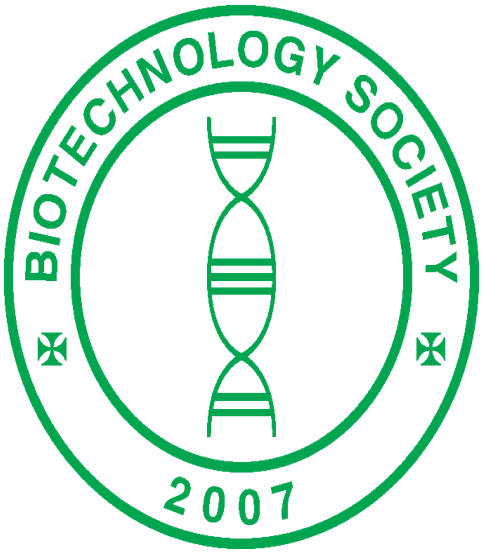
ISSN 0974-1453

BIOTECHNOLOGY INTERNATIONAL

Volume 1.1 Number 1 March 2008



BIOTECHNOLOGY SOCIETY
website: www.biotechsoc.in

2008

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2) Review articles: Review articles on Recent Topics in Biotechnology will be considered.

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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.

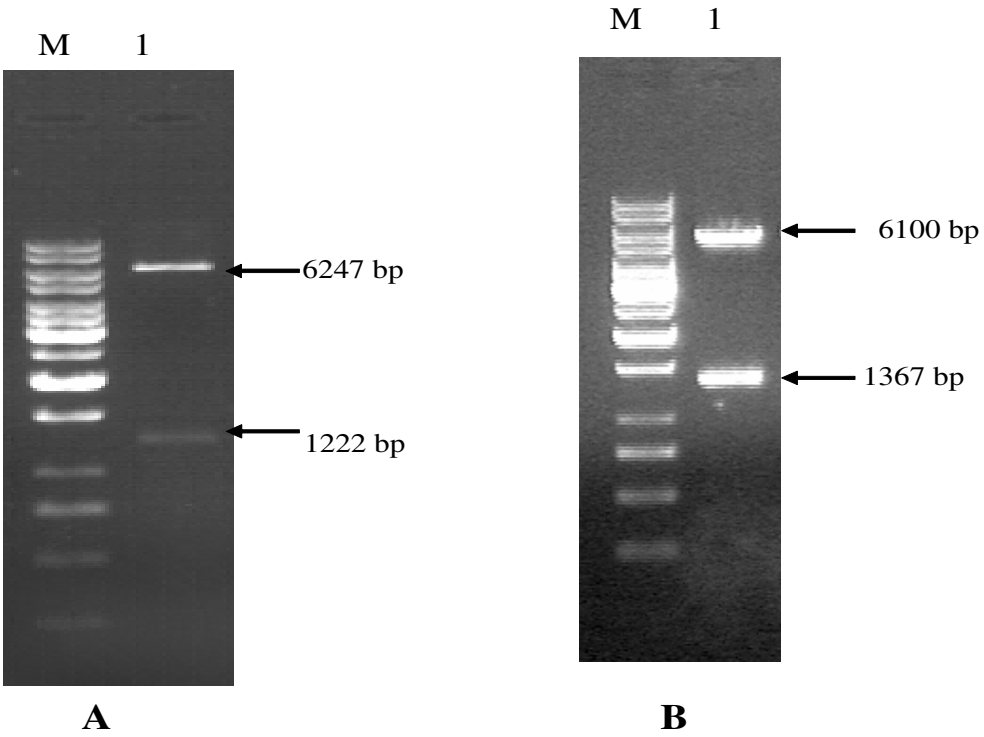


Fig1. Characterization of pIRESibdvp2s plasmid by RE analysis. A:Lane M: 1Kb DNA ladder, 1: Release of NdeI digested IBDV VP2s gene fragment; B:Lane M: 1Kb DNA ladder ,1: Release of IBDV VP2s gene from monocistronic pIRESibdvp2s plasmid digested with NheI and MluI.

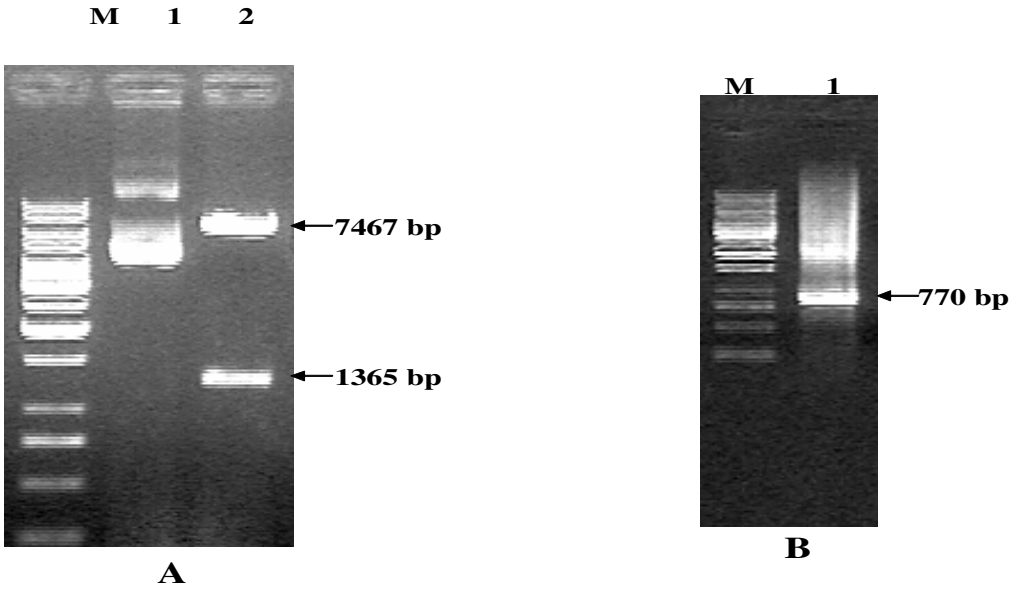


Fig2.Characterization of pIRESibdvp2s.-f plasmid by RE analysis. A.Lane M: 1 kb DNA ladder,1: uncut rplasmid, 2: EcoRI digested rplasmid showing release of 1365 bp VP2 gene insert.B: Lane M: 1 kb DNA ladder,1: PCR amplified 770 bp VP2 gene.

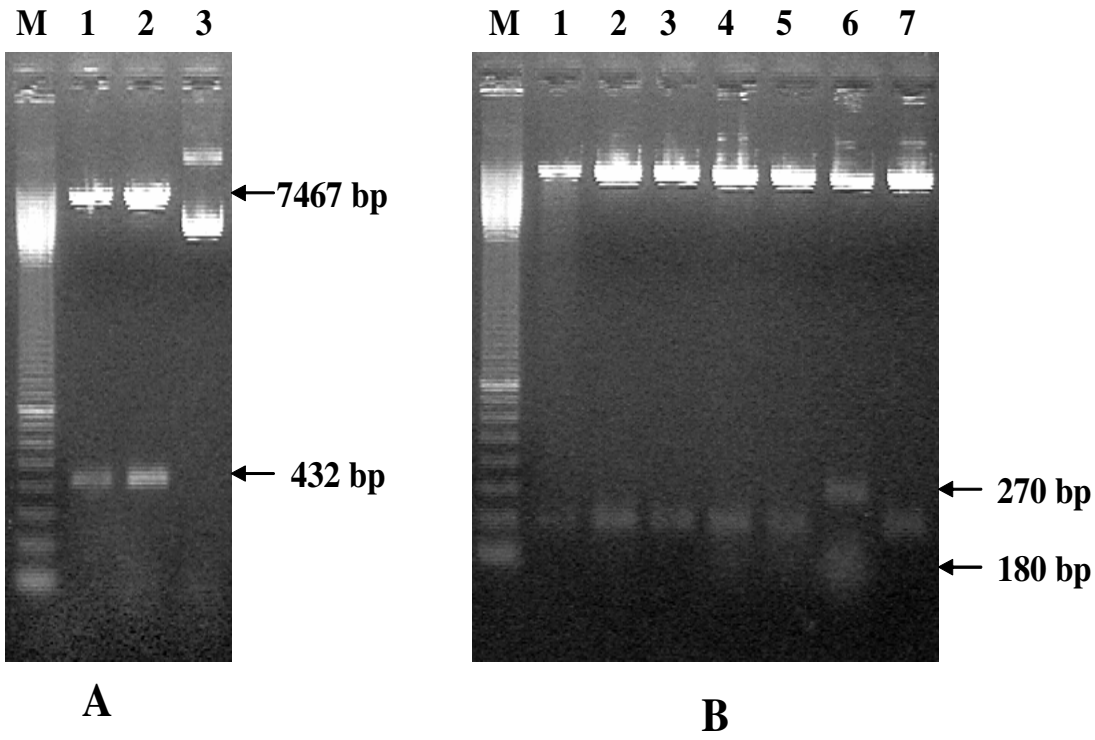
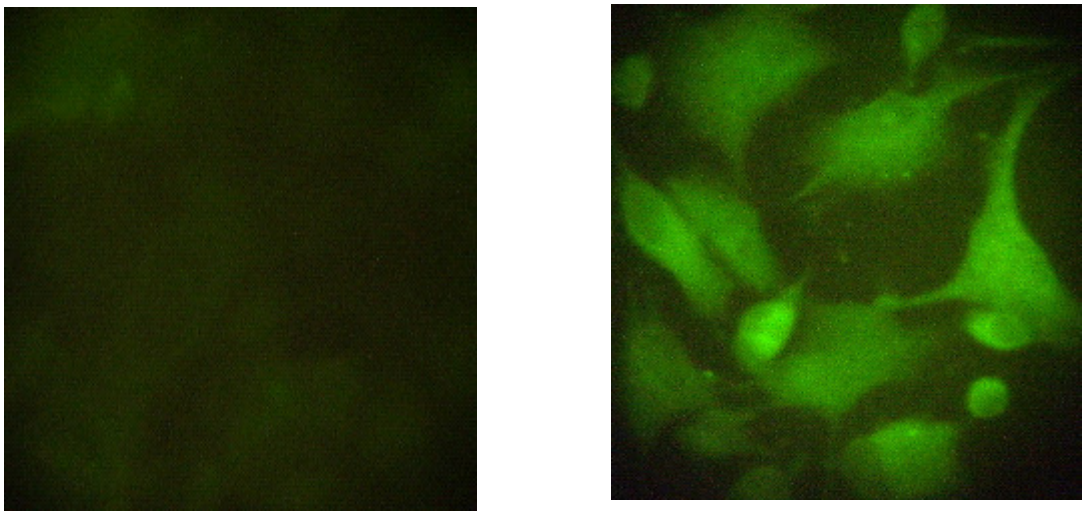


