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Research Article

Optimization of an Indirect ELISA for the Detection of Infectious Bursal Disease Virus Antibodies

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Abstract

Infectious bursal disease virus (IBDV) causes immunosuppressive disease in poultry leads to economic importance. IBD is highly infectious disease of poultry causing 60 to 100% mortality and immunosuppression in young chickens of 3 to 6 weeks age. IBD is a disease that infects the immune system of the poultry. This is characterized by the destruction of bursa of Fabricius (Primary lymphoid organ), where B cells mature and differentiate. IBDV target immature B cells and cause depletion of B lymphocytes leads to immune suppression. To provide antigen for diagnostic test, its major structural VP2 capsid protein was cloned into *Escherichia coli* pET28-a expression system. VP2 gene was inserted into pET-28a vector and purified using Ni-NTA column and protein concentration was determined by SDS-PAGE. Indirect-ELISA was optimized and developed using optimal dilution antigen and sera concentration and vaccine performance was evaluated by vaccinating the birds at day 1 by Bursaplex vaccine. Sera samples were checked for antibody titters using the developed i-ELISA kit and mean values of antibody titer was recorded as 0.39, 1.56, 2.38, 2.99, 2.67, 2.32 and 1.47 respectively. Antibody titer was increasing from day 3rd to day 7th. It was found maximum at day 7th then start decreasing. A marked decrease in antibody titer was observed at day 21st.

Keywords: *Antibody Titer; IDB; i-ELISA; Serum*

Introduction

The major contribution in global livestock share is poultry industry. The short productive span, reproductive traits, distribution throughout the world and egg production favors the use of poultry as a major protein source [1]. Poultry industry plays an important role in economic development and alleviation of poverty in Pakistan but the diseases, especially immunosuppressive viral diseases like Infectious bursal disease (IBD) is a major threat to the poultry industry.

IBD is an infectious disease of fowl which cause atrophy and necrosis of bursa of Fabricius. Infectious bursal disease (IBD) was first reported in broilers in Sussex, USA in 1962 [2]. While it was firstly reported in Karachi, Pakistan in 1971. The initial outbreaks of infectious bursal disease occurred in and around Gumboro, Delaware due to this reason IBD is also known as Gumboro disease that was described as infectious and contagious by Cosgrove in the year 1962 [3]. Due to the characteristic kidney lesion of this disease, Cosgrove referred this as avian nephritis. The causative agent of this disease was filtered from bursa of Fabricius in 1962 by Winterfield and named as infectious bursal disease virus causing infectious bursal disease in birds [4].

IBDV is non-enveloped, bisegmented dsRNA belong to Avibirnaviridae genus of Birnaviridae family mainly infecting broilers that leads to immunosuppression in birds. Virion has icosahedral symmetry, consist of single capsid of 32 capsomere having diameter of 60-70 nm. It has two serotypes, serotype 1 varies in pathogenicity and serotype 2 is non-pathogenic [5]. IBD Virus has 2 segments A and B. Larger

genome segment A (34 bp) encodes *VP2, Vp3, VP4* and *VP5* proteins and segment B is a the smaller genome of 2800 bp that encodes for *Vp1*. *VP1* is the protein which is responsible for the mRNA synthesis and replication of virus. With the infection of a cell with virus, specific ssRNA and dsRNA are synthesized. Nucleic acid replication takes place by strand displacement mechanism demonstrated by (VPg) viral genome linked proteins. Ribonucleoprotein complex formed by dsRNA genome wrapped by *VP3*, occupy the inner capsid of infectious bursal disease virus. This *VP3* linked covalently to VPg form *VP1* and during transcription this *VP1* free molecules act both as primer and polymerase [6,7].

IBD is highly infectious disease of poultry causing 60 to 100% mortality and immunosuppression in young chickens of 3 to 6 weeks age. Incidence of IBD in higher in layer birds than in broiler [8]. After infection the virus starts replicating in the macrophages and lymphocytes of GALT (Gut Associated Lymphoid Tissue). Then via blood it affects the bursa and destroys the B cells due to necrosis and apoptosis leads to the immunosuppression [9].

