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C Sworna Kumari
Ph.D., Scholar Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Vepery, Chennai, Tamil Nadu, India

TV Meenambigai
Professor, Vaccine Research Centre – Viral Vaccines, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

A Mangalagowri
Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Vepery, Chennai, Tamil Nadu, India

A Palanisamy
Dean, VCRI, TANUVAS, Tirunelveli, Tamil Nadu, India

G Sarathchandra
Dean, Faculty of Basic Sciences, Madras Veterinary College, Chennai, Tamil Nadu, India

Corresponding Author:
TV Meenambigai
Professor, Vaccine Research Centre – Viral Vaccines, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India

Cloning and expression of Vp2 protein of infectious bursal disease virus

C Sworna Kumari, TV Meenambigai, A Mangalagowri, A Palanisamy and G Sarathchandra

Abstract
The VP2 protein of highly virulent infectious bursal disease virus (hvIBDV) was cloned and expressed in pRSET A vector with high level of protein expression. VP2 is the host protective protein with major neutralizing epitopes was cloned and expressed in *E. coli* expression system BL21- DE3. The protein expression was evaluated by induction studies with IPTg and characterized by SDS PAGE and western blotting analysis. Thus, the present study focusing on expression of the immunodominant VP2 protein that may be used as a protein boost in DNA vaccine production strategy for enhanced immunity against IBDV infection in chickens in the near future.

Keywords: IBDV, VP2, cloning, expression, characterization

Introduction
The double stranded RNA of Infectious bursal disease virus (IBDV) plays a vital role in the destruction of IgM bearing B-cells in bursa of Fabricius of chicken with severe immunosuppression and mortality at the age of 3 to 6 weeks (Fatima *et al*.,2014) [4]. IBDV belongs to the genus Avibirnavirus and family Birnaviridae and is synonymously called as gumboro disease (Silva *et al.*, 2014) [5]. The genome of IBDV consists of larger segment A and smaller segment B of 3.2 kb and 2.8 kb size respectively (Bidin *et al*., 2001) [7]. The larger segment consists of two overlapping open reading frame (ORF) with different viral proteins. The most abundant encoding polyprotein is VP2, that carries major neutralizing epitopes (Hamoud *et al*., 2007, Bringhan *et al.* 2000) [6, 1]. VP2 glycoproteins are responsible for eliciting neutralizing antibodies (Heine and Boyle 1993) .IBDV is not vertically transmitted from parent to offspring.There is only horizontal transmission through infected chicken feces with symptoms of depression, whitish diarrhea, anorexia, prostration, and death. Due to a sharp decline in poultry production all over the world by the repeated exposure of IBDV outbreak, detection and protection of poultry from IBDV is necessary. Most of the successful vaccines are based on recombinant technology. Studies have been reported in developing an IBDV vaccine by expressing the immunogenic VP2 protein in various expression systems. In the present study the VP2 protein was cloned in pRSET-A vector and expressed in *E. coli* expression system. (Azad *et al.* 1986, Azad *et al.* 1991, chong *et al.* 2001) [9, 14]. pRSET-A vector was designed for high level expression of VP2 protein of IBDV with T7 promoter, 6X His tag in terminal end and ampicillin antibiotic resistance.

Materials and Method
Virus Sample and RNA extraction
The very virulent bursal tissue samples were collected and triturated in a mortar and pestle with sterile sand and PBS. 100µl of triton-x 114 was added to the triturated sample, vortexed for 1 minute followed by 3 times of freeze thawing. The supernatant was collected and processed for extraction of nucleic acid using TRI Reagent. In this method 0.25 ml of tissue supernatant was taken to which 1ml of TRI reagent was added and incubated at room temperature for 5 minutes for complete dissociation of nucleoprotein complexes. Subsequently 200 µl of chloroform was added and mixed vigorously for 10 -20 seconds and incubated at room temperature for 5 minutes, followed by centrifugation at 4 °C for 10 minutes at 14,000rpm.The RNA containing aqueous layer was carefully transferred into a fresh tube and equal volume of isopropanol was added and kept for 10 minutes at room temperature. The samples were centrifuged at 13,000 rpm for 10 minutes.

