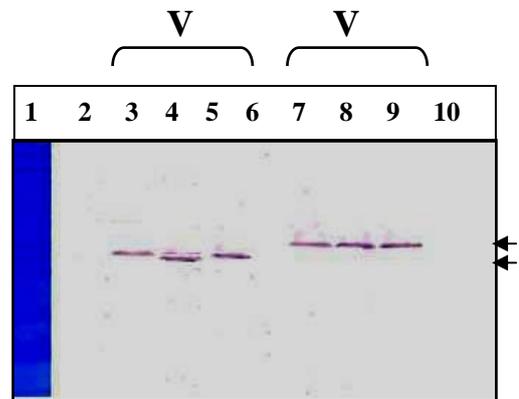
immunizations were performed with two weeks intervals. Blood samples were collected from each group at day 0, 14, and 28 post-immunization. Two weeks after the third immunization, chickens in groups B, C, D, and E were challenged orally with 104.8 EID50 of a very virulent IBDV isolate outbreaked in Iran (SDH1). Chickens in the Group A did not receive virus and acted as negative control. Chickens were observed daily for mortality.

**Antibody production assay.** An enzyme linked immunosorbent assay (ELISA; SYNBIOTIC, USA) was used to assay the antibody levels produced to IBDV using the methods recommended by the manufacturer. The antibody titers were calculated based on the following calculation,  $\log_{10} \text{titer} = 1.172 (\log_{10} \text{SP}) + 3.614$ . Statistical analyses were performed using Prism software (Graph Pad software, Inc).

**RESULTS**

**Cloning and expression of VPX and VP2 in *E. coli*.** The cloned 6xHis-VPX and 6xHis-VP2 were sequenced using Automated cycle sequencing and no mutations were observed. Both VPX and VP2 proteins were expressed as recombinant protein with the 6xHis epitope at their amino termini in *E. coli*. Expected bands of 51 kDa and 48 kDa were detected when the membrane was probed with the monoclonal anti-His antibody (Figure 1).

**Induction of humoral immune response to VP2 and VPX.** To test humoral immune response to the recombinant VP2 and VPX, the anti-VP2 and VPX antibodies were measured by ELISA at days 0, 14, and 28 post-vaccination. The mean IBDV antibody titers and standard deviation values were calculated for each Group (Table 1). Compared to negative control (Group B), Groups C, D, and E chickens seroconverted to VP2 and VPX at 2 weeks post-immunization and the antibody titers increased over the following 2 weeks, significantly ( $p < 0.05$ ) (Figure 2, Table 1). Chickens immunized with recombinant bacteria-VPX +VP2 + adjuvant (Group E) had the antibody titers higher than the other groups ( $p < 0.05$ ).



**Figure 1.** Western blot analysis of expressed recombinant VPX (Lanes 3-5) and VP2 (Lanes 7-9) proteins probed with monoclonal antibody to the 6x His epitope . Arrows indicate the expected product size, ~51 kDa (Top arrow) and ~48kDa (Bottom arrow). Lane 1 Bench mark protein ladder (Invitrogen, USA). Lane 2: uninduced recombinant plasmid; lane 10: Wild type pRSET B.

**Virus Challenge.** Two weeks after the last vaccination, all chickens were healthy with no adverse clinical sings suggesting that the vaccine preparations were safe. Chickens in Groups B, C, D, and E were challenged orally with hvIBDV. As shown in Table 1, 8 out of 9 chickens (89%) in Groups C and D survived virus challenge while all chickens (100 %) in Group E survived. All chickens in negative control group (Group B) died 3-4 days post-challenge while none of non-vaccinated chickens (Group A) died.

**Table 1.** Effects of different immunization cocktails on protection of chickens from challenge with hvIBDV. VPX (Group C), VPX+VP2 (Group D), and VPx+VP2+adjuvant (Group E). Chickens in Group B (control) were not immunized. The exact antibody titres displayed in Figure 2 have been presented in the Table.

