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Research Article

Infectious bursal disease virus cloning and structural protein (VP2) expression in *Escherichia coli*

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Abstract

Immunosuppressive diseases like infectious bursal disease (IBD) are serious threat to the poultry meat industry. IBD virus consists of two genomic segments in which segment A encodes all structural and non-structural proteins (*VP2, VP3, VP4*) and segment B encodes RNA dependent RNA polymerase *VP1*. In IBD the host immune response is due to the *VP2* capsid protein of the virus that leads to the immunosuppression of the birds. In the current research the partial *VP2* clones were developed using cRNA based reversed genetic system to understand the molecular determinants of infectious bursal disease virus for pathogenic phenotype and virulence. For the expression analysis, the cloning primers were designed for amplification containing *NdeI* and *EcoRI* restriction sites for the attachment of restriction enzymes. *VP2* of the IBDV was expressed in *E. coli* cells with the utilization of pET28a expression vector. Expression analysis of bacterial lysate on SDS-PAGE after IPTG induction of 5 hours with 1mM concentration, 30 KDa *VP2* protein band was observed. This study suggests the use of recombinant *VP2* (*rVP2*) as a subunit vaccine.

Keywords: *Escherichia coli*; IBDV; I-ELISA; RT-PCR; rVP2

Introduction

Gumboro is highly contagious disease of poultry birds. Its causative agent is infectious bursal disease virus that belongs to Avibirnaviridae group of Birnaviridae family. Infectious bursal disease virus (IBDV) is dsRNA non-enveloped virus with a genome having 2 segments A and B with diameter of 55–60 nm. Two serotypes of IBDV have been known of which only serotype-I causing disease in birds. Serotype-II infects several avian species and turkeys but no disease has been reported. Self-cleaved protease encodes the segment A of IBDV that consists of *VP4* and other structural proteins like *pVP2* and *VP3*. *pVP2* further yields the *VP2* as a major capsid protein. A wide variety of pathotypes of serotype-I have been classified as clinical virulent, subclinical virulent and very virulent groups **[1]**.

IBD is known from 1957 and now it is prevalent all over the world. It spreads very quickly in young commercial flock causing immunosuppression that leads to great economic losses. IBD target organ is bursa of Fabricius, where lymphoid cells are mainly infected by the virus that results in reduced feed efficiency and increased mortality. By three to six weeks of age the bursa of Fabricius reaches to its maximum development, at this stage the birds are highly susceptible to this disease and infection results in depletion of lymphoid cells and destruction of bursa [2]. Diagnosis of this disease is possible by virus isolation, Immunofluorescence assay, enzyme linked immunosorbent assay and monoclonal antibody assay. DNA hybridization has been developed for the detection of IBD virus using radioactive and non-radioactive cDNA probes [3]. However, the detection and amplification of the IBD virus can also be done using RT-PCR. Use of two oligonucleotide primers in PCR allow DNA polymerase to produce number of copies of viral DNA by utilizing the template DNA present at the 3′ ends of the primers [4]. IBDV is present in clinical and subclinical forms and birds recovered from virus remains carrier. Virus is resistant to inactivation and there is no control for this disease but vaccination and biosecurity [5].

Considering the above problems in the detection and screening of infectious bursal disease, we aim to produce the *rVP2* protein for the development of indirect ELISA and subunit vaccine through recombinant DNA techniques. This article describes the molecular cloning of *VP2* genome of infectious bursal disease virus.

Materials and methods

Isolation of Infectious bursal disease virus

Bursa samples infected with infectious bursal disease were collected from outbreaks in the poultry farms located in different areas of district Faisalabad. Suspected bursa tissues from birds showing disease symptoms were triturated in pestle mortar adding 1ml PBS. Homogenate was subjected to three cycles of freeze and thaw at -20℃ to break the cells and centrifuged at 10,000 x g for 10 minutes at 4℃. Supernatant was collected from the top layer [6].

