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Bicistronic DNA vaccine containing two VP2 genes of infectious bursal disease virus confers enhanced immunity and protection

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Summary: Infectious bursal disease (IBD) is a highly contagious immuno-suppressive viral disease of young chicken. The vaccine currently being used to protect the birds causes bursal lesions and mild immunosuppression. The DNA vaccine which elicits comprehensive immune response offers several advantages. In the present study, two VP2 genes of IBDV were cloned in a bicistronic vector for enhancing immune response in birds. IBDV VP2 genes were subcloned from pVAX1ibdvp2s and pcDNAibdvp2$_f$ in different multiple cloning sites of pIRES, a bicistronic mammalian expression vector. Bicistronic DNA plasmid encoding IBDV VP2 gene and chicken IL-2 was also constructed by subcloning chicken IL-2 from pGEMT.IL-2 into recombinant pIRESibdvp2$_s$ plasmid. The recombinant clones were characterized by restriction enzyme analysis and used to transfect MDCK cells to detect expression of VP2 and IL2 proteins *in vitro* in MDCK cells which was confirmed by IFAT, IPT, SDS-PAGE/Western blotting. *In vivo* DNA vaccination study in birds showed 80% protection by bicistronic pIRES.ibdvp2s.f with CMI response being higher than humoral response, 70% by pIRESibdvp2$_s$IL-2 with good humoral immune response and 60% in monocistronic pIRES.ibdvp2 group.

Key words: IBD, DNA vaccine, cloning, immunity, VP2 gene, bicistronic vector

Introduction

Infectious bursal disease (IBD) is highly contagious immunosuppressive viral disease of particularly young chicken of 3-6 weeks age caused by IBD virus (IBDV).The IBDV primarily affects bursa of Fabricius resulting in lymphoid depletion. In the classical form of the disease, the mortality rate may range generally from 1 to 50% but it may go upto 90%.Very virulent strains are reported to cross maternal antibody barrier. IBDV which belongs to genus *Avibirnavirus* of *Birnaviridae* family, contains double stranded bi-segmented RNA genome. Segment A has two open reading frames, the larger ORF encodes for polyprotein VP2-VP4-VP3 which is proteolytically cleaved to two structural proteinsVP2 and VP3 and a protease, VP4, the other ORF encodes for nonstructural VP5 protein; segment B codes for RNA polymerase. Among structural proteins, VP2 contains major virus neutralizing epitopes and has been utilized for developing recombinant/subunit vaccines. Two distinct serotypes of IBDV are known, out of which only serotype 1

viruses are pathogenic in nature with varying virulence. Infection with serotype 1 strains are not protected with existing conventional vaccines and to control these strains, vaccine strains with intermediate virulence are used which cause mild bursal lesions and atrophy resulting in immuno-suppression which interferes with other vaccinations (Tsukamoto *et al.,* 1999). In addition, the live attenuated vaccines have the risk associated with the potential for reversion to a virulent phenotype. Inactivated vaccines are typically safe but less effective than attenuated vaccines (van den Berg, 2000). To overcome these disadvantages, newer effective method of DNA vaccination was developed. In the present study, two copies of VP2 gene were cloned in bicistronic vector and used as DNA vaccine against IBD with or without IL-2 gene in order to achieve improved protection.

Materials and Methods

Vectors

pIRES cloning vector (Clontech, BD Biosciences) was used for cloning two copies of VP2 genes ($VP2_s$- synthesized in lab; $VP2_f$- amplified from Indian field isolate) for use as DNA vaccine. The recombinant plasmids pVAX1.ibdvp2_s containing synthetic VP2 gene and pcDNA.ibdvp2_f containing VP2 from IBDV field isolate, available in the laboratory, were used as source for these VP2 genes. The recombinant plasmid pGEM.IL2ch , available in the lab was used as a source for chicken IL-2 gene.

Chicken

One-day-old broiler chicks were procured from the Experimental Hatchery Section of Central Avian Research Institute, Izatnagar-243 122, UP, India for immunization with recombinant plasmids.