Fig3. Characterization of pIRESibdvp2s-il-2ch plasmid by REanalysis.A:Lane M: 1 kb DNA ladder, 1&2 : EcoRI digested rplasmid showing release of 432 bp IL-2 gene insert,3: uncut rplasmidB:Lane M: 1 kb DNA ladder; 1-7 NotI and XhoI digested rplasmid.



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.

Acknowledgement

Authors thank the Director, Indian Veterinary Research Institute, Izatnagar-243 122, UP for providing necessary facilities to carry out the work.

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Expression and modeling of human interleukin-2 protein

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Summary: The recombinant plasmid pTargeT.IL-2hu was observed to express IL-2 protein in Vero cells as detected by indirect fluorescent antibody and immunoperoxidase techniques. The modeling of the IL-2 protein using DNASTAR and SWISS-MODEL softwares revealed the structure and epitopes of the protein.

Keywords: Interleukin-2, human, protein modeling

Introduction

Interleukin-2 (IL-2) is a potent cytokine that activates multiple compartments of the immune system including T helper cells, cytotoxic T cells, B cells, macrophages and NK cells (Farrar *et al*., 1981; Kawase *et al*., 1983). The gene is 465 bp in length and encodes a protein of 154 amino acids. Co-administration of plasmids encoding human IL-2 and hepatitis antigen resulted in enhancement of both humoral and cell mediated immune response to hepatitis DNA vaccine (Chow *et al*., 1997). Also IL-2 gene has been used for therapy in various cancers and AIDS to achieve desired immune response (Shah *et al.,* 2006). In this study, we have analyzed the expression of human IL-2 gene in vitro and modeling of the protein.

Materials and Methods

Gene expression

For expression analysis, the recombinant plasmid pTarget.IL-2hu (Saxena et al, 2007) was isolated using Qiagen plasmid mini kit (Qiagen), trasfection was carried out in Vero cells using polyfect transfection reagent (Qiagen) according to manufacturer's instructions and the expression was analysed by indirect fluorescence antibody (IFAT) and immunoperoxidase tests.

Indirect florescence antibody test

Vero cells in 24-well plate with approximately 80% confluent monolayer were transfected with plasmid pTargeT.hu IL-2 and the cells were incubated at 37°C and 5% CO_2. After 48 hours, the cells were fixed with 80% chilled acetone. Mock-transfected vero cells were also fixed as a control. To the

fixed monolayer, 1:50 diluted mouse anti-human IL-2 hyperimmune serum was added and incubated at room temperature for 1 h. After the incubation, the wells were washed twice with PBS and again incubated with goat anti-mouse FITC conjugated secondary antibody and incubated further for 1h at room temperature. Cell monolayer were washed with PBS, mounted in 50% glycerol in PBS and examined under fluorescent microscope.

Immunoperoxidase test

The protocol was similar to IFAT except that, goat anti-mouse HRP as the secondary antibody conjugate was used. The color was developed with DAB (1mg/ml in PBS with 1µl/ml H_2O_2) at room temperature for 5 min.

Protein modeling

The amino acid sequence of human IL-2 was submitted to SWISS-MODEL website online for modeling and the results obtained were visualized using spdbv and Rasmol software. (Kopp and Schwede, 2004; Schwede et al, 2003; Guex Peitsch, 1997; Peitsch, 1995). DNASTAR was also used for modeling.

Results and Discussion

The pTarget.IL-2hu transfected Vero cells showed good immunofluorescence while control healthy cells did not show any fluorescence (Fig.1-2). Similarly the Vero cells transfected with the recombinant plasmid DNA showed enhanced staining reaction in immunoperoxidase test while control cells did not show any staining (Fig.3-4). Modeling of human IL-2 using Protean program of the DNASTAR showed that the IL-2 protein contains alpha, beta, turn and coil regions and has quite distinct antigenic epitopes and has compact helical structure (Fig. 5-6).The SWISS-MODEL software used online produced a very authentic 3-D model which was visualized using spdbv software using pds format files.This recombinant protein is 154 amino acid long with an isoelectric pH 5.55, 117 polar amino acids indicating that the protein is highly hydrophilic. It was then visualized with rasmol software and different models like ribbon, strand, spacefill, ball and stick and back bone were constructed (Fig.7-10). The model in various forms showed the reactive sites, epitopes, groups, secondary structure and various amino acid groups.The protein structure was submitted to database TrEMBL and was assigned the accession # QOGK43.

The expression of the human IL-2 gene in Vero cells showed that the mammalian expression vector pTarget is highly efficient in expressing the gene cloned in it. It further demonstrated that it would express in appropriate host when injected. The modeling of the human IL-2 protein provided through insight into the structural details of the proteins, which included different groups, epitopes, reactive sites and antigenic surfaces.

It has been reported earlier that IL-2 is a potent cytokine that activates multiple compartments of the immune system including T helper cells, cytotoxic T cells, B cells, macrophages and NK cells (Farrar et al., 1981). Wang et al., (1993) reported immune response against HIV after gene inoculation since co-administration of human IL-2 and hepatitis antigen resulted in enhancement of both humoral and cell mediated immune response to hepatitis DNA vaccine (Chow et al., 1997). Several DNA vaccines for animal and human have been developed including rabies (Rai, 2007). In parallel with the earlier attempts to enhance the immune response of vaccines, it can be advantageously used as adjuvant with rabies DNA vaccine for humans. Human IL-2 has also been found beneficial when used in cancer patients and thus it can be used in cancer and related conditions. This study finds that the recombinant human IL2 is expressed in vero cell line is similar to its normal native protein found in body. The topographical studies reveal that the epitopes and hydrophilicity are maintained.

Fig1. Control Vero cells showing no fluorescence.

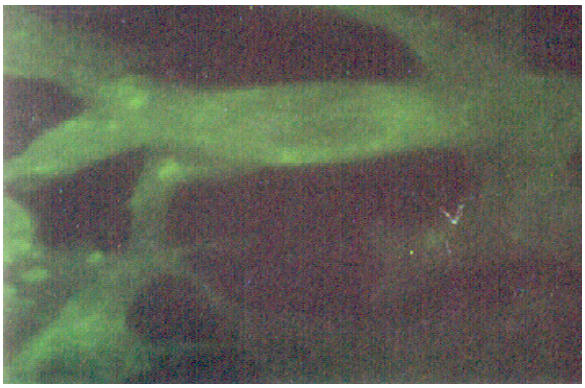

Fig 2. Recombinant plasmid transfected Vero cells showing fluorescence.

