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Expression of immunogenic protein of infectious Bursal disease virus in mammalian vector pVAX.1

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Abstract
The immunogenic protein VP2 of highly virulent infectious Bursal disease virus (hvIBDV) strain were cloned and expressed in mammalian expression vector pVAX.1. The pVAX.1 vector, has high-copy number replication in *E. coli* with high-level transient expression of the protein of interest in most mammalian cells due to the presence of human cytomegalovirus (CMV) promoter. VP2 protein was transformed in *E. coli* BL21 DE3 host system and characterized by SDS PAGE and western blotting. The present paper focuses on the strategies of cloning and expression of the immunodominant protein of IBDV which in future can be used as a DNA vaccine to control IBDV infection in chickens.

Keywords: IBDV, VP2, Immunodominant, cloning, expression, pVAX

Introduction
Infectious Bursal disease is an acute contagious immunosuppressive disease targeting young chicken with severe immunosuppression and mortality at the age of 3 to 6 weeks (Fatima *et al*., 2014) [5]. It belongs to the genus Avibirnavirus and family Birnaviridae (Silva *et al.*, 2014) [6]. The genome of IBDV consists of smaller segment and large segments (Bidin *et al*., 2001) [8]. The larger segment encodes the polyprotein with VP2 (Heine H.G. and Boyle D.B. 1993) [4], the major glycoprotein in trimer structure with main host-protective antigen (Hamoud *et al*., 2007, Birghan *et al*., 2000) [7, 1]. IBDV infected chicken show symptoms of depression, whitish diarrhea, anorexia, prostration and death (Kibenge *et al.*, 1997) [3]. Diagnosis of IBD is dependent on clinical signs, differential diagnosis, gross lesions, histopathological lesions, virus isolation, serological and molecular diagnosis. Although confirmatory diagnosis of IBD is traditionally performed with serological methods such as ELISA or virus neutralization, a number of molecular diagnostic methods are based on RT-PCR. (Meenambigai *et al.*, 2020). Through these different identification methods more appropriate control measures could be developed in future. The preliminary control measures is through vaccines. Hence novel vaccines based on recombinant DNA technology becomes the need of the hour.
In the present research study, VP2 protein of IBDV was cloned and expressed in *E. coli* expression system with pVAX expression vector. The *E. coli* and Vero cell expression systems are best known system to express the targeted protein to yield large amounts of recombinant protein (Azad *et al.*, 1986, Azad *et al.*, 1991, Chong *et al.*, 2001). pVAX vector, which allows high-copy number replication in *E. coli* with high-level expression of the protein in most mammalian cells, due to the presence of Human cytomegalovirus immediate-early (CMV) promoter, Bovine growth hormone (BGH) polyadenylation signal for efficient transcription termination and Kanamycin resistance gene for selection in *E. coli*. Thus the pVAX vector in BL21 (DE3) *E. coli* and VERO host results in the expression of the immunodominant VP2 protein that may be used as a DNA vaccine against IBDV infection in chickens in the future.

Materials and Method
Amplification of VP2 gene
The bursal tissue samples (very virulent) were collected and the nucleic acid was extracted using TRI Reagent. The extracted RNA was used to synthesize cDNA with reverse transcriptase. And the targeted VP2 genes were amplified using the forward primer: 5' CCC AAGCTTA ATATG GTCC TTCT GATG CCAA CAACC 3' and Reverse primer 5' CCGGAATTCCTA ATG ATG ATG ATG ATG ATG TTACCTCCTTATGGCCCG 3' with EcoRI and Hind III restriction sites. PCR was carried out with the reaction mixture of dNTPs, Taq polymerase and reaction buffers along with target cDNA.

Amplification was carried out at 95 ˚C for 5 minutes followed by 35 cycles of 95 ˚C for 45 seconds, 52 ˚C for 45 seconds and 72 ˚C for 1.30 minutes with final extension at 72 ˚C for 10 minutes. (Sworna Kumari *et al*., 2020).