The RNA pellet was washed with 70% ethanol, air dried and resuspended in 15 µl of RNase free water and quantitated. (Adamu *et al.*, 2013).

Polymerase Chain Reaction (PCR)

The extracted total RNA was used to synthesize cDNA with reverse transcriptase. PCR was carried out utilizing the primer sequences of very virulent IBDV. Forward primer -5'-GGTAACGTCCTCAGCTTA Reverse primer- 5'-GTTCAGGATTTGGGATCAGC-3'. The reaction mixture was subjected to initial denaturation of 95 ˚C for 5 minutes followed by 35 amplification cycles of 95 ˚C for 45 seconds, 51 ˚C for 45 seconds and 72 ˚C for 1.30 minutes with final extension at 72 ˚C for 10 minutes. (Meenambigai *et al*., 2017) [10]

Amplification of VP2 gene

Primers were designed with appropriate restriction enzyme sites for aiding expression. The forward primer: 5' GGA AGATCT AGCCTTCTG ATG CCA ACA ACC GG 3' and Reverse primer 5' CCC AAGCTT ATCTGTCAG TTCACTCAGGC 3' with BglII and Hind III restriction sites were designed. PCR was carried out with the reaction mixture consisting of dNTPs, Taq polymerase and reaction buffers along with target cDNA and was amplified at 95 ˚C for 5 minutes followed by 35 amplification cycles of 95 ˚C for 45 seconds, 54 ˚C for 45 seconds and 72 ˚C for 1.30 minutes with final extension at 72 ˚C for 10 minutes.

Cloning of vp2 gene fragment in Prset a vector

The amplified VP2 gene was purified and cloned into pRSET A expression vector at BglII and EcoRI restriction enzyme sites. The enzyme digested vector and VP2 protein product were ligated with T4 DNA Ligase. The ligation mixtures were transformed into competent cells of E. coli BL21/plySS. Eight clones were picked randomly from the LB plates and screened for positive clones by colony PCR. The recombinant plasmid was confirmed by restriction digestion with BglII and Hind III enzymes.

Analysis of Vp2 Protein Expression

A positive clone was selected for the expression of VP2 protein in LB broth containing 100 µg/ml ampicillin. Expression was induced with IPTG for 5 hours. The expression of VP2 protein was confirmed by SDS-PAGE analysis. Briefly, the cells from IPTG induction at 0th hour to 5th hour were centrifuged at 3500 g for 10 min and the pellet was resuspended in 6X SDS PAGE loading buffer. The mixtures were subjected to 12% sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).The gels were then stained with Coomasie brilliant blue and destained with methanol. The expressed VP2 protein was characterized by Western blot analysis. The separated proteins from SDS-PAGE were transferred to Nitrocellulose (NC) membrane. The membrane was blocked with 3% BSA at 37˚C for 2 hours followed by triple washes in phosphate buffered saline with Tween-20, The NC membrane was then incubated with primary antibody at room temperature for 1 h. The membrane was washed thrice and incubated with conjugated secondary antibody at 37 ˚C for 2 hours. The Nitrocellulose membrane was then developed using DAB substrate. The enzyme reaction on the membrane was terminated by washing with distilled water.

Result and Discussion

Identification of Very virulent IBDV strains

Virus samples were initially screened by reverse transcriptase polymerase chain reaction using primers for the specific identification of very virulent IBDV strains. A 633 bp product (Fig – 1) was detected in positive samples, which was compared with 100bp DNA molecular weight. marker.