Construction of Bicistronic plasmid

The $VP2_s$ gene was excised from recombinant pVAX1.ibdvp2_s plasmid by digesting with XbaI and NheI restriction enzymes and cloned in multiple cloning site A (MCS A) at NheI site of pIRES vector. The $VP2_f$ gene was excised from pcDNA.ibdvp2_f plasmid using NheI and NotI restriction enzymes and cloned in MCS B at NotI site in pIRES.ibdvp2.s to construct. pIRES.ibdvp2s.f. Similarly pIRES.ibdvp2s.il2 was constructed by cloning chicken IL-2 at EcoRI site in second multiple cloning site

Transfection and expression of recombinant VP2

MDCK cells (National Center for Cell Sciences, Pune, India) grown to 70% confluency was transfected with purified pIRES.ibdvp2s.f (QIAquick plasmid mini kit, QIAGEN, Germany) using Lipofectin® Reagent (Invitrogen, USA) following manufacturer's protocol. At 72 h post transfection, medium from transfected cells was discarded and cells were washed with phosphate buffered saline, rinsed once with 80% acetone (in water), fixed with 80% chilled acetone at room temperature for 30 min and incubated

with 2% BSA for 1 h at room temperature. It was then incubated at 37^0C for 1 h with mouse Mabs against IBDV VP2 diluted 1:50 in 1% BSA and thereafter with goat anti-mouse IgG- FITC (1:200) diluted in 1% BSA at 37^0C for 1 h in the dark. After every incubation, cells were washed three times with PBS. The stained cells were observed under fluorescent microscope.

Dot blot assay was performed according to Sambrook and Russell (2001) to assess if VP2 was expressed. The expression was confirmed by using IBDV specific chicken polyclonal antibodies and rabbit anti-chicken HRP conjugate. After washing with PBS-T, the blot was incubated with substrate solution (3'3 diaminobenzidine-H_2O_2) till brown colour developed. The reaction was stopped by washing the membrane with water.

Immune response of the bicistronic DNA vaccine

For Immunization, recombinant plasmids isolated using QIAGEN HISpeed plasmid purification kit were used to immunize chicks as given below. These birds were immunized with 50 µg plasmid DNA intramuscularly in the quadriceps muscles of hind leg at 10 days of age. Before, immunization serum from birds were assessed for presence of maternal antibody.

Group	No of chicks	Vaccine	Plasmid type
I	20	Bicistronic IBDV Vaccine	pIRES.ibdvp2s.$_f$
II	10	Bicistronic IBDV VP2 and IL2Vaccine	pIRES.ibdvp2$_{s.il}$-2
III	10	Vector Control	pIRES
IV	10	Monovalent IBDV VP2$_{(s)}$ Vaccine	pIRES.ibdvp2$_s$
V	10	Monovalent IBDV VP2$_{(f)}$ Vaccine	pcDNA.ibdvp2$_f$
VI	10	Commercial IBD Vaccine	
VII	10	Healthy Control	

Serum neutralization test (SNT)

Sera samples from all the groups of the birds were assayed by SNT using different dilutions of sera and 100TCID$_{50}$ cell culture adapted IBDV in 96 well plate as per the procedure of OIE (Anon, 2004). Reciprocal of highest dilutions of sera showing complete neutralization of the virus was taken as end point titre.

Peripheral blood lymphocyte proliferation assay

The lymphocyte proliferation test was performed as per Bounous et al (1992) to assess the CMI response. . The buffy coat from chicken blood were separated by centrifugation for 30 min at 2500 rpm on histopaque 1.077 density gradient. Lymphocytes at the interface were collected, washed three times in RPMI-1640 and resuspended in RPMI-1640 medium supplemented with 10% FBS. For proliferation assay, 4 x10^5 cells in 50µl were placed in each well of 96-well flat-bottomed tissue culture

plates and 200 ng of Concanavalin A (ConA, Sigma) in 5 µl was added to each well except the negative control and virus control wells. Plates were incubated at 37^{0}C in a humidified incubator for 72 h, 100 µg (in 20 µl) MTT was added to each well, plates were further incubated for 4 h at 37^{0}C under 5% CO_2 and then 150 µl DMSO was added to each well. After mixing, the plate was read at 550 nm with reference reduction at 655 nm using a microtiter plate reader (Biorad , USA). The stimulation Index (SI) was calculated by the formula: SI=mean OD of ConA or virus stimulated cells/ mean OD of unstimulated cells.