Natural host of infectious bursal disease virus is domestic fowl. Turkeys do not show clinical signs but considered to be affected with IBDV and serological evidence was observed in ducks and penguins. Dogs are considered as a carrier or reservoir of infectious bursal disease virus if fed on infected birds and IBD virus is detected in its feces indicates that it is capable of spreading infection to healthy birds [10]. Microscopically lesions are observed in bursa with inflammation signs leading to hyperemic, hypertrophic and edematous bursa. Mucosal surface of bursa gives yellowish colour accompanied by petechial hemorrhages. Earlier bursa becomes enlarged that comes to normal by 5th day and reduces to less than 3rd of normal size by 8th day [9].

IBDV is extremely resistant and can survive in poultry farm pens and feed for several weeks to months. No evidence has been present still for the vertical transmission i.e. in eggs [11] but infectious bursal disease spread horizontally either direct contact or indirectly through fomites, equipments, farm workers and contaminated feed, water and litter [12]. Primary method to control infectious bursal disease virus is immunization achieved through maternal antibodies in young chicks [13].

For the evaluation of vaccine programme and diagnostic purposes, detection of infectious bursal disease virus antibodies is a valuable tool. Virus neutralization assay is a specific tool for the detection of antibodies particular to a specific strain but this is laborious, time taking and expensive assay to perform in laboratory [14]. Infectious bursal disease virus can be identified through ELISA. Enzyme Linked Immunosorbent Assay is very sensitive assay to measure changes in enzyme activities utilizing enzyme conjugated antibodies with antigen or antibody involved in immune reaction [15]. Its application is the highly sensitive detection of disease related antibodies and its quantification [16]. The development of ELISA has played important role in the detection of IBD virus using monoclonal antibodies. The main focus of this study is the antigenicity of VP2 protein to detect anti-IBDV antibodies in enzyme linked immunosorbent assay, which will serve as safe, chemically defined and non-infectious serodiagnosis method.

Materials and Methods

Isolation of virus

Infected bursa was taken from field outbreaks and confirmed through histopathological examination. Viral RNA was extracted using FavorPrep® Nucleic Acid Extraction kit (Favorgen® Biotech).

Amplification of IBDV-VP2

The VP2 was amplified with the use of reverse transcriptase polymerase chain reaction (RT-PCR) kit by ABM® (Applied Biological Materials). The primers BGF: 3´- CATATGTGCAGCCGATGATTACC-3´and BGR: 3´- CCTGGAATTCTCAGGGGAGAGTTG -5´ amplified the partial VP2 from base 751 to base 1449 of genome segment A. The start codon in forward primer is underlined. For the VP2 reaction the PCR was at initial denaturation at 94°C for 4 minutes followed by 35 cycles of 94°C for 45 seconds, 57.6°C for 45 seconds and 72°C for 90 seconds while the final polymerization is at 72°C for 10 minutes.

Preparation of IBDV gene clones

Plasmid was isolated from *Escherichia coli* by alkaline lysis method. After restriction digestion of plasmid and insert DNA with EcoRI and NdeI, the 699-bp product of VP2 was inserted into pET-28a vector and ligation reaction was incubated overnight at 16°C. Plasmid was used to transform the *E. coli* strain BL21. Transformed bacteria was grown on LB-agar containing 100 µg/ml and incubated at 37°C overnight. White transparent colonies were confirmed by colony PCR and visualized on 1% agarose gel.

Expression and purification of recombinant VP2 protein

The VP2 protein was subjected to SDS-PAGE and the purification of 6x His-tagged protein was done by Ni-NTA purification system. Ni-NTA column has the affinity for six tandem histidine residues containing recombinant fusion protein. It uses nitrilotriacetic acid (NTA) in a cross linked 6x agarose matrix that binds Ni ions by four coordination sites.