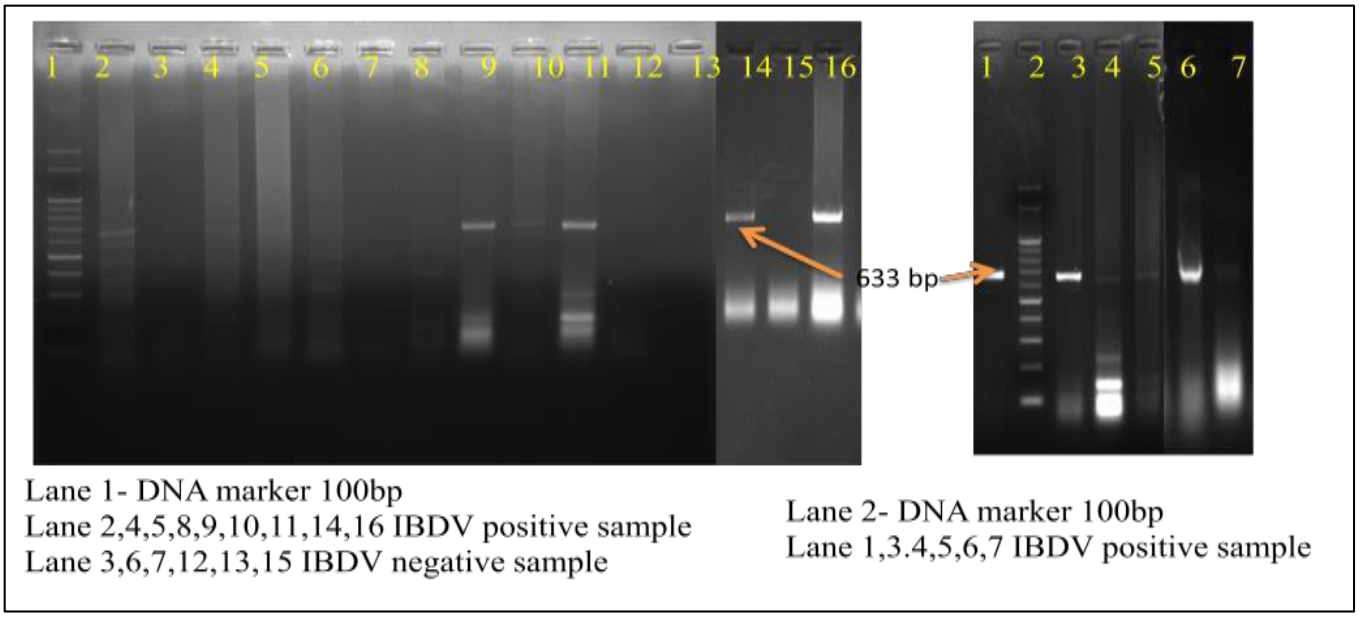

Fig 1: Identification of Very virulent IBDV strains

Amplification of VP2 Protein

The positive vvIBDV isolate was used to amplify the VP2 protein to be expressed in expression vector. The amplicon was 366bp in size. (Fig – 2) The VP2 protein was produced in bulk and purified.