Flow Cytometry

FACS was carried out as per the protocol described by manufacturer (Serotec, USA) manual. Briefly, whole blood collected aseptically and incubated with anti-chicken CD8, CD4, CD3 and Bu+1a (Serotec,) antibody conjugated with rhodamine and FITC for 15 min. RBCs were lysed using NH_4Cl and the remaining pellet were resuspended in PBS after centrifugation at 2500 rpm for 5 mins. CD3 conjugated with FITC conjugate were screened by FL-1 filter while CD8 and CD4 were screened by FL-3 filter in FACS Calibre (BD Biosciences, USA) by taking 10000 events for a single sample.

Challenge test

All the vaccinated birds along with control were challenged 35 days post immunization with 10^6 ELD_{50} virulent IBDV. The birds were observed for 10 days for IBDV specific symptoms and mortality and % protection was determined by formula, % Protection = number of birds showing no symptoms or death X 100/ number of birds in a group. The birds showing symptoms like whitish diarrhea, vent peking, ruffling of feather, closed eyes and recumbency were isolated and examined for gross bursal lesion. Bursa lesion scores were prepared depending on gross picture of bursa : 0- No lesion, 1- slight change, 2- scattered or partial bursal damage, 3- 50% or less follicle damage, 4- 51 to75% follicle damage, 5- 76 to100% bursal damage. Small representative pieces (5 mm thickness) of bursa from respective groups were collected in 10% formalin, fixed for 3-4 days , and processed , for histopathological examination .

Results

The $VP2_s$ was successfully ligated to linear pIRES vector between blunt and NheI sites in multiple cloning site A (MCS A). Digestion of recombinant pIRESibdvp2_s with NdeI produced distinct 1222 bp and 6247 bp fragments (Fig. 1A) whereas pIRESibdvp2_s was digested with NheI and MluI released 1367 bp vp2_s gene was released (Fig.1B). The $VP2_f$ gene was was ligated in to MCS B of linear pIRESibdvp2s vector between XbaI and NotI sites. The recombinant pIRES.ibdvp2s.$_f$ was digested with *Eco*RI enzyme to confirm the right orientation of VP2 gene wherein 1365 bp fragment was released (Fig. 2A) . This was further confirmed by nested PCR with T3 reverse primer and gene specific forward primer that produced a distinct band at 770 bp (Fig. 2B). The 432 bp IL-2ch gene released from pGEMT.IL-2ch plasmid could be successfully ligated to the MCS B of pIRES.ibdvp2s.$_f$

from which ibdVP2$_f$ had been released by EcoRI digestion. The resultant recombinant DNA pIRESibdvp2s.IL-2ch digested with EcoRI released the IL-2 gene (Fig. 3A) . Digestion with NotI and XhoI confirmed the right orientation of the IL-2 gene as digestion with NotI and XhoI produces distinct 280 bp in right orientation. (Fig.3B).

The immunoflourescence analysis revealed diffuse cytoplasmic fluorescence in the MDCK cells transfected with pIRES.ibdvp2s.$_f$ while no fluorescence was seen in mock transfected cells confirming the gene expression (Fig 4 A,B)). The expression was further confirmed by SDS-PAGE and immunoblotting and also by dot blot analysis of transfected MDCK cells which showed clear dots as compared to control in which no dot was seen. Lymphocyte proliferation assay showed marked stimulation of lymphocytes collected after second booster from DNA immunized chicks. There was a marked increase in stimulation index of virus stimulated cells as compared to ConA stimulated cells (Table 1). Similarly, flow cytometric analysis of CD4 and CD8 clearly showed the proliferation of CD8 cells in pIRES.ibdvp2s.f group while CD4 response was prominent as compared to CD8 in pIRES.ibdvp2s.IL-2ch. The pIRES.ibdvp2s.$_f$ showed 46% CD4 while pIRES.ibdvp2s.IL-2ch showed 59% CD8 cells in 35 day post -vaccination blood sample. Post-challenge examination of birds revealed hemorrhages and enlargements in non-vaccinated groups whereas no such changes were seen in the birds vaccinated with pIRES.ibdvp2s.f. The hemorrhages in the bursa were also evident in birds of other groups. Challenge of birds with virulent IBDV revealed 80% protection in the birds vaccinated with pIRES.ibdvp2s.f followed by 70% in birds vaccinated with pIRES.ibdvp2s.IL-2ch and 40-60% in groups vaccinated with rplasmid containing single IBDV vp2 gene (Table 1).

Table1: Immune response of birds vaccinated with different recombinant plasmid constructs 32 days post-vaccination.