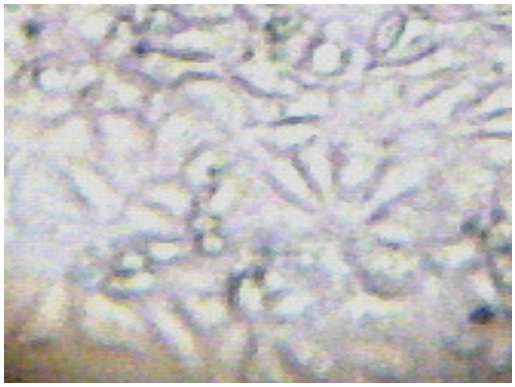

Fig 3. Control Vero cells showing no IPT reaction

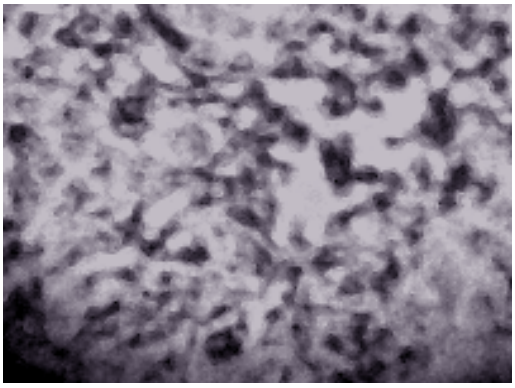

Fig 4 Recombinant plasmid transfected Vero cells showing positive IPT staining.

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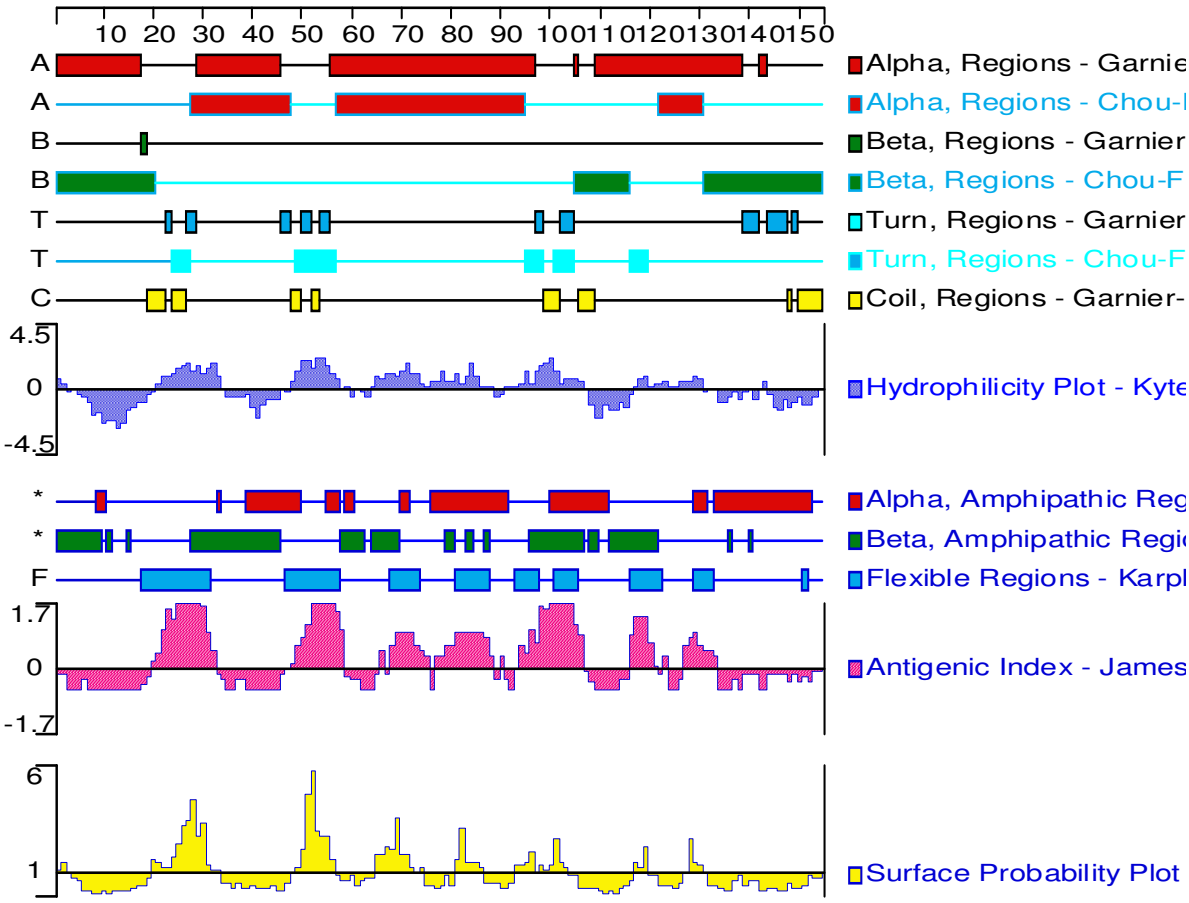

Fig 5. Modeling of human IL2 by protean program of DNASTAR software.

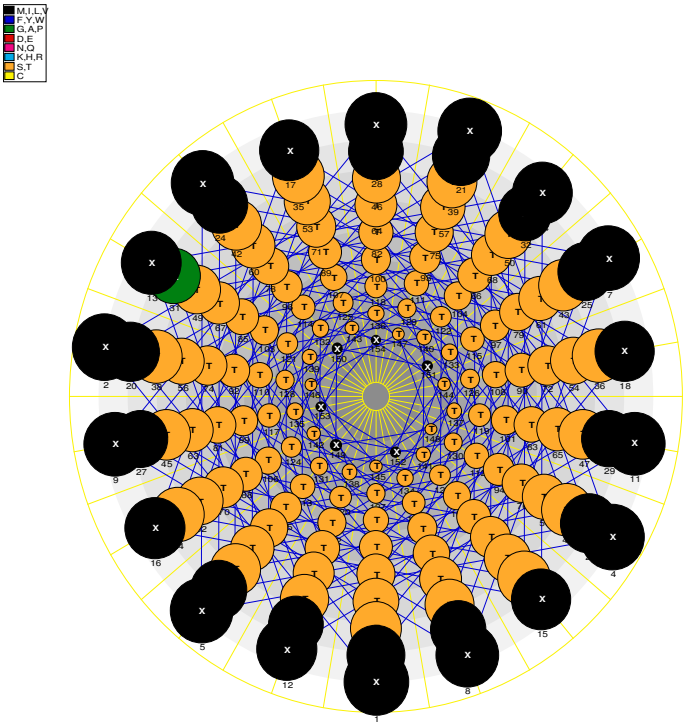

Fig 6. Modelling of human IL2 by protean program of DNASTAR software.showing helical model.