Cloning of VP2 gene fragment in pVAX vector

The amplified VP2 gene was eluted from the agarose gel using the gel elution kit (Bio Basic) and cloned into pVAX.1 vector (Invitrogen) using EcoRI and Hind III restriction enzymes (NEB BIOLABS). The restricted pVAX.1 and the VP2 PCR product were ligated using T4 DNA Ligation kit (Thermo fisher scientific). The ligated products were transformed into competent *E. coli* BL21/plySS cells. Eight clones were picked randomly from the LB plates and screened for positive clones with VP2 gene insert using colony PCR. The recombinant plasmid was confirmed by restriction digestion using the same restriction enzymes. (Pradhan *et al.*, 2012) [2].

Analysis of VP2 protein expression

A positive clone was selected for the expression of VP2 protein in LB broth containing 100 µg/ml Kanamycin and induced with sterile IPTG for 5 hours. The expression of VP2 protein was preconfirmed by SDS-PAGE analysis. Briefly, the cells from IPTG induction 0th hour to 5th hour were centrifuged at 3500g for 10 min and the pellet was resuspended in 6X SDS PAGE loading buffer and the resolved protein was processed for further confirmation.

Characterization of protein

Western blot analysis was performed for the characterization of immunogenic protein. The separated proteins from SDS-PAGE were transferred onto Nitrocellulose (NC) membrane the membrane was blocked with 3% BSA at 37 ˚C for 1 to 2 hours followed by washing with phosphate buffered saline-Tween-20 and incubated with primary antibody at room temperature for 1 h. The membrane was washed three to four and incubated with conjugated secondary antibody at 37 ˚C for 2 hours. The Nitrocellulose membrane was then developed using 3,3'-Diaminobenzidine (DAB) substrate.

Expression in VERO cell lines

The DNA vaccine construct pVAXVP2 was transfected into Vero cells to confirm expression of the encoded protein. In a six well plate, vero cells were seeded 24 h before transfection. 70-80% confluent cells were used for transfection. The transfection mixture contained 100µg plasmid DNA and 10µL of lipofectamine reagent in 750µL serum free medium. Before transfection, the transfection mixture was incubated at room temperature for 45 min. The cells and transfection mixture incubated for 6 h at 37 ℃. After removing transfection mixture, 1.5 mL Dulbecco's modified eagle's medium with 20% Fetal Bovine Serum was added to the cells and replaced with medium for every 24 hour. After 48 hours of post-transfection, the total RNA was extracted using Trizol. cDNA was synthesized and was amplified at 95 ˚C for 5 minutes followed by 35 amplification cycles of 95 ˚C for 45 seconds, 52 ˚C for 45 seconds, 72 ˚C for 1.30 minutes and final extension at 72 ˚C for 10 minutes. The resulting PCR products were electrophoresed on 1.2% agarose gels. (Pradhan *et al.*, 2014).

Results

Amplification of VP2 gene of IBDV

The positive vvIBDV subjected to polymerase chain reaction with designed primers was amplified with a product size of 366bp. The amplified VP2 fragment was bulk purified from 1.5% agarose gel.