Groups	Lymphocyte stimulation index		No of birds showing no bursal lesions		
	by ConA	by virus		SN antibody titre of sera#	% Protection** on challenge*
pIRES.ibdvp2s.$_f$	1.206818	1.447727	11/20	256	80 (16/20)
pIRES.ibdvp2$_{s.il}$-2	1.118568	1.243848	4/10	256	70 (7/10)
pIRES	1.006742	1.173034	1/10	8	10 (1/10)
pIRES.ibdvp$_s$	1.052632	1.210526	4/10	16	60 (6/10)
pcDNA.ibdvp2$_f$	0.956848	1.090056	1/10	4	40 (4/10)
Control	0.80625	1.075	0/10	4	0 (0/10)
Live attenuated vaccine	1.0025	1.13959	3/10	8	60 (6/10)

*The birds were sacrificed on 10 days post challenge and bursa of Fabricius was examined grossly and histopathologically

#dilution of sera that completely neutralized the IBD virus in cell culture.

** figures in parenthesis denote number of birds protected/total number of birds in group.

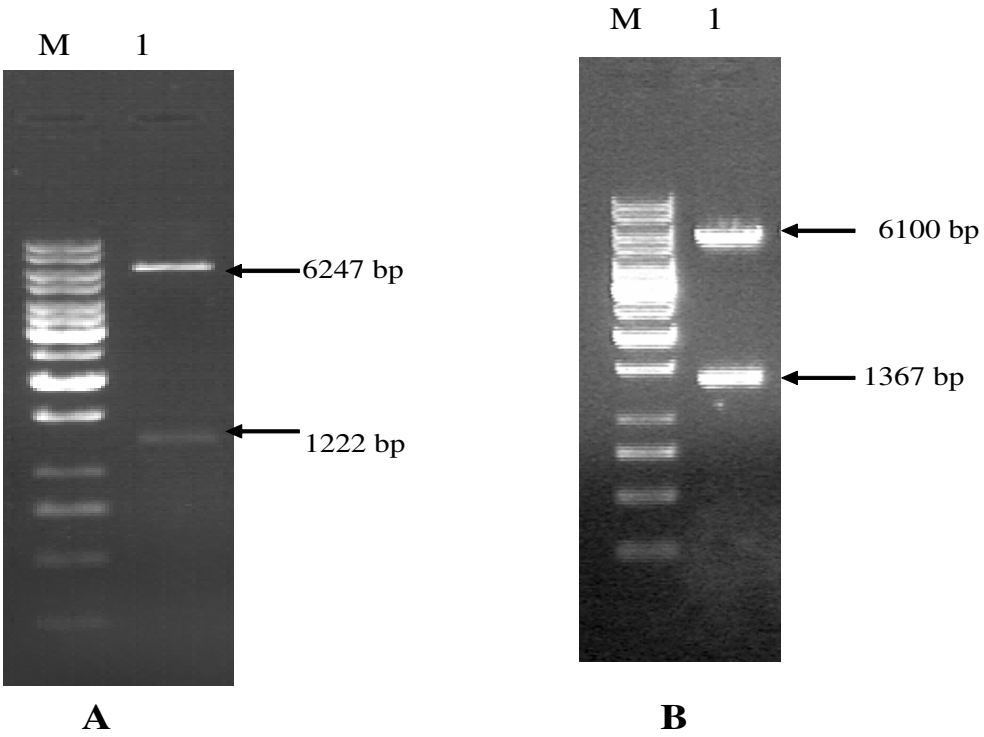


Fig1. Characterization of pIRESibdvp2s plasmid by RE analysis. A:Lane M: 1Kb DNA ladder, 1: Release of NdeI digested IBDV VP2s gene fragment; B:Lane M: 1Kb DNA ladder ,1: Release of IBDV VP2s gene from monocistronic pIRESibdvp2s plasmid digested with NheI and MluI.