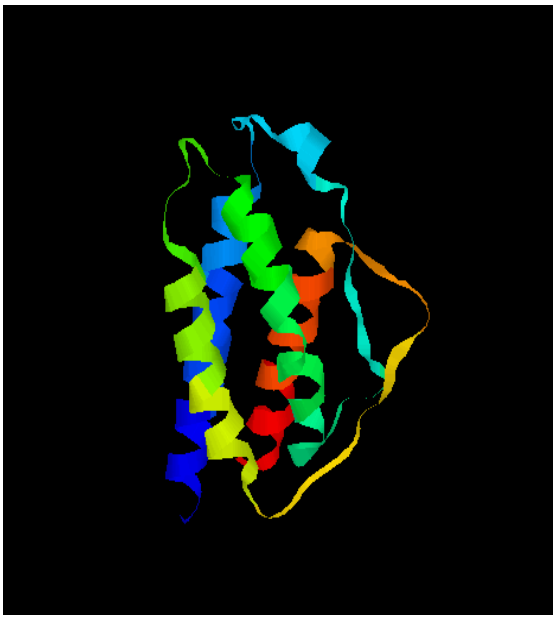

Fig7. Human IL2 model, ribbon, Rasmol

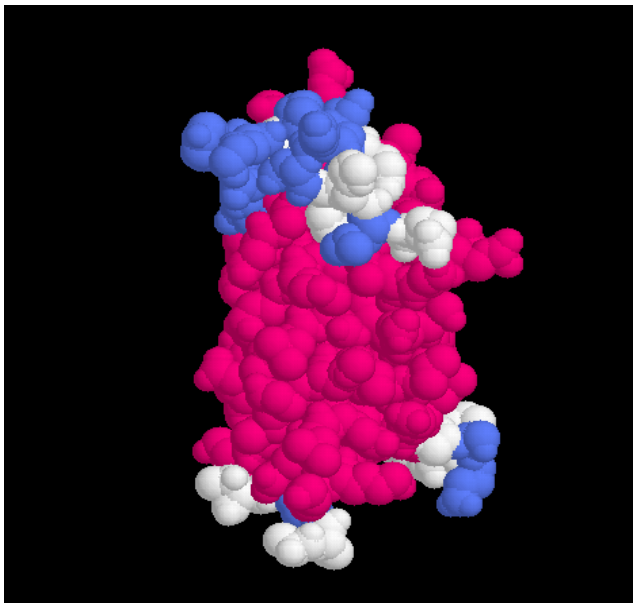

Fig8. Human IL2, strand, Rasmol

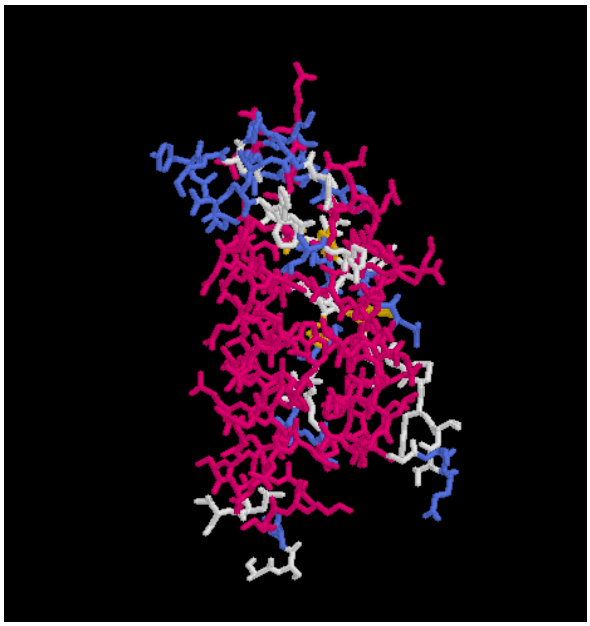

Fig 9. Human IL2,spdbv

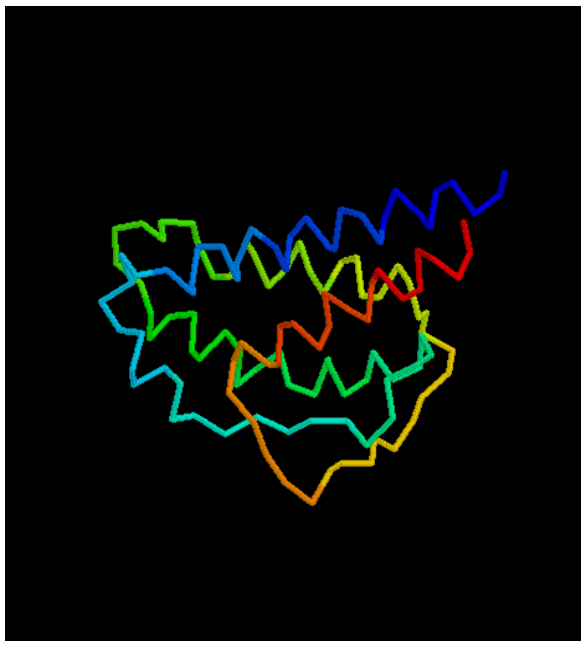

Fig 10. Human IL2, spacefill,Rasmol

Acknowledgements

The authors thank the Director, Indian veterinary research Institute, Izatnagar for providing facilities to carry out this work.

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Cloning of canine adenovirus type 1 hexon gene in mammalian expression vector and analysis of its immunogenicity

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Summary: Canine adenovirus 1 hexon gene was amplified and cloned in pTargeT mammalian expression vector. The expression of cav1 hexon proteins was detected by IFAT, IPT, SDS PAGE and Western blotting. The humoral immune response was detected by serum neutralization and ELISA tests. All the vaccinated dogs showed seroconversion in ELISA tests while the control nonimmunized healthy dogs remained serologically negative. The lymphocyte proliferation test showed that rplasmid induced the cell mediated immune response. FACS analysis revealed the increase of CD4+ count from 48% to 61% in immunized dogs which along with the SN titre substantiates that strong humoral immune response was induced by the rplasmid. The Real time PCR analysis with four cytokines viz. IL-2, IL-4, IL-6 and IFN gamma showed that the rplasmid induced both humoral and cellular immune responses.

Keywords: Canine adeno virus1, infectious canine hepatitis, DNA vaccine, cloning

Introduction

Infectious canine hepatitis (ICH) is an acute viral disease of dogs, coyotes, foxes and other Canidae and in Ursidae (bears) (Greene, 1990; Whetstone et al., 1988). The overall mortality rate from the disease is estimated to be approximately 10 percent and is principally from puppies in the younger age group. Adenoviruses are linear double stranded DNA viruses that infect a wide variety of mammals and birds. Two adenoviruses have been identified in the dog, canine adenovirus type 1 (CAV-1) which infects most of the major organs causing hepatitis (Koptopoulos and Cornwell, 1981). Infectious canine hepatitis (ICH) is an acute highly contagious disease of dogs and foxes, caused by CAV-1. The typical signs include high rise in temperature, vomiting, diarrhea and convulsions. The disease is prevalent in many countries in the world and is also prevalent in epizootic proportions in India. Inactivated vaccines do not produce lesions in dogs but must be given frequently to provide protection equal to modified live vaccines while modified live virus CAV-1 and CAV-2 vaccines afford lifelong immunity.

Although vaccine associated ocular and renal diseases have been observed in dogs after CAV1 modified live virus vaccine, while CAV-2 causes respiratory signs (Willis, 2000).

The DNA vaccination offers the potential for further advancements in the production of effective vaccines (Robinson *et al*., 1993). DNA vaccines induce strong humoral and cellular immune responses conferring long lasting immunity associated with memory cells against a variety of bacteria, viruses and parasites (Hanlon *et al*., 2001; Fan *et al*., 2002; Serezani *et al*., 2002). The DNA vaccine for canine adenoviruses has not yet been attempted.

The main targets of specific antibodies are the adenoviral structural proteins hexon and fibre (Norby, 1969). The neutralizing antibody response is directed to hexon alone. The hexon is the largest and most abundant structural protein in the icosahedral adenovirus capsid and the neutralizing antibody response is directed to hexon alone. The purified hexon antigen prepared from CAV-1 conferres complete protection in dogs against severe challenge with virulent CAV-1 virus (Tribe and Wolff, 1973). Verkhovskaia *et al* (1990) have shown hexon gene as major diagnostic antigen and immunogen of CAV-1.

Two types of CAV1 vaccines are available viz. inactivated and modified live attenuated virus vaccines. Inactivated vaccines are prepared from infected cell cultures which do not produce any lesions in vaccinated dogs but they must be given frequently along with adjuvant to make them more immunogenic to equal the protection afforded by modified live virus vaccines. In contrast, modified live virus vaccines can provide lifelong immunity with a single dose. A potential disadvantage of modified live virus vaccines, however has been that vaccine virus localizes in the kidney and causes mild subclinical interstitial nephritis and persistent shedding of vaccine virus (Willis, 2000). The dsDNA encodes approximately 30 proteins. Adenoviruses family consists of linear molecule of double-stranded DNA viruses with non-enveloped, icosahedral nucleocapsid. The hexon of mammalian adenoviruses contains a cross-reacting group antigen (Davison *et al*., 2003). The hexon is the major capsid protein of the adenovirion and is composed of three identical large polypeptide chains and containing approximately 1000 residues, which require about 8% of the coding capacity of the viral genome (Jornvall et al., 1981). It is the largest and most abundant of the structural protein in the adenovirus capsid (Rux and Burnett, 1999; San Martin and Burnett, 2003). It accounts more than 83% of the capsid proteins. The main targets of type specific antibodies are the adenovirus structural proteins like hexon and fiber. As antifiber antibody neutralizes virus infectivity only in vitro (Wohlfart C, 1988) and not in vivo (Gall *et al*., 1996), the neutralizing antibody response is directed to hexon. Purified hexon antigens prepared from CAV1 virus conferred complete protection in dogs against severe challenge with virulent CAV1 virus. Hexon protein is a potent adjuvant for activation of a cellular immune response (Molinier *et al*., 2002).

Verkhovskaia *et al* (1990) reported that hexon gene is major diagnostic antigen and immunogen of CAV1. Keeping above in view, hexon is a candidate for use in DNA vaccine development. Several reviews on DNA vaccines in veterinary medicine are available (Krishnan, 2000; Dunham, 2002; Babiuk et al 2000, 1998).

Material and methods

The pTargeT™ plasmid (Promega, USA) was used to clone hexon gene (2736 bp) and construct recombinant mammalian expression vectors for use as a DNA vaccine. Primers were designed using the sequence NCBI GenBank database accession no. AC_000003 and got custom synthesized from Bangalore Genei (India) and from Operon (Germany).The primers used were forward 5'GCC ATA TTA GTT TAG GTC GTG GTG GCG TTG 3'(27 mer) and reverse 5'TGA GAA GAT GGC AAC TCC GTC GAT G 3' (25 mer).