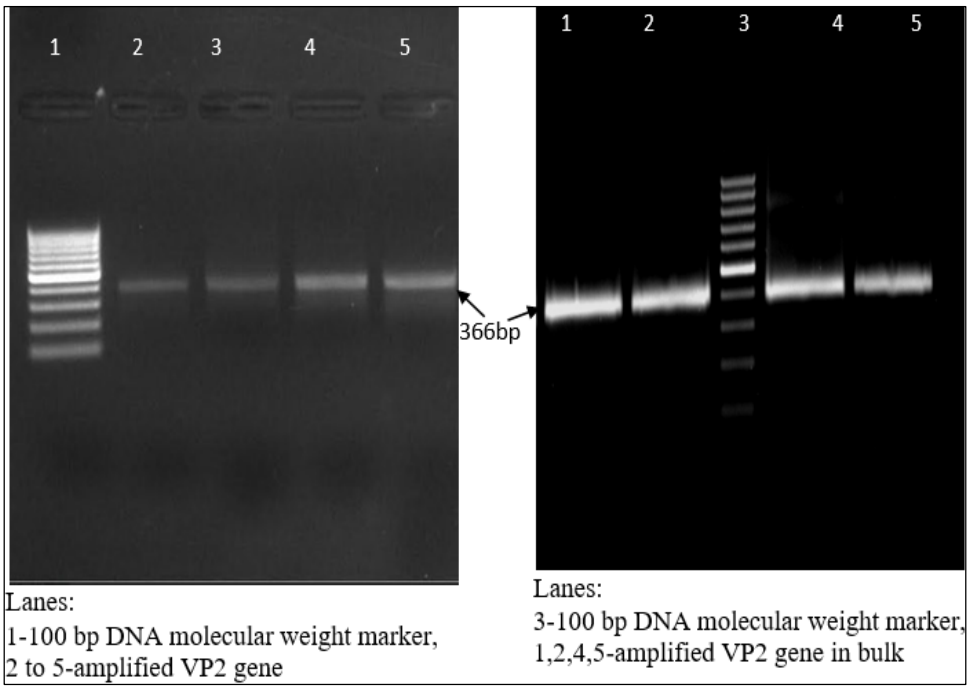


Fig 1: RT-PCR Amplification of VP2 Gene Fragment

Cloning of VP2 gene fragment in pVAX.1 vector

The purified VP2 gene and pVAX.1 vector were digested separately with EcoRI and Hind III restriction enzyme, followed by ligation with T4 DNA ligase enzyme. The ligation mixture was transferred to *BL21 (DE3) E. coli* host cells and plated into LB agar plates with kanamycin antibiotic (100µg/ml).The transformants were confirmed by colony PCR and orientation PCR using gene specific primers and T7 promoter primers, which results in the 366 bp product and 517 bp (Fig 3) respectively. Further the positive clones were

subjected to restriction digestion using the same EcoRI and Hind III restriction enzymes. The restriction digested products results in two bands one of vector (2.9kb) and other of product (targeted 366 bp size product-Fig 4). The positive clones were transferred into 3ml LB broth with Kanamycin antibiotic overnight.