Extraction of IBDV RNA

Viral RNA was extracted using FavorPrep® commercial RNA extraction kit following manufacturer's protocols. Added 150µl isolated viral supernatant in Eppendorf tube and mixed with 750µl VNE buffer. Vortexed and incubated at room temperature for 10 minutes. Absolute ethanol was added @ 750µl and tube was vortexed again. VNE column from the kit was fixed in the collection tube and all the mixture was centrifuged through the column at 8000 x g for 1 minute. The flow through was discarded and washed the column with 500µl of wash solution I (available in the kit). Centrifuged and discarded the flow though. The column was washed two times with 750µl wash buffer I I and centrifuged to discard the flow through. The column was fixed in storage tube and eluted the RNA by carefully adding 50µl elution buffer in the center of the column and incubated for 2 minutes at room temperature. Centrifuged at 8000 x g for 2 minutes to collect the eluted RNA in storage tube and stored at -80℃ [7].

Amplification of VP2 fragment of IBDV

To get the cDNA of IBDV-VP2, 5µl of RNA template was mixed with 1µl random primers, 1µl dNTP mix, 7.5µl reaction buffer, 0.5µl RNase ribonuclease inhibitor and 1µl RTase to the final volume of 20µl. Reaction was incubated at 25℃ for 5 minutes and 42℃ for 50 minutes respectively. The reaction was stopped by incubating at 85℃ for 5 minutes. *VP2* cDNA was amplified through PCR using partial *VP2* primers following manufacturer's instructions. The primers BGF: 3′-CATATGTGCAGCCGATGATTACC-

3´and BGR: 3´-CCTGGAATTCTCAGGGGAGAGTTG -5´ amplified the partial *VP2* in segment A of the IBD virus from amino acid sequence 751 to 1449. PCR was run by adjusting the amplification conditions to initial denaturation for 4 minutes at 94℃ and repetition of 35 cycles including denaturation for 45 seconds at 94℃, annealing for 45 seconds at 57.6℃, extension for 90 seconds for 72℃ and final extension of 10 minutes at 72℃ [3]. The amplified segment corresponding to *VP2* was separated on 1.0% agarose gel, the expected size of the band was 699 bp **[8]**.

Molecular cloning of *VP2* into bacterial expression vector pET28a

The amplified *VP2* fragment was cleaved with *NdeI* and *EcoRI* at the restriction sites introduced by oligonucleotide primers during synthesis. The isolated *VP2* fragment was ligated to bacterial transfer vector pET58a that had also been digested with the same restriction enzymes. The ligated DNA construct pET28a-Vp2 was transformed into *E. coli* (BL-21) cells and the reaction was carried out at 16℃ overnight **[9]**.

Colony PCR

White transparent colonies were picked from all the plates one by one and mixed in 5µl double distilled water in microcentrifuge tubes. PCR reaction mix were prepared in 10 separate microcentrifuge tubes by adding ddH_2O (35.5µl), PCR buffer with magnesium (5µl), Magnesium sulphate (3µl), Taq-DNA polymerase (0.5 ul), dNTP mix (1ul), F-primer (1.2µl), R-primer (1.2µl) and DNA template (2.5µl). After pulse centrifugation, the PCR conditions were set as described above. PCR product was run on gel and observed in gel documentation system **[10]**.

Expression analysis of rVP2 protein through SDS-PAGE

Recombinant *VP2* was inoculated on Luria Bertani broth and incubated in shaking incubator at 150 rpm for four hours. The cells were harvested by centrifugation at 1000 x g for 5 minutes and resuspended in the solution containing 50% glycerol, 0.6ml Tris HCl, 0.5ml B-Mercaptoethanol, 2ml of 10% SDS and 1ml of 1% bromophenol blue. To separate the cytoplasmic and nuclear fractions, the cells were passed five times through a 25.5-gauge needle. After centrifugation at 14,000 rpm for 15 minutes and diluted with sample buffer, cytoplasmic and nuclear fractions were separated by SDS-PAGE and presence of recombinant *VP2* was visualized by Coomassie brilliant blue staining **[11]**.