PCR and cloning

The PCR was carried out in 50 ul volume with 5 ul viral DNA as template, 50 pmol of each forward and reverse primers, 1 ul of 10 mM dNTPs mix, 6 units of Taq DNA polymerase in 1X PCR buffer. The hexon gene was amplified following initial denaturation at 94^0C for 10 min, 35 cycles of denaturation at 94^0C for 30 sec, annealing at 55^0C for 1 min, amplification at 72^0C for 4 min and a final extension at 72^0C for 10 min. After amplification, an aliquot of 5 ìl was subjected to 1% agarose gel electrophoresis along with 1kb DNA marker at 60 volts for the analysis of PCR product. The gel extraction of DNA fragments was done using MinElute Gel extraction kit (Qiagen, Germany) following manufacturer's instruction. The linear plasmid vector (approximately 25 to 50 ng) was ligated with insert in 1:10 ratio using 1U of T4 DNA ligase (Promega, Madison) in a 10µl reaction volume containing 1µl of of ligation buffer (pH 7.5). The reaction mixture was incubated at 16^0C in a water bath overnight. Ligation control reaction was also set up with all the components except that the insert was not added to the reaction.The 5ìl ligation reaction product was used to transform 200ul competent DH5 cells. The recombinant colonies were screened based on blue white screening and ampicilin resistance (ampicillin 100ìg/ml, 0.5 mM IPTG and 80 ìg/ml of X-gal). (Sambrook and Russel, 2001) with modification. Ultra pure plasmid was isolated for transfection using Qiagen plasmid mini kit (Qiagen, MD). Supercoiled plasmid DNA for immunization was isolated using Qiagen HiSpeed Plasmid Purification kit following the manufacturer's instructions.

Restriction endonuclease digestion

The plasmid DNA (5 to 10 µg) was incubated overnight with appropriate 1X restriction endonuclease buffer and 3 to 5 U restriction endonuclease at 37^0C. The digested product was analysed on 1% agarose (SRL, Mumbai) and visualized under UV-transilluminator.

The presence of gene insert in the recombinant plasmids was also confirmed by PCR using T7 primer as forward primer and gene specific reverse primer. The PCR reaction mixture (50 ìl) contained 100 ng of recombinant plasmid, 50 pmol each of gene specific reverse primer and T7 promoter specific primer and 3 units of Taq DNA Polymerase in 1X PCR buffer. The hexon gene was amplified following initial denaturation at 94 ^{0}C for 5 minutes, 35 cycles of denaturation at 94 ^{0}C for 45 seconds, annealing at 55 ^{0}C for 1 minute, and amplification at 72 ^{0}C for 4 minutes and a final extension at 72 ^{0}C for 10 min. The recombinant plasmid was further confirmed by sequencing with T7 sequencing primer.

Transfection of MDCK cells

The MDCK cell were seeded in 6 well plates for IPT and IFAT analysis and in 25 cm^2 tissue culture bottles for SDS-PAGE and western blot. The Qiagen mini kit purified plasmid DNA was transfected to 60 to 80% confluent MDCK cells using Polyfect Transfection Reagent (Qiagen, Germany) according to manufacturer's instructions.

SDS-PAGE and western blot

Transfected MDCK cell monolayer in 25cm^2 flask was lysed with 1ml 1X SDS- PAGE sample buffer and SDS-PAGE and western blot analysis was done according to Sambrook and Russel (2001).

Indirect fluorescent antibody test (IFAT)

The cells grown in 6 well plates were fixed with 80% chilled acetone. A mock-transfected MDCK cells were also kept as a control. To the fixed monolayer, 1:50 diluted mouse anti-hexon hyperimmune serum was added and incubated at room temperature for 1 h after which the wells were washed with PBS and again incubated with goat anti-mouse FITC conjugated secondary antibody and incubated further for 1h at room temperature. Thereafter, cell monolayer were washed with PBS, mounted in 50% glycerol in PBS and examined under fluorescent microscope (Nikon) and photographed using digital camera.

Immunoperoxidase test (IPT)

The transfected cells were washed with PBS and fixed with 80% chilled acetone. The PBS washed cells were treated with 2% H_2O_2 for 10 min and again washed with PBS. The cells were first incubated with 1:50 dilution of anti-mouse adenovirus hexon hyperimmune serum raised in mouse for 2h at room temperature, washed three times with PBS and then incubated with rabbit anti-mouse HRPO conjugate (1:400diluted) at room temperature for 1 hr. The cells were again washed thrice with PBS and incubated with DAB (1mg/ml in PBS with 1 ìl/ml H_2O_2) at room temperature for 5 min to develop colour. Once the colour developed, the cells were washed with PBS, dried in air and were observed under microscope.

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Assay of immune responses of recombinant plasmid

The vaccination trials were carried out in 2 months old pups. These pups were first treated with antihelminthetics a week before the experiment to reduce the parasitic load and then were tested for antibody against the canine adenoviruses and found to be serologically negative. The dogs were first immunized with 100 µg of rplasmid per pup intramuscularly in hind leg thigh muscle and then a booster immunization after 28days. All groups were evaluated for the immune response induced by the DNA vaccines. Blood and sera samples were collected on 14, 21 and 28 day of primary immunization. Three groups of dogs were maintained namely group 1 (vector control), group 2 (pTargeTcav1hex) and group 3 (non immunized).

ELISA

ELISA was performed as per Gupta *et al*.(2005). Briefly, ELISA plate wells were coated with 1 ìg purified cell culture grown canine adenovirus antigen diluted in 0.05M carbonate-bicarbonate buffer (pH 9.6). The plates were incubated at 4 ºC overnight. After washing once with PBS-T, the coated plates were blocked with 5% skimmed milk in PBS-T for 2 hours at 37 ºC. The plates were then washed with PBS-T and then various dilutions of dog serum in 50 ìl volume were added in duplicate wells and plates were again incubated at 37 ºC for 1 hour. The plates were washed thrice with PBS-T and 50 ìl 1:1000 dilution of rabbit anti-dog IgG HRP conjugate (Promega, Madison) was added to each well. The plates were incubated for 45 minutes at 37 ºC. Washing was done three times with PBS-T and colour was developed with OPD substrate. After 20-30 minutes incubation at room temperature, enzyme activity was stopped by adding 1N sulphuric acid and plates were read at 490 nm. For end point titre determination, a positive was scored for any sample with an absorbance two fold or more as compared with absorbance from control sera. The ELISA titres were defined as the reciprocal of the highest serum dilution positive in ELISA.

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Serum Neutralization Test (SNT

Serum neutralization was was done using sera from dogs following the method described earlier (Gupta et al., 2005). Briefly, these assays were performed after heat inactivating all serum samples at 56^0C for 30 min. Assays were performed in 96 well micortitre plates by mixing 0.05 ml of serial two-fold dilution of sera in cell culture medium (DMEM), with 0.05 ml of CAV suspension containing 100$TCID_{50}$ virus diluted in DMEM in triplicate. The serum virus mixture was then incubated for 2 hours at 37^0C, after which 100 ì l MDCK cell suspension in cell culture maintainence medium was added into the mixture and incubated for 4 days at 37^0C under 5% CO_2. The microtitre plates were then examined under inverted microscope and the neutralizing antibody titre was calculated as the reciprocal of the highest dilution that neutralized 50% of the virus.

Lymphocyte Stimulation Test

The lymphocyte proliferation assay was done following the method described by Bounous *et. al.,* (1992) with slight modifications. After 72 hours of incubation, 15 ìl MTT (5 mg/ml) was added to each well and the cells were incubated for another 4 hours at 37 ºC in 5% $CO_{2.}$ After incubation, the cultures were removed from the incubator. The resulting Formazen crystals were dissolved completely by gentle pippetting up and down. Absorbance was measured spectrophotometrically using microtitre plate reader (Biorad, USA) at 550nm. Background absorbance of multiple plates was measured at 655nm and substracted from the 550 nm measurement. The stimulation index (SI) was calculated according to the formula: SI MTT = mean OD of stimulated culture / mean OD of unstimulated culture

Real time PCR

cDNA was made from pBMCs isolated from the all the three groups of pups using MMLV reverse transcriptase enzyme (Bangalore Genei,, India).. The cDNA was quantified and used for the study of the cytokines (IL-2, IL-4, IL-6 and IFN gamma) using real time PCR with specific primers for each cytokine using Brilliant SYBR® Green QPCR master mix (Stratagene, USA) and Mx3000P spectroflourometric thermal cycler operated by MxPro™ QPCR software. Each QPCR was put in duplicate in a total volume of 25 µl using template cDNA 1 ul, forward and reverse primer 10 pmol each, and 2X SYBR Green QPCR master mix 12.5 ul. Forty cycles of amplification were performed. Non template Control (NTC) were done in which cDNA was not added. The comparative Ct method was employed for relative quantification where amount of target amplicon was normalized to an endogenous reference (house keeping gene). The Ct value indicates that fractional cycle number at which the fluorescence of the amplified target exceeds background fluorescence and äCt represents

the difference in the threshold cycles between the target and housekeeping genes (beta actin). Relative quantification of cytokine mRNA was represented by äCt values.