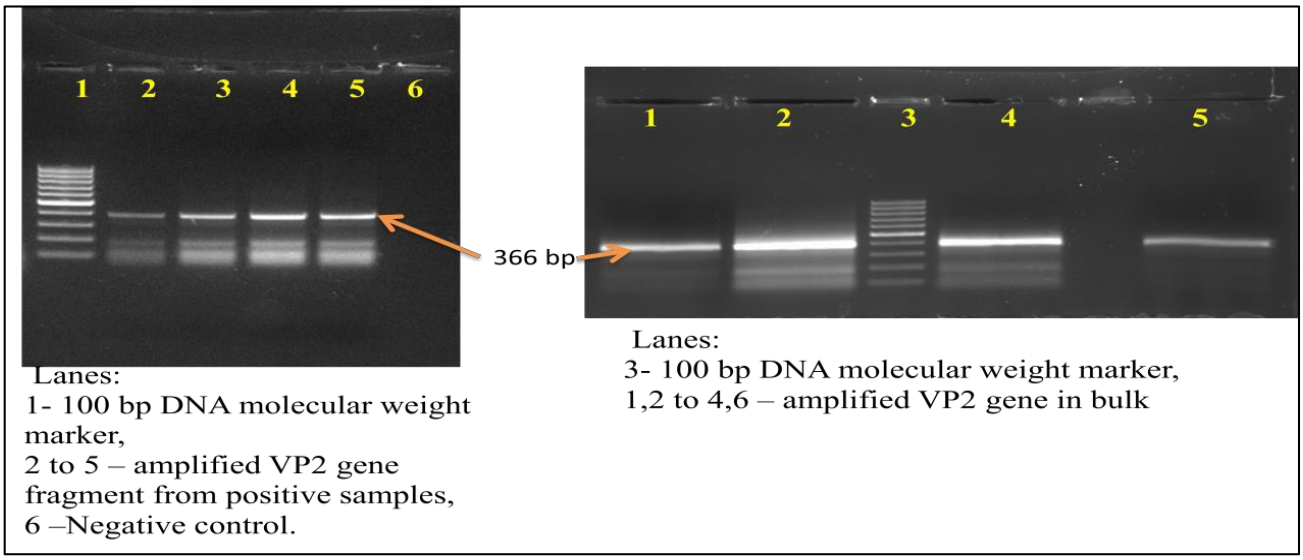

Fig 2: Amplification of VP2 protein

Cloning of Vp2 Gene Fragment in pRSET a vector

The purified VP2 gene and pRSET A vector were digested separately with Bgl II and HindIII restriction enzymes. The digested product was ligated using the T4 DNA ligase enzyme. The ligation mixture was transferred to BL21(DE3) *E. coli* host and plated in LB plate with ampicillin antibiotic 100µg/ml. Recombinant white colonies were detected with Vp2 protein insert. (Fig – 3). The transformants were confirmed by colony PCR (Fig – 4). Further the positive clones were subjected to restriction digestion with Bgl II and HindIII restriction enzymes to confirm insert release. (Fig – 5).