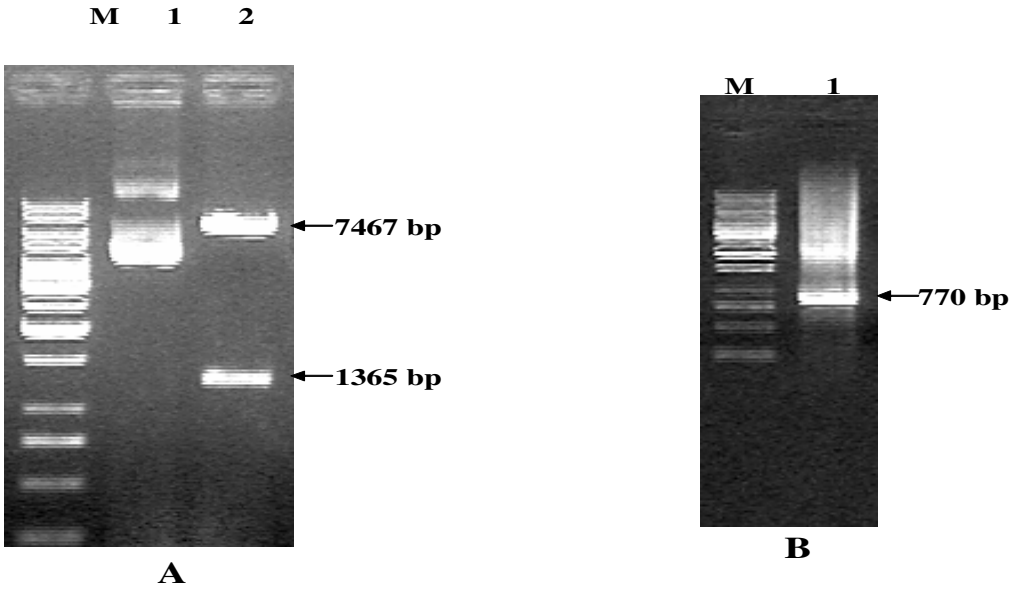


Fig2.Characterization of pIRESibdvp2s.-f plasmid by RE analysis. A.Lane M: 1 kb DNA ladder,1: uncut rplasmid, 2: EcoRI digested rplasmid showing release of 1365 bp VP2 gene insert.B: Lane M: 1 kb DNA ladder,1: PCR amplified 770 bp VP2 gene.

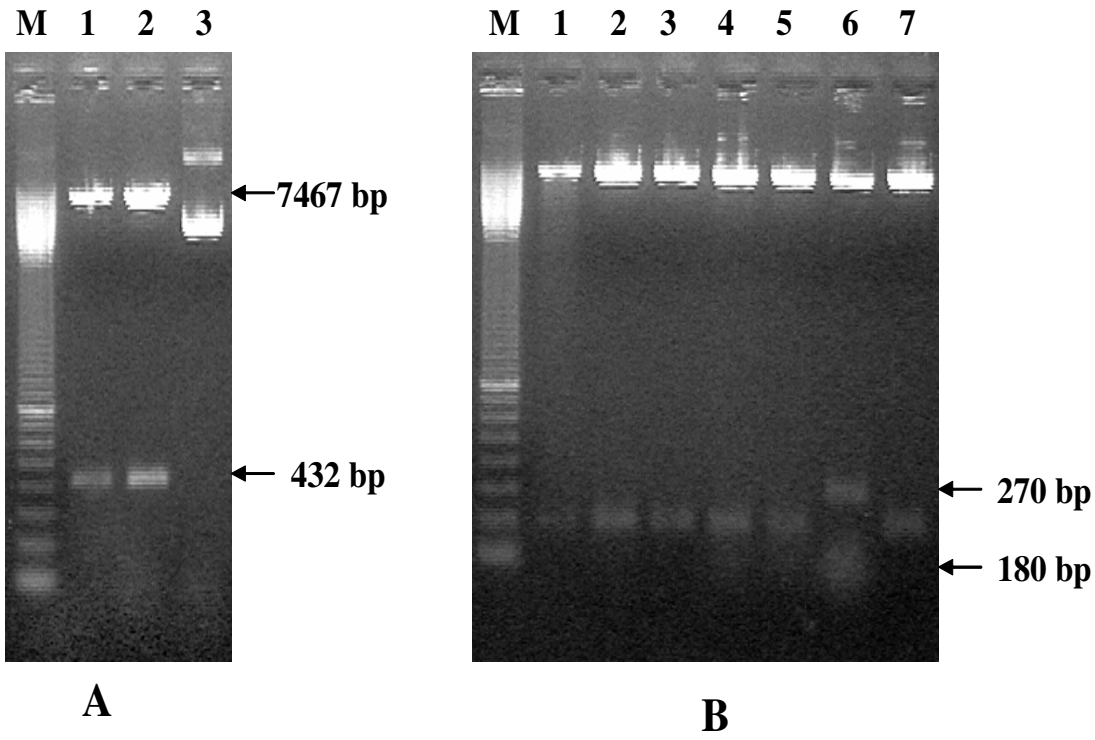
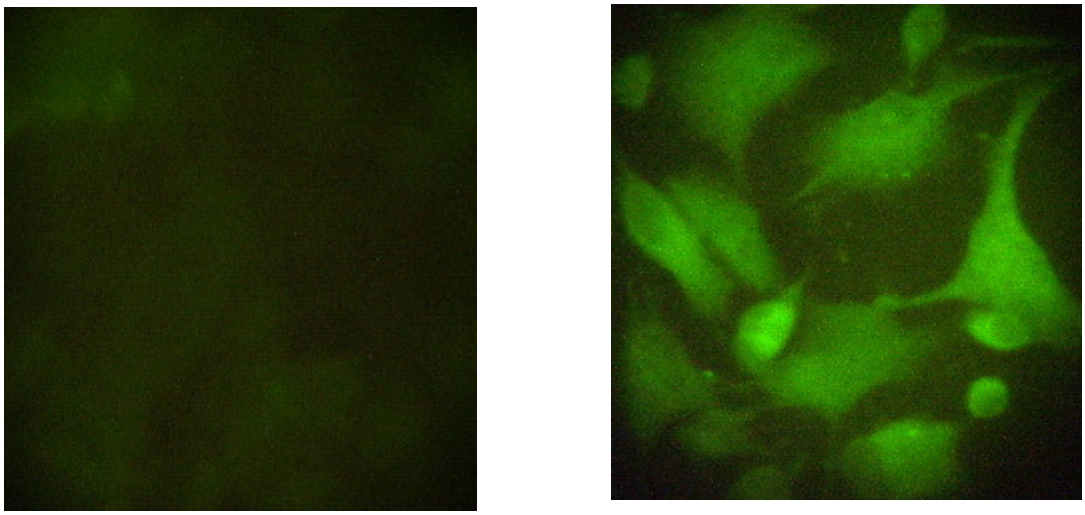


