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**1. Umbilical cord blood: A promising source of stem cells**

**by Ashwin Ashok Raut and Bandu Mane 41-49**

**2. Cloning of canine adenovirus type 2 hexon gene in mammalian expression vector for use as DNA vaccine**

**by S S Salunkhe, Anant Rai, Sudarshan Kumar, Ankur Saxena 50-67**

## **Cloning of canine adenovirus type 2 hexon gene in mammalian expression vector for use as DNA vaccine**

**S S Salunkhe, Anant Rai, Sudarshan Kumar, Ankur Saxena,  
Division of Animal Biotechnology, Indian Veterinary Research  
Institute, Izatnagar, Bareilly-243122, UP, India**

### **Abstract**

The cav2 hexon gene was amplified from the genome of cav2 from a commercial vaccine and cloned in pTarget vector. The resultant recombinant plasmid pTarget.cav2hex was then characterized by restriction enzyme analysis by EcoRI, NheI and NarI, and sequencing. Expression of recombinant clone was confirmed by transfection in MDCK cell line. *In vitro* expression of protein was confirmed by positive immunofluorescence and immunoperoxidase test. Cell lysate from cells transfected by recombinant clone showed expected bands of 101 kDa in SDS PAGE and was further confirmed by western blot analysis. The sequence obtained was assigned the GeneBank accession numbers **DQ839392**.

The immunological studies of recombinant plasmid demonstrated that the SN titre was high in cav2 hex vaccine (128). The adjuvant effect was seen in cav1hex and cav2hex immunization along with canine IL-2 which elicited a high SN titre of 64 and 128. Thus, either two immunizations with pTarget.cav2hex or IL-2 along with pTarget.cav2hex will confer the protection against cav infection in dogs. All the vaccinated dogs showed seroconversion in ELISA tests while the control nonimmunized healthy dogs remained serologically negative. The lymphocyte proliferation test showed that DNA vaccine induced the cell mediated immune response. induced both humoral and cellular immune response and the IL-2 when used as adjuvant enhanced the immune response.

**Key words:** Canine adenovirus type 2, hexon gene, DNA vaccine, cloning, expression

## **Introduction**

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 causes hepatitis and canine adenovirus type 2 (CAV-2) that causes respiratory enteric disease (Koptopoulos and Cornwell, 1981). ILT is a highly contagious respiratory infection of dogs caused by CAV-2 which occurs after oronasal contact. The virus replicates in the epithelium of the nasal mucosa, pharynx, tonsillar crypts, trachea, bronchii and in nonciliated epithelium. CAV-2 has been associated with outbreaks of respiratory disease in kennelled dogs and classical form of CAV-2 infection is known as Kennel cough. Ditchfield *et al* (1962) showed that the new virus, named Toronto A26/61, was an adenovirus antigenically similar to CAV-1, but, antigenically distinguishable from it. Recognition of CAV-2 as antigenically distinct from CAV-1 is based more on the results of hemagglutination inhibition (HI) test than on those of serum neutralization (SN) or complement fixation (CF) tests (Wigand *et al.*, 1982). CAV-2 vaccines have been developed as an alternative in the prevention of ICH. Modified live virus of CAV-2 vaccine rarely, if ever, produces ocular or renal disease when given intravenous or subcutaneous, although the vaccine virus may localize in and be shed from the upper respiratory tract. DNA vaccine for canine adenoviruses type 2 has not yet been attempted. The present study attempts to see the efficacy of recombinant CAV 2 as DNA vaccine in the prevention of ILT in pups

## **Material and methods:**

### **PCR amplification and preparation of recombinant clones carrying the CAV2 hexon gene**

The PCR reaction was set up in 50  $\mu$ l volume containing 50 pmol each of gene specific forward and reverse primer, 6 units of Taq DNA Polymerase in 1X PCR buffer. The template DNA was 5  $\mu$ l of vaccine purified DNA for CAV2. The amplification conditions were kept as initial denaturation at 94 C for 10 minutes followed by 35 cycles of each denaturation at 94 C for 30 sec, annealing at 55C for 1 minute, extension at 72 C for 4 minutes with a final extension at 72 C for 10 minutes. After checking the integrity and specific size amplification product on 1% agarose gel electrophoresis, the amplified product was gel purified using MinElute Gel extraction kit (Qiagen, Germany) following manufacturer's instruction. Ligation reaction was put in 10  $\mu$ l volume with T4 DNA ligase keeping the ratio of insert to vector (pTarget Promega, Madison) approximately 1:10. The ligated mixture was used to transform competent DH5 alpha cells following the protocol mentioned in Sambrook ( ) and the recombinant colonies were identified based on X gal and Ampicillin resistance screening.