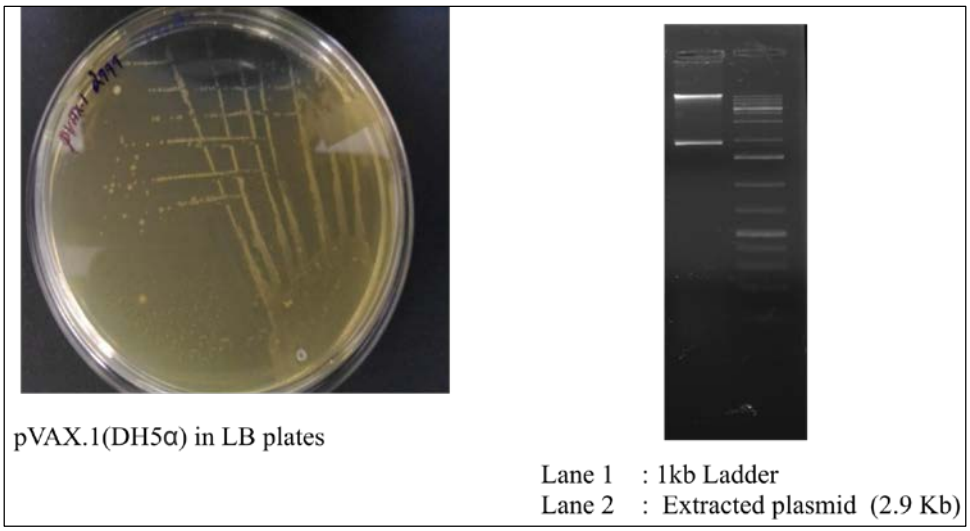


Fig 2: Plasmid extraction

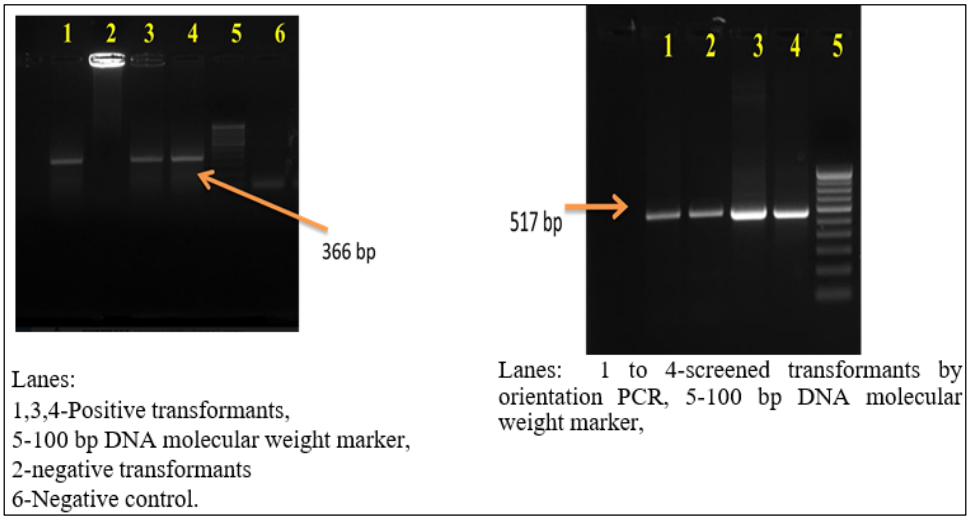


Fig 3: Screening of transformants by colony PCR. The 366 bp insert in the pVAX.1-VP2 was amplified by PCR using insert specific primers

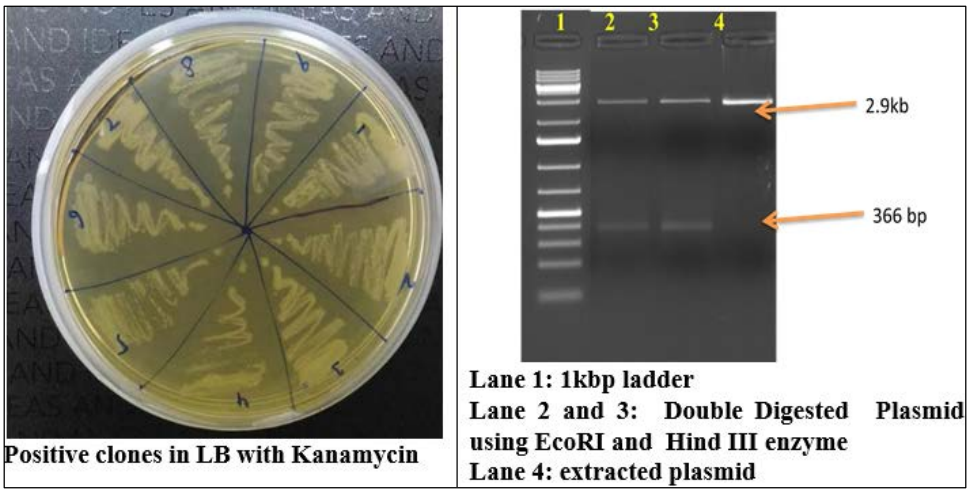


Fig 4: Double digestion using ECORI and hind III enzyme

Analysis of VP2 protein expression

The overnight culture approximately 1ml was inoculated into 100ml LB broth for IPTG induction. The uninduced and induced 0th hour, 1st hour, 2nd hour, 3rd hour, 4th hour, 5th hour and