Results

Molecular cloning of VP2 of IBDV

Virulent strains of infectious bursal disease virus were isolated during outbreaks in district Faisalabad. A search for the DNA sequence homology between various strains present at database and the sequence of *rVP2* (to be published separately) revealed the short stretched of highly conserved sequence present at the amino terminal ends. Restriction sites were introduced to enable the convenient cloning of the *VP2* fragment into bacterial expression vector pET28a (Figure 1). cDNA was prepared corresponding to the partial *VP2* fragment and open reading frame of VP2 was amplified by PCR. A 699 bp amplified fragment was digested with *NdeI* and *EcoRI* and subcloned into pET28a (Figure 2) and recombinant *VP2* was isolated by colony PCR. (Figure 3).

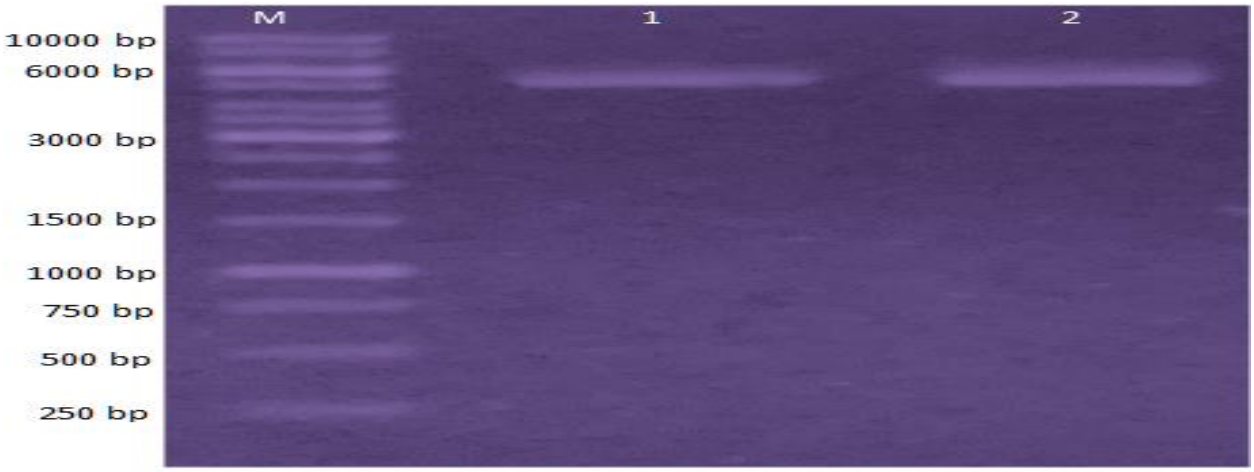

Figure 1. Lane M represents the 1kb marker while 1 and 2 represents the plasmid size of 5500bp