### **Recombinant Plasmid purification and analysis of insert in right orientation:**

High quality Plasmid DNA isolation was done with Qiagen plasmid mini kit (Qiagen, MD). 5 to 10  $\mu$ g of DNA was digested with the enzymes identified appropriate to release the cloned product. The orientation of cloning was confirmed seeing the product size on 1% agarose gel electrophoresis as well as directional PCR using T7 primer as forward primer and the reverse gene specific as reverse primer.

### **Sequencing and BLAST**

The identified recombinant plasmids assumed to be in right orientation were further confirmed by sequencing with T7 sequencing primer and using primer walking method. The sequences thus obtained were submitted to the GenBank/EMBL database.

### **Expression analysis**

#### **Transfection of MDCK cells and Indirect immunofluorescence antibody test and Immunoperoxidase test SDS-PAGE and western Blotting**

The confirmed recombinant vector DNA was tested for its expressivity invitro in MDCK cell line. Cells were seeded in 6 well plates for IPT and IFAT analysis and in 25 cm<sup>2</sup> tissue culture bottles for SDS-PAGE and western blot. Polyfect Transfection Reagent (Qiagen, Germany) was

## **Biotechnology International Vol 1, No.2, June 2008**

used for transfection according to manufacturer's instructions. Transfected MDCK cell monolayer in 25cm<sup>2</sup> flask was lysed with 1ml 1X SDS- PAGE sample buffer and SDS-PAGE and western blot analysis was performed according to the method described by Sambrook and Russel (2001). For IFAT, FITC conjugated secondary antibodies were used and for IPT rabbit anti-mouse HRPO conjugated antibodies with DAB as substrate were used. In all experiments, primary antibody were used which had been developed in mouse as hyper immune sera following repeated intramuscular injections.

### **Immunization of dogs and study of immunological changes**

For studying the immune response of rplasmids, 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 rplasmids per pup intramuscularly in hind leg thigh muscle and then a booster immunization after 14 days for cav2hex. All groups were evaluated for the immune response induced by the DNA vaccines (Table 3.4).

**Table 3.4: Groups for immunization in dog pups for pTarget.cav2hex**

Group I	Vector control
Group II	pTargetcav2hex
Group III	pTargetcav2hex+ pTarget.IL2
Group IV	Non-immunized

Pups were given booster with the respective plasmid DNA with same route and dose on 14<sup>th</sup> day of primary immunization. Blood and serum samples were collected from pups on 14<sup>th</sup>, 21<sup>st</sup> and 28<sup>th</sup> day of primary immunization for determination of immune status of vaccinated dogs.

### **Anti Hexon antibody detection in sera by ELISA**

ELISA was performed for detection of anti-hexon antibodies in dogs following the method described earlier (Gupta *et al.*, 2005). 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.

### **Serum Neutralization Test (SNT)**

Virus titration was done in MDCK cell line to calculate TCID<sub>50</sub>. Serum neutralization was performed using sera from dogs following the method described earlier (Gupta *et al.*, 2005).

## **Biotechnology International Vol 1, No.2, June 2008**

The assays were performed after heat inactivating all serum samples at 56°C for 30 min. Assays were performed in 96 well microtitre 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 100TCID<sub>50</sub> virus diluted in DMEM in triplicate. The serum virus mixture was then incubated for 2 hours at 37°C, after which 100 µl MDCK cell suspension in cell culture maintenance medium was added into the mixture and incubated for 4 days at 37°C under 5% CO<sub>2</sub>. 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**