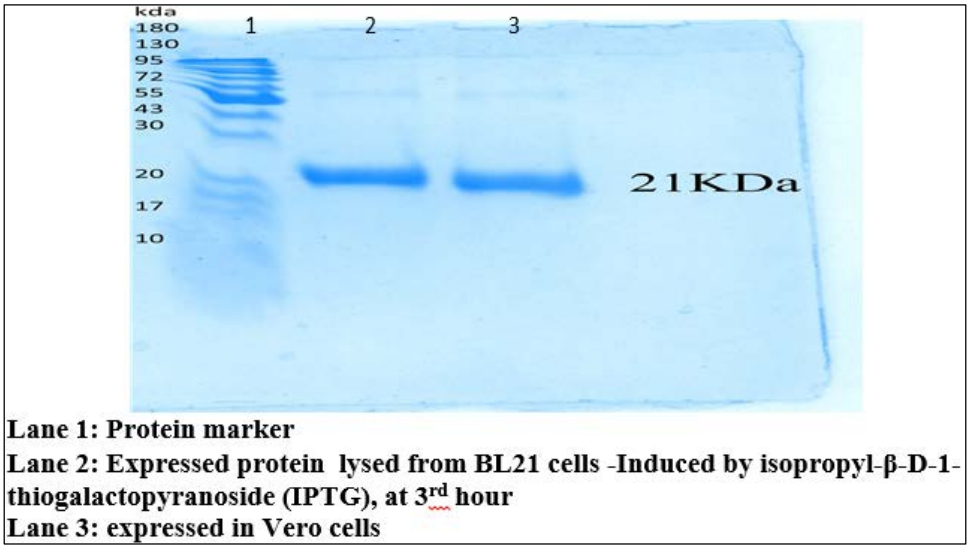
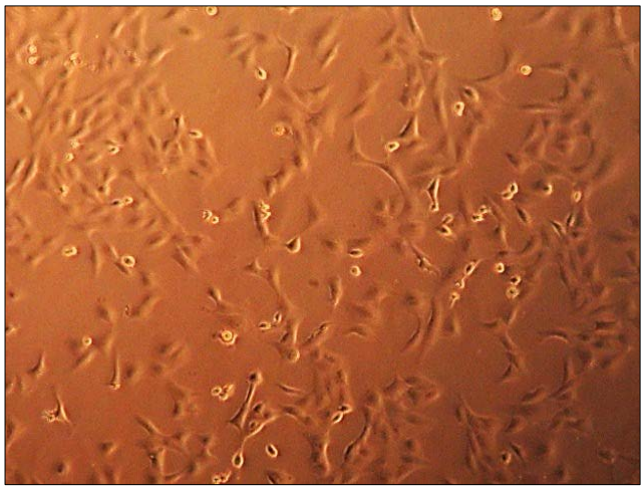


Fig 5: Induction and expression of pVAX.1VP2-IBDV in BL21 plySS and Vero cell

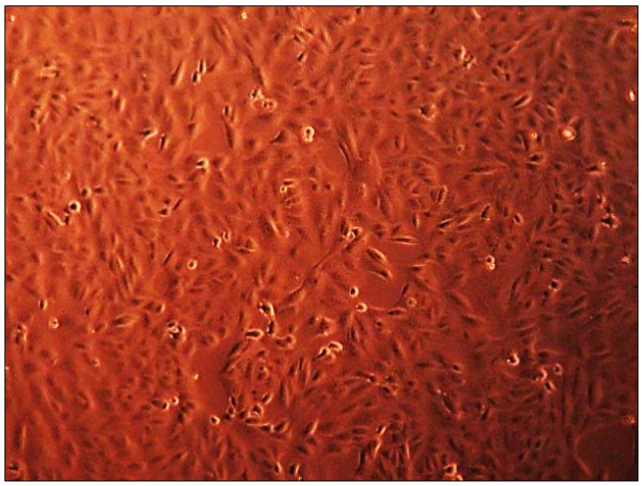
overnight samples were loaded in the well with 6X SDS loading dye for preconfirmation in 12% SDS PAGE for standardizing the exact hour of protein expression. On Next batch 3rd hour culture results in the expression of protein in abundantly, after the 3rd hour resulted in decrease amount of protein expression.

Transfection and Expression in VERO cell lines

Vero Cells were transfected with pVAXVP2 using lipofectamine reagent. The transfected cells were harvested after 48 hours. Total RNA was extracted from cells with Trizol and transcribed into cDNA. The cDNA was tested for the presence of pVAXVP2 gene in the transfected vero cell by PCR with VP2 gene specific primers. The amplification of cDNA from transfected cells yielded a PCR product of 366 bp with VP2 gene-specific primers and also the plasmid were extracted from the vero cells to confirm the transfection.



Before Transfection-vero cells with 70-80% confluency



After transfection-vero cells with positive pVAXVP2 plasmid

The vero cell line was used for the transfection of DNA vaccine construct pVAXVP2 to check the expression of VP2. The *in vitro* expression of the DNA encoded antigens was confirmed by transfection of vero cells with the vaccine constructs followed by RT-PCR and western blot analysis with IBDV-antiserum.