Fig3. Characterization of pIRESibdvp2s-il-2ch plasmid by REanalysis.A:Lane M: 1 kb DNA ladder, 1&2 : EcoRI digested rplasmid showing release of 432 bp IL-2 gene insert,3: uncut rplasmidB:Lane M: 1 kb DNA ladder; 1-7 NotI and XhoI digested rplasmid.



A B

Fig4. Indirect immunofluorescence showing expression of VP2 in the cytoplasm of **pIRESibdvp2s.-f** transfected MDCK cells. A: Mock transfected cells; B: MDCK transfected with **pIRESibdvp2s.-f**.

Discussion

IBD is a serious problem for commercial broiler production. Chicken vaccinated with IBD vaccines (mild strains like Lukert or Georgia) are not protected against variant or vvIBDV strains. Live vaccines using intermediate virulent strains such as intermediate plus, cause mild to moderate bursal atrophy and immunosuppression (Lukert and Saif, 1997, Tsukamoto *et al*., 1995). The DNA vaccination offers several advantages over conventional vaccine (Robinson *et al.,* 1993) and has successfully induced protective immunity against many pathogens in different species (Kodihalli *et al*., 1997; Fan *et al*., 2002; Serezani *et al*., 2002). The VP2, being major structural protein of IBDV contains antigenic epitopes responsible for induction of neutralizing/protective antibodies (Becht *et al.,* 1988), hence has been used by many workers for developing subunit/DNA vaccine (Chang *et al*., 2002; Chauhan *et al.,*2005). Despite the advancement of DNA vaccine technology, the protection afforded by DNA vaccines against various infectious agents is not always satisfactory. None of the DNA vaccines against IBDV by taking VP2 gene alone produced a satisfactory level of protection (Chang *et al*., 2002; Diane and Carlos, 2004). Therefore, in the present study, two copies of VP2 genes of IBDV were cloned in different multiple cloning sites in bicistronic pIRES mammalian expression vector to achieve enhanced protection. The pIRES mammalian expression vector contains internal ribosomal entry site (IRES) from encephalomyocarditis virus and cytomegalovirus (CMV) promoter. IRES allows translation of two consecutive open reading frames from same messenger RNA (Rainczuk *et al*., 2004). Bicistronic vaccine in our study showed enhanced expression in mammalian cell lines despite its low copy number which shows the high efficacy of IRES sequence. Bicistronic DNA vaccine vectors exploiting IRES sequences have been used to co-express two genes (Manoj *et al.,* 2003; Rainczuk *et al*., 2004). Polycistronic vectors (Stall *et al*., 1996) have also been used in gene therapy for enhanced immune responses (Sharma *et al.,* 1996; Guan *et al*., 2001). Workers have also used bicistronic DNA vaccine vectors exploiting IRES sequences to co express hepatitis B surface antigen and interleukin-2 in hepatitis DNA vaccine (Chow et al., 1997).