Whole blood was used to separate lymphocytes population using Histopaque (Boyum, 1976) and resuspended in RPMI-1640. For proliferation assays, cells were placed into each well (4 X 10<sup>5</sup> cells/ well) of 96 well flat bottomed tissue culture plates and Concanavalin A (ConA, Sigma) having stock of 5 µg/ml was added to each well to final concentration of 50 µg/well except in the negative control well and virus control wells. The plates were incubated at 37 °C in a humidified CO<sub>2</sub> incubator for 72 hours. The lymphocyte proliferation assay was done following the method described by Bounous *et. al.*, (1992) with slight modification. After 72 hours of incubation, 15 µl MTT (5 mg/ml) was added to each well and the cells were incubated for another 4 hours at 37 °C in 5% CO<sub>2</sub>. After incubation, the cultures were removed from the incubator. The resulting Formazan crystals were dissolved completely by gentle pipetting 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 subtracted from the 550 nm measurement. The stimulation index (SI) was calculated according to the formula:

$$SI_{MTT} = \text{mean OD of stimulated culture} / \text{mean OD of unstimulated culture}$$

### **Expression levels of interleukins by Real time PCR**

cDNA was prepared from the total RNA isolated from PBMCs of the immunized and healthy dogs. Total RNA was isolated from the PBMCs using TRIzol (Invitrogen) reagent. MMLV reverse transcriptase enzyme (Genei, Bangalore, India) was used for reverse transcription. The amplification

step was performed at 25°C for 5 min and 37°C for 1 hour. The cDNA was then tested for the presence of beta actin by PCR. The cDNA showing amplification of beta actin were further used in real time PCR. for the study of the cytokines (IL-2, IL-4, IL-6 and IFN gamma) using specific primers for each cytokine using Brilliant SYBR® Green QPCR master mix (Stratagene, USA) and Mx3000P spectrofluorometric thermal cycler operated by MxPro™ QPCR software. Each QPCR was put in duplicate in a total volume of 25 µl. Template cDNA 1 µl, Forward primer 10 pmol, Reverse primer 10 pmol, 2X SYBR® Green QPCR master mix 12.5 µl Nuclease free water to 25 µl. 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  $\Delta Ct$  represents the difference in the threshold cycles between the target and housekeeping genes (beta actin). Relative quantification of cytokine mRNA was represented by  $\Delta Ct$  values.

### **FACS analysis for CD4 and CD8 cells**

FACS was done for detection of T<sub>helper</sub> and T<sub>cytotoxic</sub> 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°C in waterbath for one hour. 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**

### **Amplification, cloning and expression of cav2hex gene**

The CAV-2 viral DNA from pentavalent vaccine was confirmed by 1030 bp PCR amplification of E3 gene of CAV (Fig 2). The primers could successfully amplify 2736 bp of cav2hex gene from template DNA (Fig 1). On digestion with *EcoRI*, gene insert cassette of 2804 bp was released because the enzyme site is present on both flanking sites of gene in MCS. To confirm the orientation of gene, *NarI* and *NheI* digestion was performed. On *NheI* digestion, plasmids having both correct and wrong orientation of gene insert were observed. When the gene is in correct orientation, the fragments released were 6038 bp, 2193 bp and 175 bp, while when the gene is in incorrect orientation; fragments generated were 7823 bp, 408 bp and 175 bp. The correct and wrong orientations were also confirmed by digestion with *NarI*; the fragments obtained are shown in Table 1. The clone was further confirmed by amplification of 93 bp product by nested PCR primers of hexon gene (Fig3-5).

**Table 1.** Restriction fragment sizes (bp) of pTarget.cav2hex plasmid after digestion with restriction endonucleases.

<b>RE enzyme</b>	<b>Correct orientation</b>	<b>Wrong orientation</b>
<i>EcoRI</i>	5602 & 2804	5602 & 2804
<i>NarI</i>	5576 & 2830	5463 & 2943
<i>NheI</i>	6038, 2193 & 175	7823, 408 & 175

The sequence obtained from sequencing by T7 primer was submitted to GenBank and accession number assigned to sequence was **DQ839392**

The SDS-PAGE (Fig 12) and western blot analysis (Fig 13) of transfected cells revealed the prominent band of approximate molecular weight 101 kDa as predicted from the cell lysate transfected with pTarget.cav2hex plasmid while there was no band corresponding to this in cell lysate from mock transfected MDCK cell control. Immunofluorescence analysis of pTarget.cav2hex plasmid transfected MDCK cells revealed fluorescence in transfected cells while there was no fluorescence in mock transfected MDCK cells (Fig 10-11) which confirmed the expression of cav2 hexon gene.