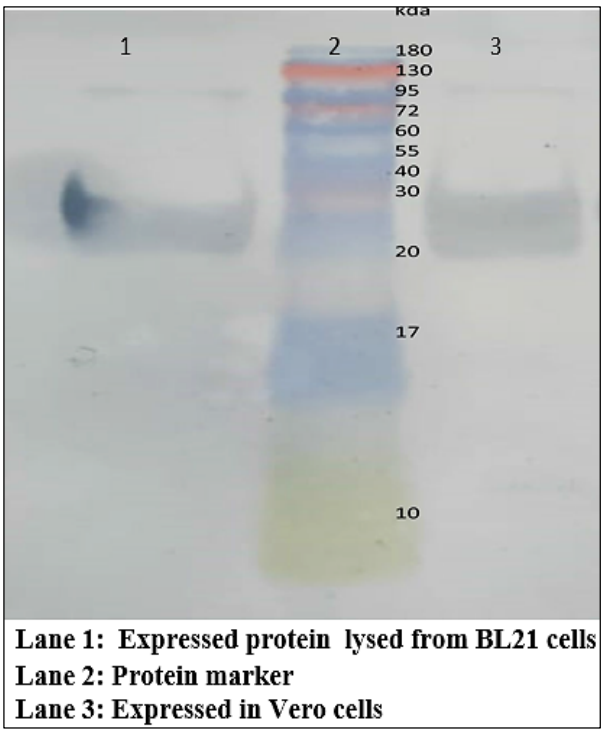


Fig 6: Characterization of VP2 protein

Discussion

Very virulent Infectious bursal disease virus is a pathogen which causes major economic loss in poultry industry worldwide. The VP2protein cloned and expressed in *E. coli* expression system and mammalian cell expression system for the large production of DNA vaccine against the IBDV virus. Here the partial VP2 protein sequences were used in this study for cloning and expression. The VP2 protein from the highly virulent IBDV strain directly amplified, cloned and expressed. In earlier studies Mahmood *et al.,* 2007 targetted the VP2 gene in *E. coli* DH5α expression system for the production of protein and also studied the efficacy of different doses of oral DNA vaccine comparing with commercial vaccine. And also reported that the antigen through this *E. coli* expression system which serve as a safe and potential vehicle for oral DNA vaccination and also offered the better protection against virulent IBDV in chicken via oral administration with induced cellular and humoral response by using the whole or purified recombinant proteins. Hosseini *et al*., 2007 and Pradhan *et al.,* 2014 confirmed that the immunodominant VP2 fragment of IBDV can be used as a potential DNA vaccine against IBDV infection in chickens. Initially they amplified the vp2 fragment and ligated in pVAX.1 vector and transfected in CHO cell line and resulted in 21kDa recombinant protein. The efficacy of the rVP2 compared with commercial vaccine resulted in better protective immune response when compared with the commercial viral vaccines. Omar *et al.*, 2006, used hvBDV Vp2 protein for studying the inappropriate expression and folding of IBDV protein by using the *E. coli* BL21cell expression system. The resultant recombinant protein used for the humoral and cell mediated response by inoculating the recombinant proteins in chickens with crude protein, purified protein and heat inactivated protein. They showed high the humoral antibody response by standard classical challenge IBDV test virus for the test of recombinant vaccines. Husharian *et al*., 2007 used pCDNA plasmid for cloning and expression of very virulent strain of IBDV, they amplified 1365bp VP2 fragment and transfected to the COS-7 cell line and they expressed the protein under the control of strong human cytomegalovirus (hCMV) immediate early enhancer and promote and the transient expression of recombinant VP2 from the constructed pCDNA4VP2 characterized by dot blotting assay with a polyclonal antibody to IBDV.

Conclusion

In the world of recombinant technology, IBDV VP2 fragment cloned in eukaryotic expression vector pVAX.1 and transfected in mammalian expression system to be used as a DNA vaccine candidate against highly virulent IBDV challenge. Our studies showed that the pVAXVP2 construct to be used in future vaccination group for eliciting the humoral and cellular responses as a DNA vaccine which can protect chickens against IBDV.

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