Optimization of i-ELISA

Initially IBDV antibody negative and positive serum (serum from normal healthy birds and serum collected post infection) was taken to determine the appropriate dilutions. Control sera were taken from twenty experimental birds vaccinated with Bursaplex vaccine. Protein was diluted in carbonate-bicarbonate buffer to 25, 50 and 100 times for coating on ELISA plates and tested against the similar dilutions of negative and positive sera for their OD_{450} values. Most suitable dilution was chosen and depending upon their highest P/N value and adopted as an optimized dilution (P/N value: Positive sera OD_{450nm} divided by negative sera OD_{450nm}).

Development of Indirect Enzyme Linked Immunosorbent Assay (i-ELISA)

Antigen (100 µl) diluted in coating buffer was dispensed in all the wells. Plate was incubated at 4°C overnight and washed three times with PBST. Blocked with 5% skimmed milk in 0.02M PBS. Serum diluted with 0.5% skimmed milk in PBST was added and incubated for 1 hour at 37°C. Peroxidase conjugated antibodies diluted with 0.5% skimmed milk was added to each well and incubated for 1.5 hour at 37°C. After incubation plate was washed with PBST three times. Chromogenic substrate OPD freshly prepared was added to each well up to 100µl. Plate was protected from light and incubated at 37°C until suitable colour development. Reaction was stopped by adding 50 µl of 2M H_2SO_4 in all the wells. Directly from the bottom of micro well plate, results were read at 450 nm using SpectraMax M2 microplate reader and SoftMax Pro software.

Results

Histopathology

Bursa samples were fixed in 10% neutral buffered formalin. The histopathology procedure was adopted and tissues sections were stained with H and E (Haematoxylin and Eosin). Examination of bursa tissues under a microscope at 4X, 10X and 40X revealed the fibrosis in bursa with severe lymphocytic depletion in cortex region and infiltration of inflammatory cells.

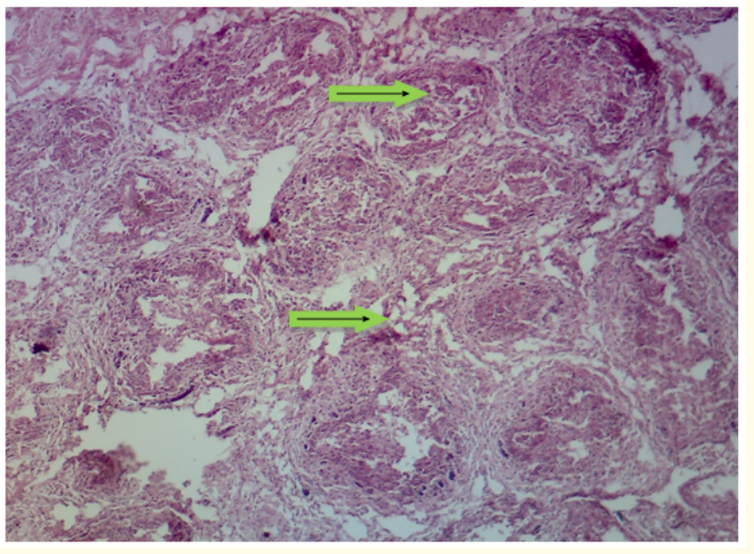

Figure 1: *Histopathology of Bursa of Fabricius shows lymphocytic depletion in cortex region.*

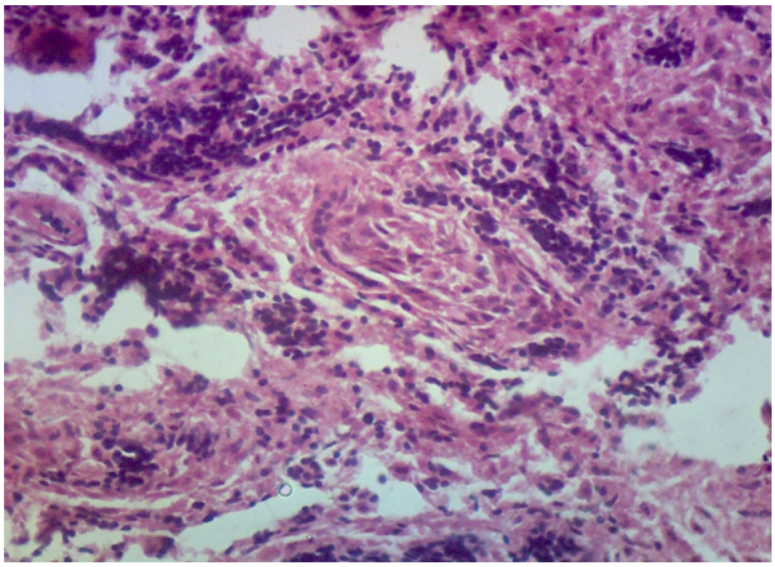

Figure 2: *Histopathology of Bursa of Fabricius shows infiltration of inflammatory cells.*