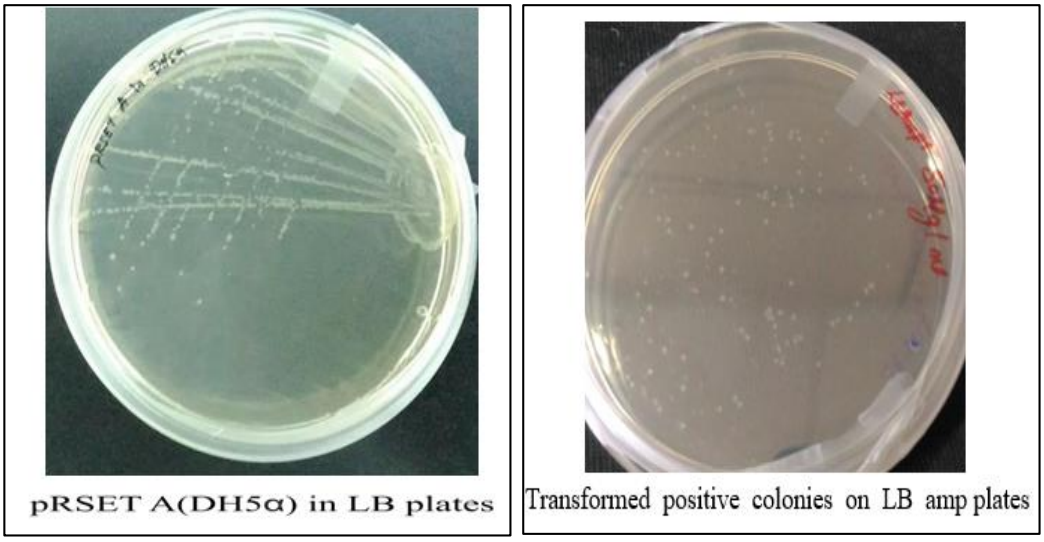

Fig 3: Recombinant white colonies of VP2 protein in LB agar plates

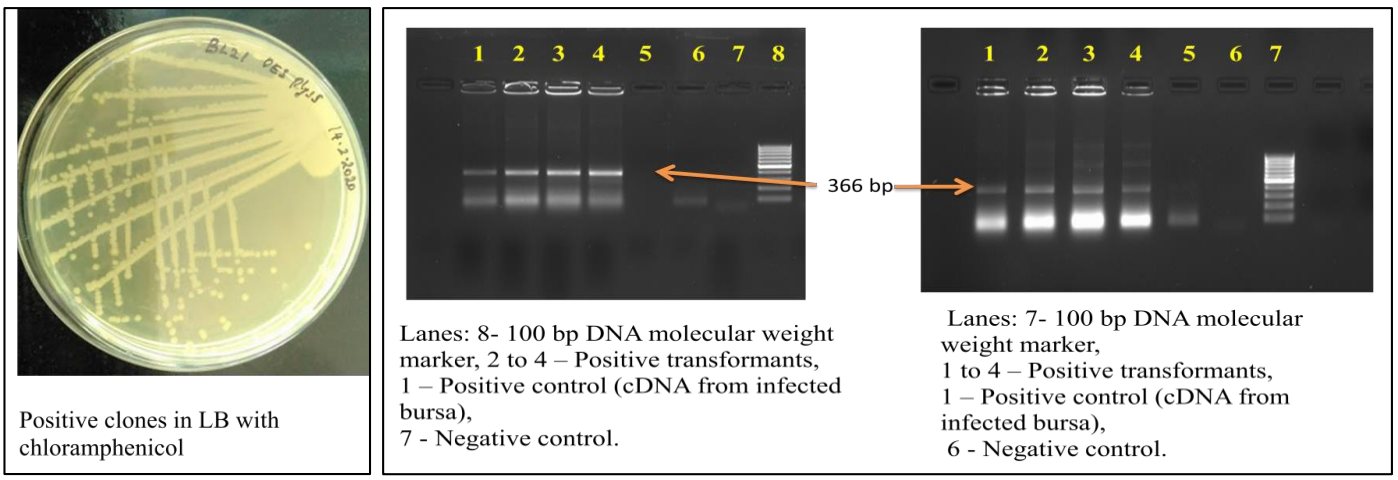

Fig 4: Screening of transformants by colony PCR. The 366 bp insert in the pRSET A- VP2 was amplified by PCR using insert specific primers