Transfection of bicistronic DNA into mammalian cells expressed the VP2 protein even after 96 h as indicated in indirect immunoflourescence and immunoperoxidase tests which showed high efficacy of the recombinant plasmid. Cell lysates from transfected cells showed expected bands of 42 kDa in SDS-PAGE which reacted with anti-IBDV seum in western blot confirming the authenticity of VP2 protein. The intensity of band was intense in pIRES.ibdvp2s.$_f$ (bicistronic) transfected cells as compared to pIRESibdvp2$_s$ (monocistronic) transfected cells indicating the level of expression being higher in the construct containing two copies of IBDV vp2 gene. High level expression was further confirmed by dot blot assay which gave clear intense dot in bicistronic group and faint ring in monocistronic group indicating lower concentration of protein expressed by single IBDV vp2 gene.

Because protection of chicken from infection with IBDV has been shown to correlate well with the antibody titre in ELISA (Tsukamoto *et al*., 1995), much of the effort with conventional vaccine for IBD has been focused on the enhancement of humoral immune response. Plasmid construct containing VP2–VP4–VP3 from classical IBDV strain STC induced low or undetectable antibodies in chicken before challenge (Chang *et al*., 2002). DNA vaccines constructed with IBDV strains GP40 and D78 induced antibody production in chickens but some of the immunized chicken, in spite of high antibody response, were not protected from the disease (Fodor *et al*., 1999). However, these two IBDV DNA vaccine studies only examined humoral immune response. Rautenschlein *et al* (2002) in a study using an inactivated IBDV vaccine, reported that T cells were critically involved in protection and antibody alone was inadequate in inducing protection in chickens. .

In our study, direct evidence was provided for involvement of cell mediated immune response in IBDV protection after DNA vaccination. The results obtained from lymphocyte transformation test of birds immunized with pIRES.ibdvp2s.$_f$ indicated significant proliferation of T lymphocytes which were further strengthened by FACS analysis which showed significant degree of CD8 (59%) cell population. while CD4 cells were dominating in pIRES.ibdvp2s.IL-2ch (46%). Tsukamoto *et al* (1999) suggested that CMI plays important role in providing protection against IBDV in chicken vaccinated with recombinant viral vector based vaccine against IBD. Chicken treated with cyclophosphamide, which caused selective destruction of B cells resulting in severely compromised antibody-producing ability, were protected against virulent IBDV (Yeh *et al*., 2002). Our finding of DNA vaccine showed marked enhancement of cell mediated immune responses thus corroborating the findings of above workers. In addition, memory T cells were retained and effectively responded to exposure to the virus (Yeh *et al*., 2002).In our study, non-vaccinated healthy birds after challenge showed typical symptoms of IBDV like whitish watery diarrhea, anorexia, ruffled feathers, trembling and severe prostration as reported by Cosgrove *et al* (1962) which were further confirmed by postmortem of diseased birds which showed hemorrhagic atrophied bursa and histopathology showed marked infiltration of RBC in bursal follicle. Such lesions were not observed in birds vaccinated with recombinant plasmid containing two copies of VP2 gene. Birds vaccinated with two copies of IBDV VP2 genes which were protected from challenge showed less bursal atrophy indicated by gross bursal lesion and histopathology of sacrificed bursa. Although the bursae of birds of bicistronic DNA vaccine group showed slight vacuolation and hemorrhages after challenge, their forms of follicles were similar to normal bursae as reported by Lukert and Saif (1997). From the present study, it can be concluded that recombinant plasmid having two copies of VP2 as well as VP2 and IL-2 genes were better in inducing protective immune response as compared to single VP2 gene.

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