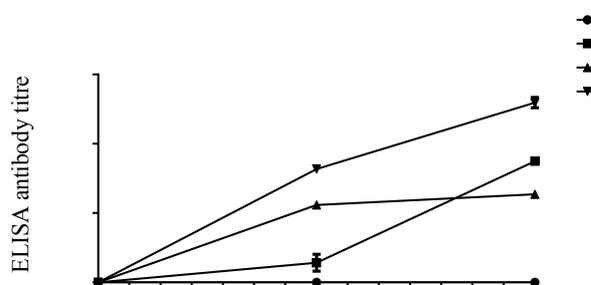
Group	ELISA antibody titres postinoculation (Days)			Mortality Rate (%)
	0	14	28	
B	165± 5.61	328± 14.62	352± 12.45	9/9 (100%)
D	165± 5.61	1997±51.63	8742 ± 5.916	1/9 (11%)
C	165± 5.61	5585 ± 80.74	6500 ± 90.08	1/9 (11%)
E	165± 5.61	8186 ± 80.87	12968 ± 375.6	0/9 (0%)

**DISCUSSION**

Several attempts have been made to produce an efficient protective vaccine for the protection of chickens against IBDV. IBDV VP2 is the major antigenic component that encodes for at least two neutralizing epitopes (Fahey *et al* 1998). Hence, numerous studies have been performed to develop an alternative IBDV vaccine by expressing the VP2

protein in various expression systems (Azad *et al* 1991, Pitcovski *et al* 2003, Vakharia *et al* 1994). Vaccination of chickens with these expression products have resulted in variable levels of active or passive protection against mortality and bursal damage. Recombinant VP2 expressed in *E. coli* reacted with a range of monoclonal antibodies (Azad *et al* 1986, Jagadish *et al* 1998). However, vaccination with the purified VP2 protein expressed in *E. coli* failed to elicit protective immunity (Azad *et al* 1991, Omar *et al* 2006). It has been reported that unpurified proteins from *Clostridium botulinum* expressed in *E. coli* induced protective immunity (Clayton *et al* 1995) suggesting that crude proteins expressed in *E. coli* may maintain their native protein structure. The current study evaluated the protective immunity induced by crude extracts of bacterially expressed unpurified VP2 and VPX proteins. First, we demonstrated that immunization with unpurified VP2 and VPX resulted in high levels of anti-VP2 antibody in SPF chickens. IBDV antibody was not detected in mock-treated negative control chickens. To further characterize the immune response to the unpurified VPX and VP2, vaccinated chickens were exposed to hvIBDV and demonstrated that 89% of chickens vaccinated with VPX and VP2 were protected. When the crude mixture of VPX and VP2 were mixed with the adjuvant, 100% of chickens were protected. In contrast to our results, purified IBDV VP2 failed to induce protective immunity in chickens (Azad *et al* 1991, Omar *et al* 2006). Therefore, the current study demonstrated the proof-of-principle that crude IBDV VP2 maintains essential epitopes for induction of protective immunity. It has been reported that a recombinant herpesvirus expressing VP2 protected 67% chickens against hvIBDV (Tsukamoto *et al* 2000) and when VP2 DNA vaccine was used only 50% of chickens were protected (Kim *et al* 2004). In conclusion, the results presented here suggest that the native form of the bacterially expressed IBDV VP2 contains neutralizing epitopes essential for induction of protective response that are lost during purification steps. Therefore, we

recommend exploring safer protocols to purify crude preparations of IBDV VP2 for its use as an effective vaccine.



**Figure 2.** Immune response to different immunizations cocktails. SPF chickens were inoculated with VPX (Group C), VPX+VP2 (Group D), and VPX+VP2+adjuvant (Group E). Chickens in Group B (control) were not immunized. Antibody titres were measured by ELISA as described in Materials and Methods. Bars indicate standard deviation values. Bars are not observed when the raw figures are not very different.

### Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

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