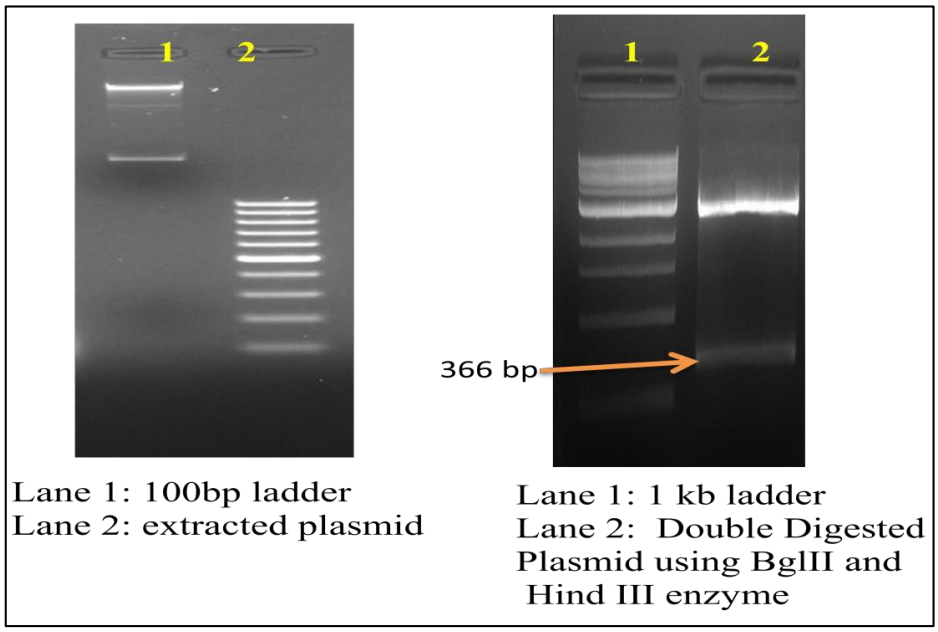

Fig 5: Further the positive clones were subjected to restriction digestion with Bgl II and HindIII restriction enzymes to confirm insert release.

Analysis of Vp2 Protein Expression

The uninduced and induced expression samples at 0th hour, 1st hour, 2nd hour, 3rd hour, 4th hour, 5th hour and overnight samples were checked by SDS PAGE. The 3rd h of induction was optimum for maximum protein expression. The 3rd hour induced sample was characterized by western blotting. The recombinant protein was purified using the NI-NTA affinity column. The His-tagged rVP2 protein was collected and dialyzed. The dialyzed eluate was analyzed initially in 12% SDS PAGE and further confirmed using western blot with Anti-his-HRP conjugate and then developed using DAB substrate. The dialyzed VP2 eluate was 21 kda in size and compared with prestained protein marker.

Discussion

Infectious bursal disease virus is a pathogen causing major economic loss to the poultry industry worldwide. The VP2 protein is the most important host protective antigen. In the present study, the partial VP2 protein sequence was explored for cloning and expression. The VP2 protein from the highly virulent IBDV strain was directly amplified, cloned and expressed. In earlier studies, Mahmood *et al.* in 2007 [12] delivered the VP2 gene in *E. coli* expression system DH5α for studying the efficacy of different doses of oral DNA vaccine comparing with commercial vaccine. They reported that the antigen delivery through *E. coli* expression system DH5α with recombinant VP2 gene in pRc vector of vvIBDV serves as a safe and potential vehicle for oral DNA vaccination and also offered better protection against virulent IBDV in chicken via oral administration with induced humoral and cellular response by using the whole or purified recombinant proteins. Hosseini *et al*., in 2007 [11], amplified the VPX and VP2 protein and cloned them in pRSET B vector with His 6 tag and expressed it in *E. coli* expression system and confirmed the recombinant protein by Western blot, using the monoclonal anti His 6 specific antibody which recognizes the tag portion of the recombinant VPX protein. Antibody titre against infectious bursal disease virus (IBDV) in chicken are tested based on enzyme-linked immunosorbent assay (ELISA). Pradhan *et al*., in 2012 [2], reported the efficacy of the immunodominant VP2 protein.

The efficacy of the rVP2 was compared with commercial recombinant fragment produced via pRSET B vector. It induced better protective immune response when compared with the commercial viral vaccines. Omar *et al*. in 2006, used highly virulent IBDV VP2 protein for studying the inappropriate expression and folding of IBDV protein. Also they studied the importance of antibody and there humoral and cell mediated response by inoculating the recombinant proteins expressed via *E. coli* expression system BL21 in chickens with crude protein, purified protein and heat inactivated protein. They showed that the humoral antibody response was associated with protection against highly virulent IBDV. Husharian *et al*., in 2007 used pCDNA with 6X histidine tag plasmid for cloning and expression of very virulent strain of IBDV, they amplified 1365bp VP2 fragment digested with ECOR I and Xba I, ligated with T4 DNA ligase and produced the pCDNA4VP2 plasmid DNA construct and transfected to the COS- 7 cell line and they expressed the protein under the control of strong human cytomegalovirus (hCMV) promoter.

Hence the VP2 immunodominant protein paves the way as a novel candidate in diagnostics and vaccine production against IBDV, enablimg effective control strategies in disease prevention.

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