### **Immunological response of pTarget.cav2hex in dogs**

The humoral and cell mediated immune response of CAV-2 DNA vaccine was studied in dogs. The serum neutralization titres showed significantly higher response as compared to healthy control. The ELISA antibody response was significantly higher in the vaccinated groups as compared to healthy control (Table 2, Fig 6&7.). There was significant difference in stimulation index of the lymphocytes in vaccinated groups as compared to control groups indicating the presence of cellular immune response against the canine adenovirus DNA vaccine (Fig 8&9).

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

**Groups No.of**

	dogs	Plasmid DNA	Ab 14 dpv			Ab 35 dpv		Ab 42 dpv	
			SN	ELISA	SI	SN	ELISA	SN	ELISA
I	6	Control	4	8	0.91	4	8	4	8
II	6	pTargeT control	4	8	0.95	4	8	4	8
III	6	pTargeT.cav2hex	32	512	1.32	28	1024	128	1024
IV	6	pTargeT.cav2hex+ pTargeT.IL2can	128	512	1.32	128	1024	128	1024

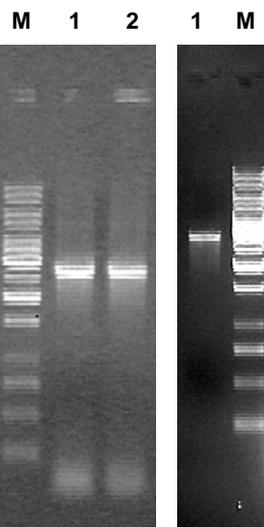
dpv=days post vaccination

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

1 2 M 3 4

Fig 2.

Lanes M: 1kb ladder, 1-4: amplified E3 gene of cav2.

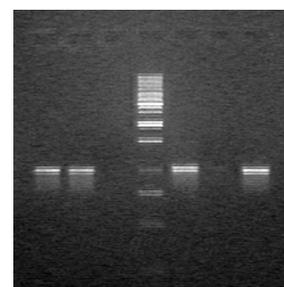


3 kb  
2736 bp

2736 bp

Elution of PCR

Fig. 1 PCR amplification of canine adenovirus type 2 hexon gene  
Lane M: 1 kb DNA ladder. Lane 1, 2: amplified hexon gene



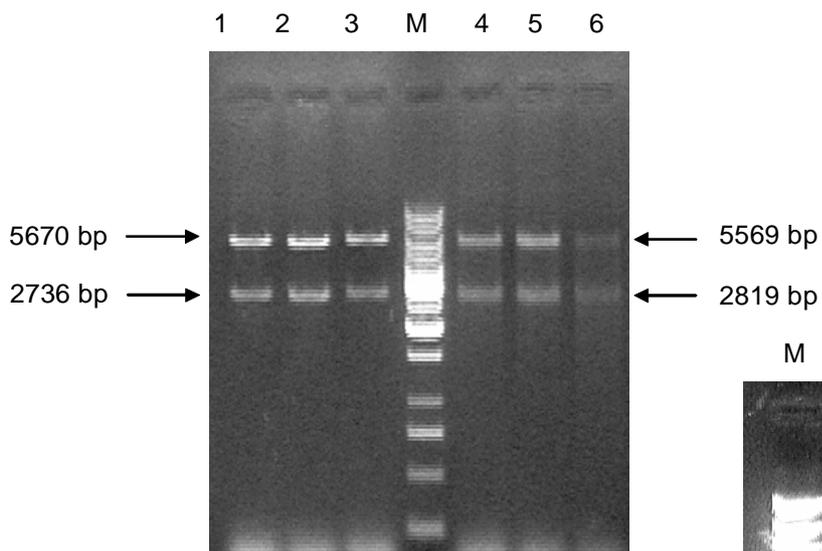


Fig.3 Restriction enzyme digestion of recombinant plasmids pTargetTcav2hex  
 Lane 1,2,3: digestion with EcoR; Lane M : 1kb DNA ladder; Lane 4,5,6: digestion with NarI