FACS analysis

FACS was done for detection of T_{helper} and $T_{cytotoxic}$ lymphocyte cells. 10 µl neat undiluted anti-canine CD3: FITC/CD4: RPE/CD8: Alexa fluor®647 (Serotec, England) was added to 50 µl blood (diluted to 100 µl by 50 µl PBS) and incubated at 37^0C in waterbath for one h. The 10 ml RBC lysis solution was added and incubated for 7 min. The cells were then washed twice with PBS, suspended in 0.5 ml PBS and finally analyzed in FACS machine (BD Biosciences).

Results

The CAV-1 was adapted in MDCK cell line in which it produced characteristic CPE of grape like clusters 48-72 hours post infection (Fig1) and the titre was 10^6 $TCID_{50}$/ml.

The cav1hex gene was successfully amplified from the template canine adenoviral DNA which was obtained from cav1 adapted in MDCK cell line as observed by the amplified band of 2736 bp in agarose gel electrophoresis (Fig.2)

The recombinant pTargeT.cav1hex plasmid was digested with *Eco*RI, *Nhe*I and *Nar*I enzymes to confirm the cav1hex gene insert. On digestion with *Eco*RI, whole cav1hex gene was released (Fig3). Digestion with *Eco*RI released a fragment of 2804 bp confirming the presence of hexon gene in recombinant plasmid. One *Nhe*I site is present in the vector in MCS and one site in initial region of cav1hex gene. When the gene is in correct orientation, two fragments were released viz. 6038 bp and 2368 bp, when the gene is in wrong orientation fragments generated were 8018 bp and 388 bp. On digestion with *Nhe*I, both types of clone were observed (Fig 4). Similarly, on digestion with *Nar*I enzyme both correct and wrong orientation of gene insert was observed (Fig 5). The sequence obtained from sequencing with T7 primer was submitted to GenBank and accession number assigned to sequence was **EF206692**

All the restriction sites were predicted in pTargeT.cav1hex plasmid using DNASTAR software (Table1)

Table1: Restriction fragment sizes (in bp) of pTargeT.cav1hex plasmid after digestion with restriction endonucleases.

RE enzymes	**Correct orientation**	**Wrong orientation**
*Eco*RI	5602 & 2804	5602 & 2804
*Nar*I	4640,2803 & 963	5462, 1981 & 963
*Nhe*I	6038 & 2368	8018 & 388

In SDS-PAGE, the cell lysate from pTargeT.cav1hex plasmid transfected MDCK cells revealed one major band of approximate molecular weight 101 kDa which was predicted to express hexon protein (Fig2). There was no band corresponding to this in cell lysate from mock transfected MDCK cell

control. The western blot revealed 101 kDa band reacted with antihexon hyperimmune serum (fig 6).

Immunofluorescence analysis of pTargeT.cav1hex plasmid transfected MDCK cells revealed fluorescence in transfected cells (fig 7), while, there was no fluorescence in mock transfected MDCK cells (fig8) which confirmed the expression of cav hexon gene. Similarly, IPT analysis revealed the immunostaining of MDCK cells (fig9) transfected with pTargeT.cav1hex plasmid while immunostaining was not observed in mock transfected MDCK cells (fig 10).

Immunological response in dog

The humoral and cell mediated immune response was studied in dogs (fig 11, table2).The serum neutralizing antibody titre was calculated using 100 $TCID_{50}$ virus (table3) which showed enhanced SN antibody level in rplasmid vaccinated dogs vaccinated with CAV1 DNA vaccine.

The canine adenovirus antibody titre in DNA vaccine immunized dogs was detected in ELISA using purified canine adenovirus antigen coated plates. All the dogs showed seroconversion with single dose of DNA vaccine. There was no seroconversion in healthy control as well as control groups receiving either pTargeT vector (Fig12 & table 4).

Table 2. Assay of immune response in dogs vaccinated with CAV-1 DNA vaccine

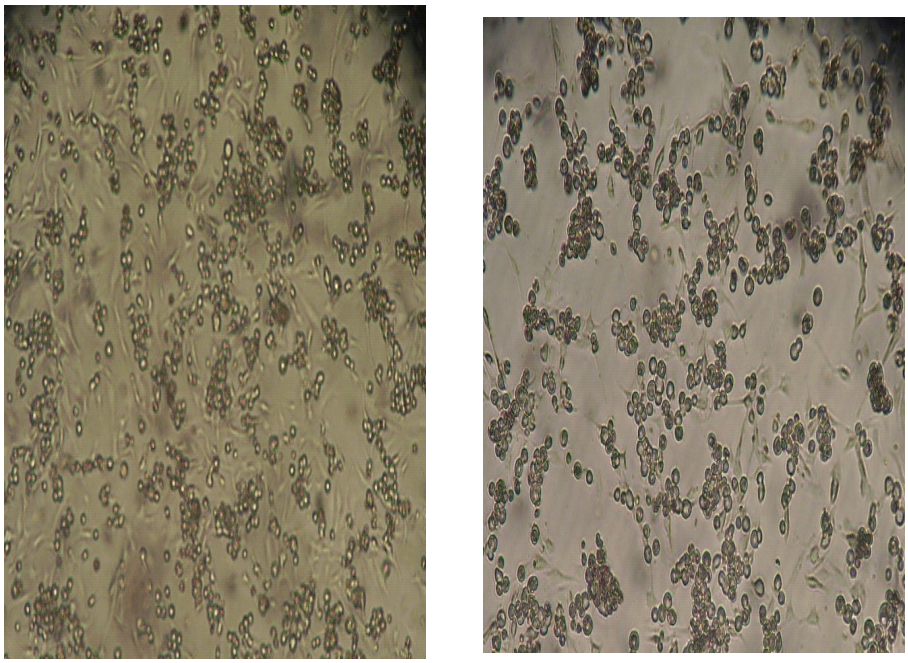
Groups	**DNA**	**No. of dogs**	**28 DPI**			**56 DPI**		
			SN Ab	**ELISA Ab**	**SI**	**SN Ab**	**ELISA Ab**	**SI**
I	Control	6	4	8	0.851	4	8	
II	pTargeT	6	4	8	0.953	4	8	
III	pTargeT.cav1hex	6	32	512	1.152	64	1024	

* The titres are shown as reciprocal of serum dilutions showing 2 times or more absorbance value compared with control unvaccinated groups.

The cell mediated immune response was measured using MTT dye at 28th day post immunization. The recombinant plasmids, Group III and IV showed significantly higher (1.15 and 1.22) proliferation than control group (0.85). The slightly higher proliferation was observed in melting curve analysis. The specific beta actin peak (81^{0}C) was observed in control and immunized cells. PCR products were analyzed by 1.5% agarose gel electrophoresis to demonstrate specificity, which confirmed the results observed in the amplification and melting plot (fig 13).The äCt values for each cytokines were obtained

by subtracting Ct values of target cytokine from Ct value of beta actin amplification. Relative expression levels of cytokines were represented as äCt values (fig1 4). The result showed increased expression level for Group II (pTargeT.cav1hex) in comparison to healthy control. Similar pattern of increase was observed in the expression of the cytokine IL-4 and IL-6.

The FACS analysis revealed the type of immune response. The control showed 48% of CD4+ cells (fig15) and 17% of CD8+ cells (fig16) of the total lymphocytes collected. The vaccinated dogs showed increase in CD4+ count to 61% (fig 17) and CD8+ (fig18) count showed slight increase to 21%.



A B

Fig 1. CAV-1 cultivated in MDCK cells showing grapelike clusters of cells72 h after infection.
A. Healthy cntrol cells and B infected cells.

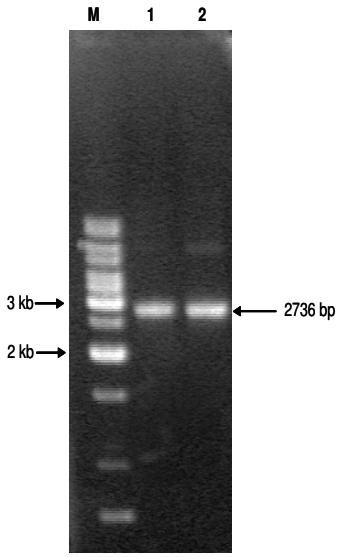


Fig.2. Agarose gel electrophoresis of amplified cav1 hex gene,**lane M**: 1kb DNA ladder, **1,2**: PCR product