RT-PCR

The Primers were designed to conserved region that amplify 699 bp fragment of IBDV VP2 gene. RT-PCR was conducted and the final product was run on gel to get the desired bands.

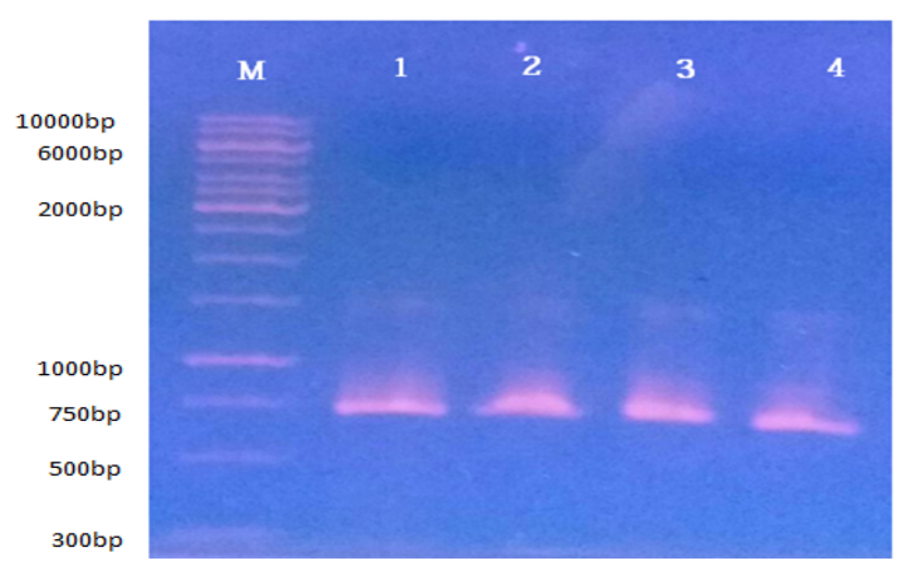

Figure 3: *Lane M shows GeneRuler DNA marker; Lane 1-4 shows 699 bp amplified PCR Products.*

Expression of VP2 protein

Recombinant proteins expression was analysed in supernatant and sediments. Heated the samples at 90 °C for 5 minutes and 30 µl form each samples was loaded. Gel was run at 150V current and the gel was subjected to Coomassie staining and visualized. Proteins were over expressed as inclusion bodies (Figure 4).

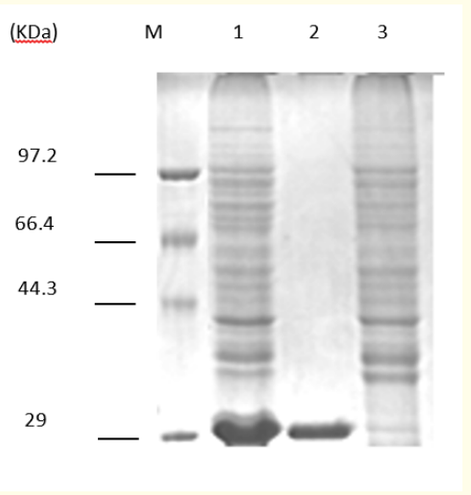

Figure 4: *SDS analysis of Crude and purified protein of recombinant VP2. M is the molecular weight marker; Lane 1 shows pET-28a Transformed VP2 cell lysate, IPTG induced E.coli; Lane 2 Shows VP2 Purified Protein; Lane 3 shows Empty cell lysate from pET-28a.*

Optimization of Protein and serum dilutions for ELISA

IBDV negative and positive serum samples were compared to determine the appropriate dilutions. The optimized condition for i-ELISA was 25 µg/ml for coating protein and 100 times serum dilution of samples.