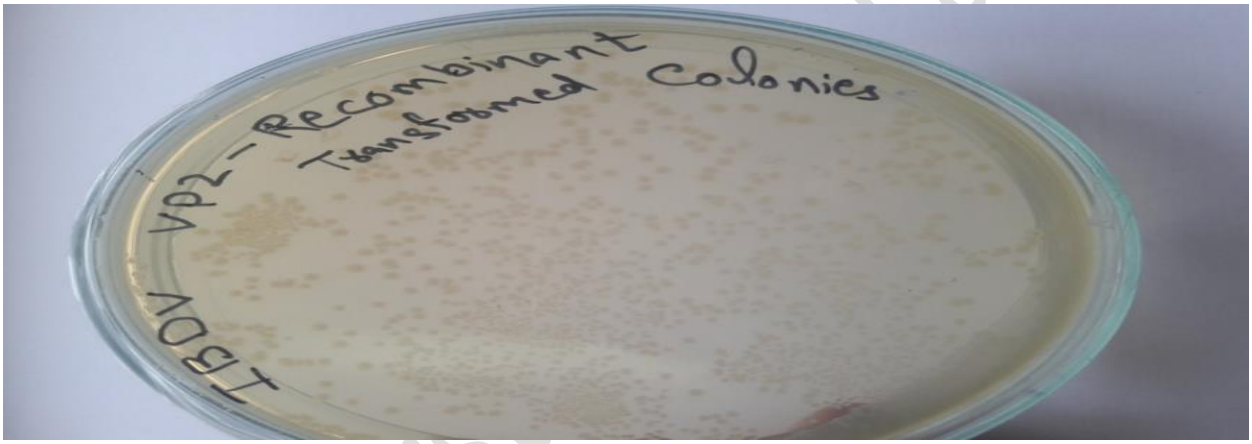

Figure 2. Represents the white transparent colonies *rVP2* in *E. coli* cells

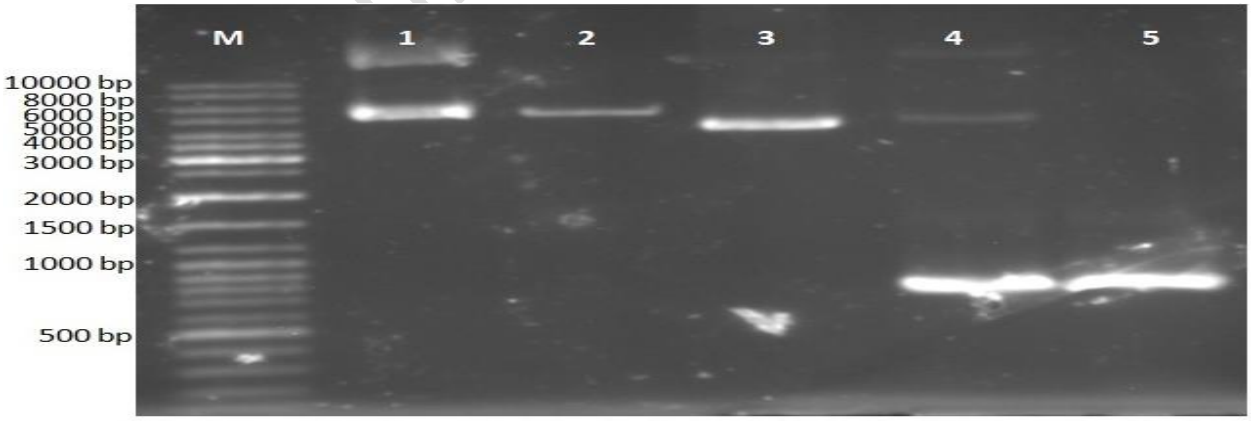

Figure 3. Lane M shows 1kb molecular weight marker (Gene Ruler™); Lane 1 shows pET-28a (Restricted/ Digested); Lane 2 shows recombinant pET-28a; Lane 3 shows pET-28a plasmid (uncut); Lane 4 shows recombinant plasmid after double digestion which emits a 699 bp clone fragment and Lane 5 shows 699 bp PCR product of partial *VP2* region (control)

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Recombinant *VP2* Expression analysis by SDS-PAGE

Expression analysis in sediment and supernatant of *VP2* protein was done by SDS-PAGE. Sample (30ul) was loaded in the wells of SDS gel after heating for 5 minutes at 90^{o}C. A current of 150V was passed through the gel and Coomassie brilliant blue stain was used to visualize the band with molecular mass of about 30 KDa [13,14]. Following the precipitation of the protein fractions with 25% ammonium sulfate, a strong band of *VP2* was detected in cytoplasmic fraction while no *VP2* was detected in nuclear fraction of the cells (Figure 4).