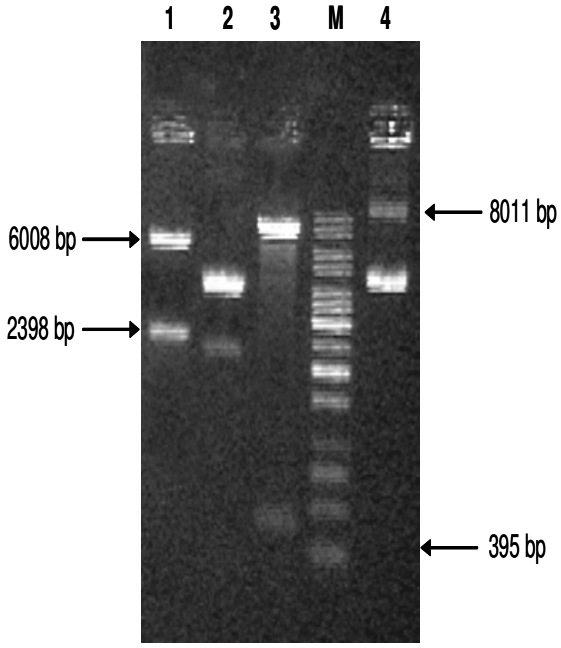


Fig 3. RE analysis of pTargeTcav1hex with Nhe1, **Lane M**: 1kb DNA ladder, **1:** Recombinant clone with correct orientation, **2:** Vector alone without clone, **3:** Recombinant clone with incorrect orientation, **4:** Uncut recombinant plasmid

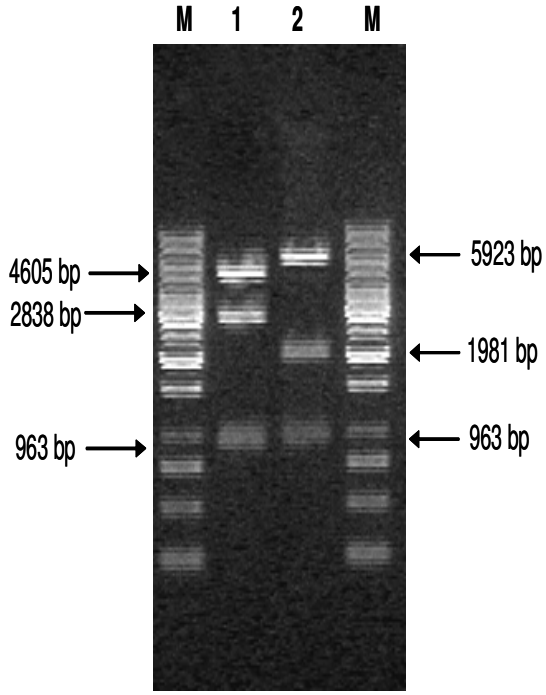


Fig.4. RE analysis of pTargeTcav1hex with Nar1,**Lane M:** 1kb DNA ladder, **1:** recombinant clone with correct orientation, **2:** recombinant clone with incorrect orientation

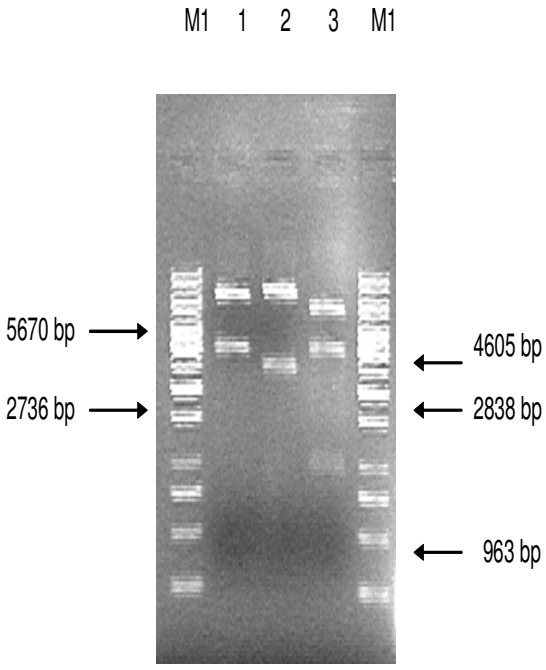


Fig.5.Restriction enzyme analysis of pTargeTcav1hex,Lane M1, M2: 1kb DNA ladder,Lane 1: Digestion with EcoR1,Lane 2: Digestion with Nhe1,Lane 3: Digestion with Nar1

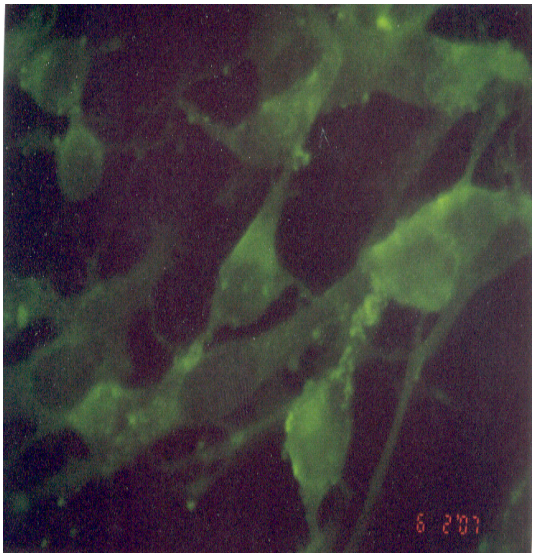


Fig. 6a. MDCK cells transfected with recombinant plasmid pTcav1hex showing fluorescence in FAT.

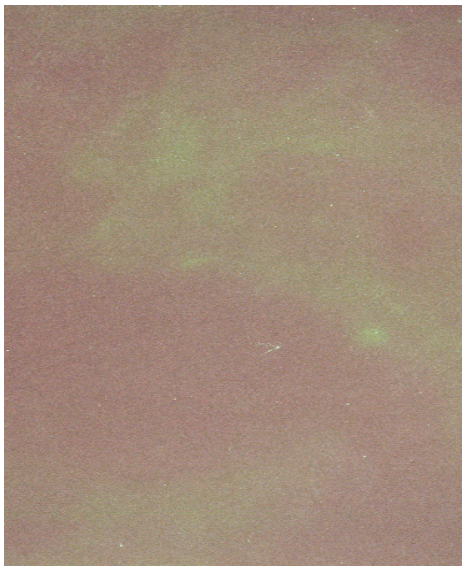


Fig 6b. Mock transfected cells showing no fluorescence in FAT.

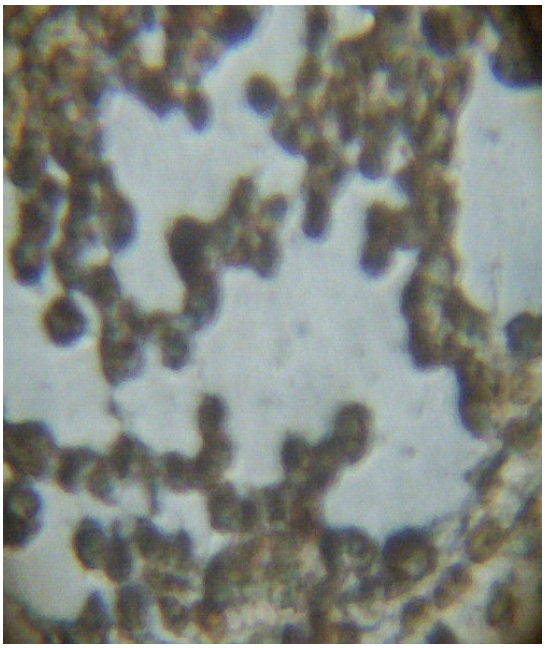


Fig.7a . MDCK cells transfected with recombinant plasmid pTargetcav1hex showing brown colour development in IPT.

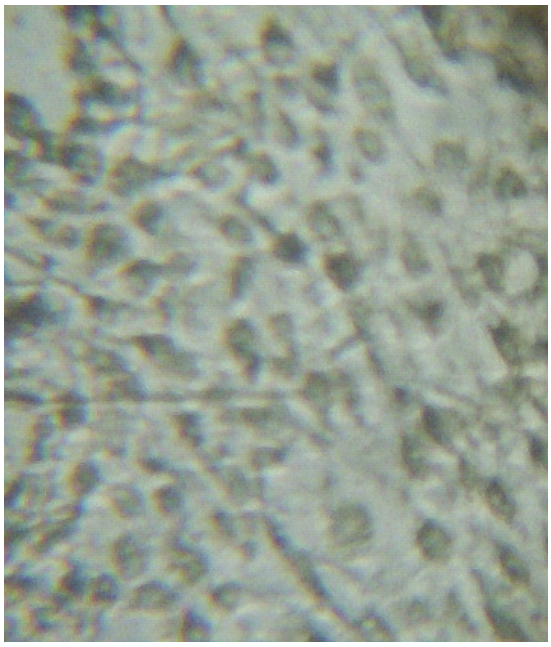


Fig.7b Mock transfected cells showing no colour in IPT.