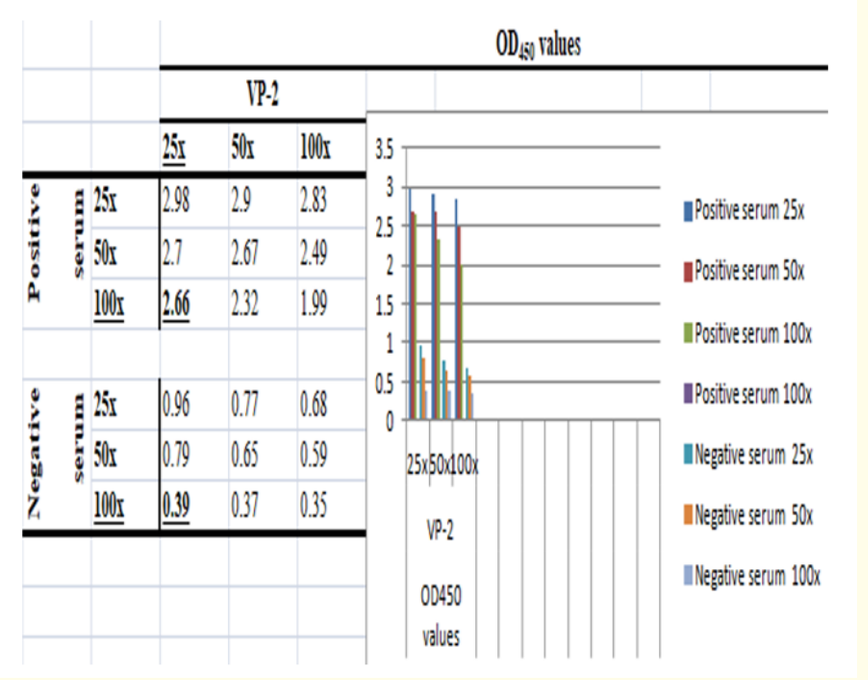

Figure 5: *Optimization of i-ELISA kit using positive and negative serum samples.*

Determination of antibody titer by i-ELISA developed using recombinant VP2-IBDV

Developed i-ELISA kit was evaluated with the positive sera from the 50 poultry birds. One day old chicks were tagged and vaccinated with commercial vaccine Bursaplex (Zoetis, USA). 0.2 ml of the vaccine was injected subcutaneously. Sera samples were collected at days 3rd, 5th, 7th, 10th, 14th and 21st. ELISA was performed using positive samples while negative run as a control. Result showed: (1) VP2 was more antigen and suitable for coating as an antigen for i-ELISA; (2) Sera samples at different days were tested and substantial difference was observed; (3) The vaccinated sera samples at different days were collected and tested with developed i-ELISA kit expressed the titer as OD_{450} values were higher at day 7th. Then a marked decrease was observed after day 7th to 21st.

	OD_{450} values
	VP2-IBDV
IBDV Anti-serum	2.66
Negative Serum	0.39
Day 3	1.56
Day 5	2.38
Day 7	2.99
Day 10	2.67
Day 14	2.32
Day 21	1.47