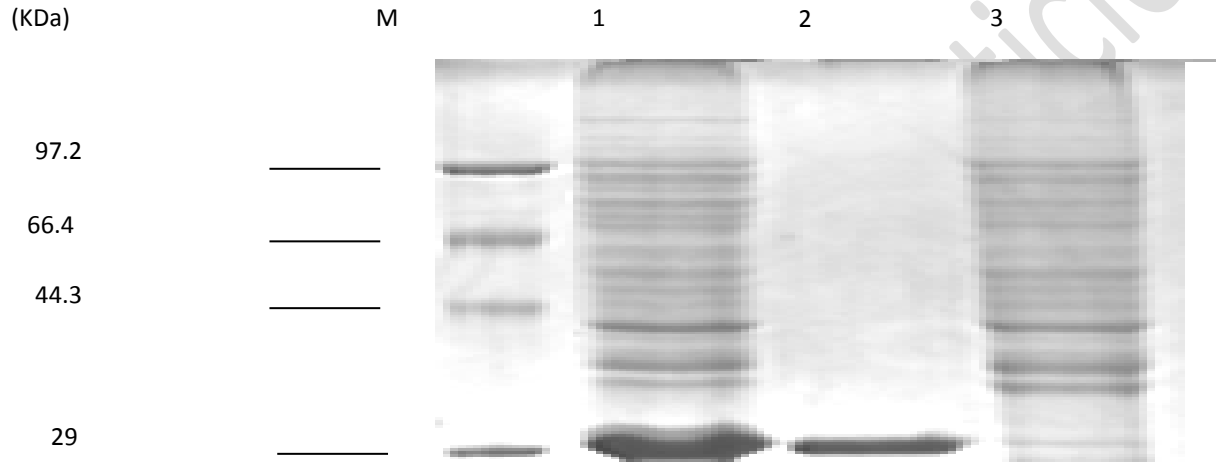

Figure 4. Represents the SDS analysis of Crude and purified *rVP2*. M is the molecular weight marker; Lane 1 shows pET-28a Transformed *VP2* cell lysate, IPTG induced *E. coli*; Lane 2 Shows *VP2* Purified Protein; Lane 3 shows empty cell lysate from pET-28a

Discussion

The economic importance of the IBDV in the world's poultry has made it a major pathogen of the poultry birds that leads to immunosuppression due to B cell destruction and secondary bacterial infection. IBD virus consists of two segments includes A constitutes *VP2, VP3* and *VP4* while the smaller segment B encodes only *VP1*. IBD is highly infectious disease and the only control is vaccination **[12,13]**. *VP2* segment of the infectious bursal disease virus considered as the most immunogenic region and could be used in the development of vaccine for the effective control of infectious bursal disease in poultry birds to eliminate the major economic losses. Moreover, the detection and screening of IBD is very crucial for the sake of protection of birds. Therefore, the recombinant DNA techniques can be effectively utilized **[14, 15]**.

This study focuses on the cloning of *VP2* region of infectious bursal disease virus in bacterial expression system. For this purpose, the suspected bursa samples were collected from the outbreaks at poultry farms located in the district Faisalabad. The infectious bursal disease virus was isolated and partial *VP2* gene was amplified by Polymerase chain reaction using cloning primers. The purified product was restricted using restriction enzymes and ligated in pET28a expression system. The ligated product was transformed in *Escherichia coli* cells. SDS-PAGE

analysis was done for the expression of *rVP2* region. The size of *rVP2* protein was expected to be larger than the native protein due to the addition of extra amino acids at the carboxyl terminus for cloning procedure.

The *rVP2* could be useful in the production of vaccine and the recombinant protein would eliminate the danger inherit in the use of killed or attenuated virus vaccine that is the outbreak of the disease as the result of changes in virus used in vaccine or its incomplete preparation. Immunization with the recombinant vaccine involves the minimum number of antigens essential to stimulate the formation of neutralizing antibodies while preventing the exposure of the antigen that might suppress the immune system. The *rVP2* enables the rapid production of a subunit vaccine and it could be easily modified for the rapid response to the virus drift by inserting the gene encoding *VP2* of the new variant in to bacterial expression system. Thus, a subunit vaccine against infectious bursal disease virus produced in bacterial expression system may be of great advantage to the poultry industry by stimulating the immediate production of protective antibodies. Another advantage of *rVP2* protein is the development of indirect ELISA for the early and possible detection of infectious bursal disease virus antibodies in the serum to determine the immune status of the poultry birds.

Conclusion

In this study we have expressed the *VP2* gene of IBDV in *Escherichia coli*. Through SDS-PAGE analysis, the expected band of 30KDa was detected after gel staining. This recombinant *VP2* region is involved in the formation of virus neutralization epitopes and this formation aids in the development of recombinant infectious bursal disease virus vaccines for poultry birds.

Authors' contributions

Conceived and designed the experiments: S Sajid & S Rahman, Performed the experiments: S Sajid & IU Khan, Analyzed the data: S Nayab, Contributed materials/ analysis/ tools: A Javeed, Wrote the paper: S Sajid.

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