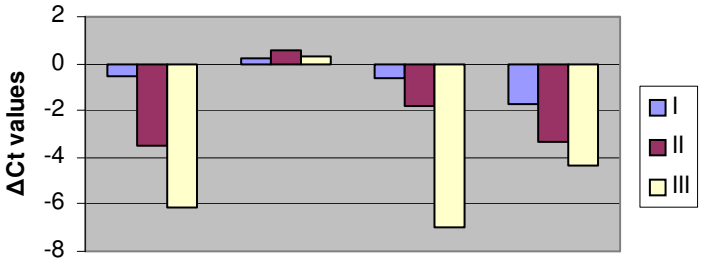
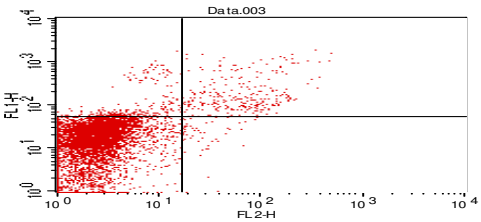


Fig.8 Relative expression showing ÄCt values of four cytokines analyzed by real time PCR.Group I: Healthy control; Group II: dogs immunized with pTargeT.cav1hex; Group III: dogs immunized with pTargeT.cav1hex and pTargeT.IL-2can



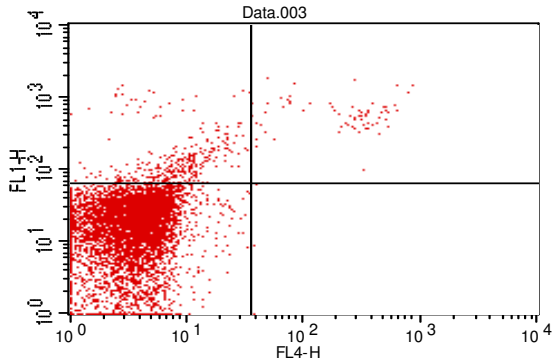
Quadrant Statistics

Log Data Units: Linear Values
Gated Events: 6002
X Parameter: FL2-H (Log)

Gate: G1
Total Events: 10000
Y Parameter: FL1-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	269	4.48	2.69	6.36	5.27	163.15	99.73
UR	252	4.20	2.52	85.06	63.83	293.03	187.54
LL	5432	90.50	54.32	2.70	2.29	19.62	13.95
LR	49	0.82	0.49	31.44	27.92	26.11	20.47

Fig .9a. FACS analysis of lymphocytes from healthy control



Log Data Units: Linear Values
Gated Events: 6002
X Parameter: FL4-H (Log)

Gate: G1
Total Events: 10000
Y Parameter: FL1-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	333	5.55	3.33	9.42	7.46	197.65	136.33
UR	69	1.15	0.69	274.95	200.98	652.58	567.42
LL	5598	93.27	55.98	3.87	3.29	20.49	14.43
LR	2	0.03	0.02	36.69	36.68	5.07	3.02

Fig .10a FACS analysis of lymphocytes from the healthy dogs

Discussion

Clinical cases of ICH are reported continuously in India since first report in 1955 (Nair, 1955). Vaccination is the most effective method to control and prevent ICH in dogs. Currently available live modified vaccines of CAV-1 can produce interstitial nephritis and ocular disease (blue eye) in dogs. The killed inactivated virus vaccines do not produce lesions in dogs but must be given frequently to provide protection equal to modified live vaccines. It has been just over 10 years since the first reports regarding DNA immunization were published (Tsang et al., 1992; Cox et al., 1993; Ulmer et al., 1993). Since then numerous reports of this technique have been applied to develop DNA vaccines against bacteria, viruses and parasites and also against cancer (Babuik et al., 2000; Lewis and Babuik, 1999). DNA vaccines have been successfully used to induce protective immunity against many pathogens in different species with varying efficacy (Kodihalli *et al*., 1999; Fan *et al*., 2002; Serezani *et al*., 2002, Ahi, 2006; Kumar, 2006; Chauhan et al., 2005; Gupta et al., 2005; Rai et al., 2002, 2005a, 2005b; Patial et al., 2007). Though a lot of work has been done to develop DNA vaccines against different diseases, no literature can be cited for canine adenoviruses. The neutralizing antibody response is directed against the protein alone and the purified hexon protein has shown to be protective in dogs against the canine adenoviruses (Tribe and Wolff, 1973). Thus, the hexon protein is obvious choice for the development of DNA vaccine and therefore work was undertaken for amplification, cloning of hexon and evaluation of the immune response against the recombinant DNA constructs. The hexon gene of CAV1 was amplified using a single set of primers by Taq DNA polymerase. The PCR products were gel purified and were used for cloning in T/A cloning based pTargeT™ mammalian expression vector. The pTargeT™ vector system is convenient system for cloning and expression of PCR products in mammalian cells because it has 5' T overhang at both ends which helps in ligation. The pTargeT™ vector carries the human cytomegalovirus (CMV) immediate-early enhancer/ promoter region and the chimeric intron upstream of the gene insert to promote high level of constitutive expression of heterologous gene in mammalian cells. Recombinant clone can be identified by blue/white screening due to presence of the LacZá region in the MCS.

Clones containing cav1hex in right orientation were designated as pTarget.cav1hex. The selected clones were sequenced using T7 sequencing primers. Confirmation of gene expression by *in vitro* testing is essential to use recombinant construct as DNA vaccine. The expression of recombinant clones was confirmed by transfection in MDCK cells. After 72 hours of transfection, the expression of protein was successfully demonstrated by IFAT and IPT. The SDS PAGE and western blots showed prominent band of 101 kDa confirming the expression of the protein. In cav1 infection, the serum neutralizing antibody titre mounted by the host after exposure determines the outcome of infection. A high titre clears virus from the infected animal tissues, a low titre results in disseminated disease, and an intermediate titer is associated with immune complex disease (Willis, 2000). The dogs vaccinated with pTarget.cav1hex showed SN antibody titre of 32 and 64 after first and second immunization respectively. DNA vaccines are known to be effective in inducing broad spectrum of cell mediated immune responses (Ulmer et al., 1993). Lymphocyte proliferation was clearly observed in PBMCs isolated from vaccinated dogs after stimulation in lymphocyte proliferation test, with heat killed virus as an antigen revealing the cell mediated immune response stimulated by recombinant plasmids. It showed that recombinant DNA vaccine construct induced cell meditated immune response. Stimulation index data showed that rplasmids pTarget.cav1hex (SI = 1.15) used as DNA vaccine stimulated the cell mediated immune response. The flow cytometry permits counting of different subpopulations of lymphocyte CD3+, CD4+ and CD8+ which reveals the immune status of the animal. The proportion of peripheral blood CD4+ and CD8+ count in control dogs was found to be 48% and 17% respectively which is within the normal range of CD4+ (42-45%) and CD8+ (18-28%) count (Bourdoiseau *et al*, 1997, Byrne *et al*, 1999). The 11% increase in CD4+ count in dogs vaccinated with pTarget.cav1hex indicates strong humoral response elicited by the rplasmid. It was also substantiated by high SN antibody titre. The slight increase in CD8+ count (4%) to 21% is within the normal range and is not immunologically significant.

Real-time PCR has been recognized as accurate and sensitive method of quantifying messenger RNA (mRNA) transcripts (Bustin, 2000, 2002) as this method allows the detection of amplicon accumulation as it is formed rather than by conventional end-point analysis. Real time measurement of amplicon accumulation also allows determination of reaction efficiency and thus permits the selection of more sensitive assays. Hence, the cytokine profiling was done using real time PCR to assess the immune response produced by pTargeT.cav1hex. The cytokines studied

were of two types viz. Th1 (IL-2, IFN gamma) related to cellular immune response and Th2 (IL-4, IL-6) types related to humoral immune response to detect the orientation of the immune response. The cytokine mRNAs were quantified using the syber green dye which revealed that the IFN gamma concentration (Ct = 30.17) was more in the dogs with canine IL-2 plus pTargeT.cav1hex and pTargeT.cav1hex alone (Ct = 32.47) as compared to healthy control (Ct = 34.62). Similarly, the concentration of IL-4 and IL-6 was highest in the dogs immunized with canine IL-2 plus cav1hex (Ct for IL-4 = 29.33; Ct for IL-6 = 31.97) as compared to cav1hex alone (Ct for IL-4 = 34.16; Ct for IL-6 = 32.67) while the healthy control had the lowest concentration of the same cyokines (Ct for IL-4 =34.53; Ct for IL-6 =33.36). This result indicates that co-immunization with IL-2 enhances both the Th1 and Th2 response after vaccination which is in agreement with recent findings that IL-2 acts as an adjuvant that enhances both the humoral and cellular immune responses (Bu *et al*., 2003; Jianrong *et al*. *2004*).

The work provides preclinical evidence of the potential advantages of the DNA vaccine for the induction of cellular and humoral immune response. It demonstrates that both Th1 and Th2 responses against viral antigens can be raised by DNA immunization. It also demonstrates the immunogenecity of hexon gene as shown by all the tests, and especially by SNT and FACS analysis and the adjuvant effect of IL-2 in enhancing immune response. The high SN antibody titre on single immunization with pTargeT.cav1hex can shows the humoral immune response in vaccination and the high concentration of IFN gamma, IL-4 and IL-6 It demonstrates that the recombinant plasmids can be used as DNA vaccines against CAV-1 diseases in canines.

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