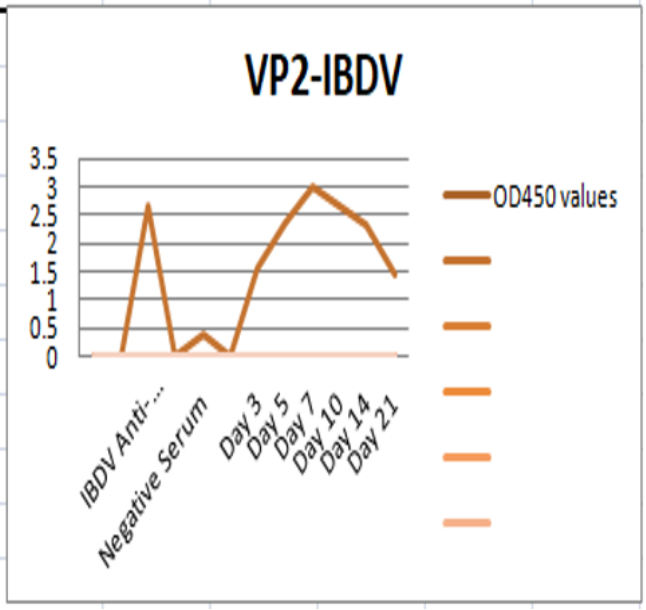

Figure 6: *Vaccination of birds with Bursaplex and the estimation of antibody titer using developed i-ELISA Kit (Mean Values).*

Discussion

The poultry industry of Pakistan has reached to the level of self-sufficiency during the last few years. Fast growth of the poultry industry has encouraged a lot of people to invest and adopt the poultry farming as business and source of income. However, inspite of favorable conditions and availability of high producing birds, the poultry sector is a witness of several problems and many lethal diseases like infectious bursal disease virus [17].

IBDV has great economical importance. The target organ of this disease is bursa of Fabricius which destroys B lymphocytes that leads to immunosuppression [18]. Infectious bursal disease virus is very difficult to isolate, laboratory diagnosis are based on serological testing and molecular methods. The primary control of infectious bursal disease is vaccination. One of the major reasons of genetic drift of

field viruses is the failure of vaccine that leads to the divergence of variant strains [19]. Infectious bursal disease is the immunosuppressive disease of poultry that accompanied by high level of specific anti IBDV antibodies. Successful immunization required reliable vaccine and determination of vaccine performance is very important as to determine the antibody titer of the birds.

In this study VP2 region was selected since, it is variable region and provide genetic data regarding variability among stains and best evolutionary clue [20]. This study was designed to develop an indirect ELISA kit using recombinant VP2 protein of infectious bursal disease virus. The purpose of study was to optimize the test conditions for i-ELISA, evaluation of vaccine performance and the determination of antibody titer using the developed ELISA kit. Virus was isolated form infected bursa samples and amplified by self designed primers to get the bands. Primer set was used to amplify the partial VP2 region form 751 bp - 1449 bp to get the 699 bp product. Viral DNA was inserted and transformed in BL21 expression system and clone was confirmed through colony PCR. Protein was expressed by SDS-PAGE analysis. After purification of protein through affinity column this protein was coated on ELISA Plates. Different dilutions of protein and sera samples were used to optimize the test conditions for i-ELISA. 25x dilution containing 25 µg/ml of protein were used for coating on ELISA plates with 100 time dilution of serum. For the evaluation of vaccine performance and to check the antibody titer, 50 birds were kept. At day 1 all the birds were vaccinated with Bursaplex vaccine with the dose rate of 0.2 ml/bird subcutaneously. Sera samples were collected at day 3, 5, 7, 10, 14 and 21. Sera samples were checked for antibodies using the developed i-ELISA kit and mean values of antibody titer was recorded as 0.39, 1.56, 2.38, 2.99, 2.67, 2.32 and 1.47 respectively. Antibody titer was increasing from day 3 to day 7. It was found maximum at day 7 then start decreasing. A marked decrease in antibody titer was observed at day 21.

Conclusion

This study concluded that VP2 was more immunogenic as compared to VP1, Vp3 and Vp4 region. VP2 is more suitable for coating as an antigen for the development of indirect ELISA as compared to other proteins and whole IBDV because it has advantage of having antigenic region which functions as neutralizing antibodies while VP3 and VP4 produce non-neutralizing antibodies. In hyper immune sera the polyclonal antibodies raised by VP2 are more immunogenic and suitable for detection hence, used for the development of indirect ELISA.

Future Aspects

Only one shortcoming in this study is that, it is very difficult to isolate and amplify the DNA of infectious bursal disease virus as it is double stranded RNA virus. But using a suitable primers and optimization of PCR it could be sorted. Future improvisations could be the development of subunit vaccine against infectious bursal disease virus using the recombinant VP2 protein.

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