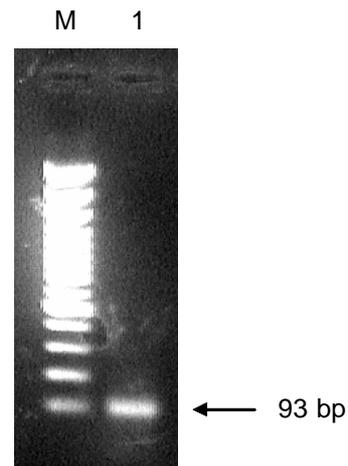


Fig.4 Confirmation of pTargetT.cav2hex by nested PCR. Lane M: 100 bp ladder; Lane 1: 93 bp Nested PCR product

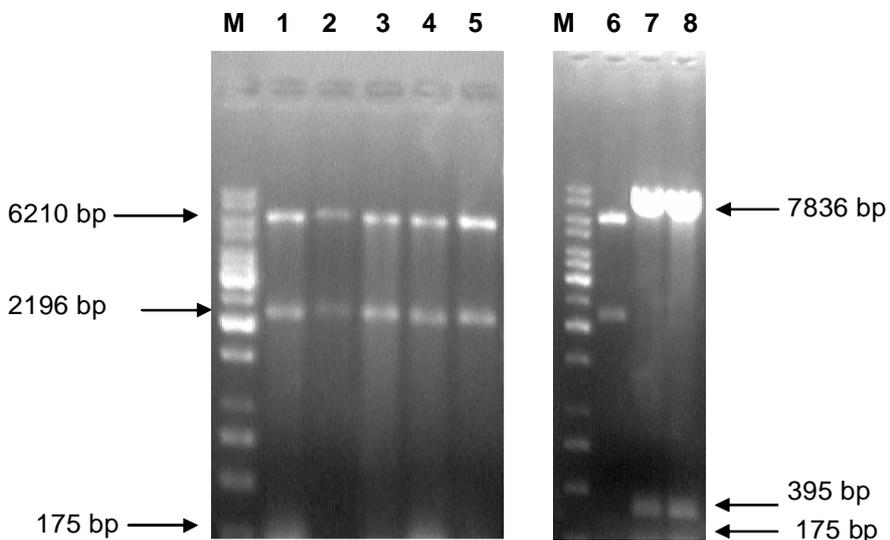


Fig.5 Restriction enzyme digestion of recombinant plasmids pTargetTcav2hex with NheI  
 Lane M : 1kb DNA ladder; Lane 1,2,3,4,5,6 : Recombinant clone with correct orientation; Lane 7,8: Recombinant clone with incorrect orientation

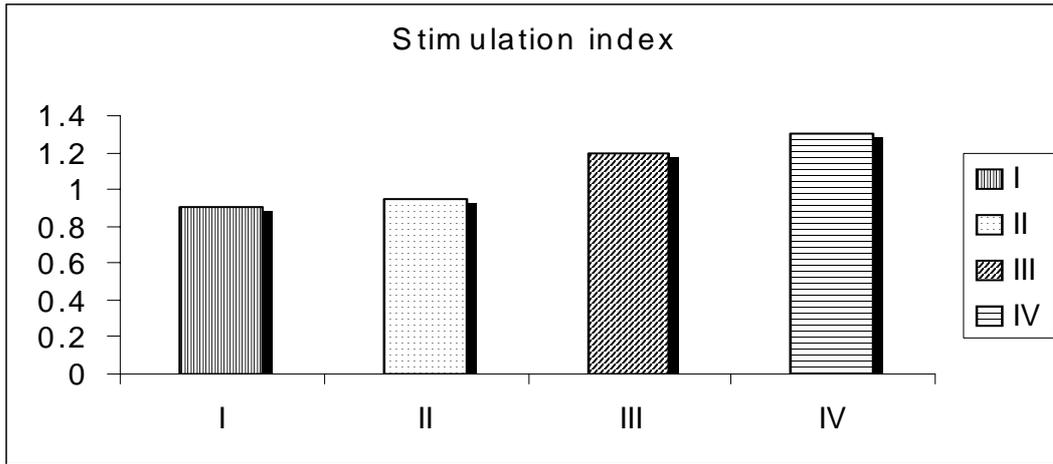


Fig.6 .Lymphocyte proliferation assay of PBMCs of pTarget.cav2hex. Stimulation indices were calculated by dividing OD value of stimulated cells with that of unstimulated cells.

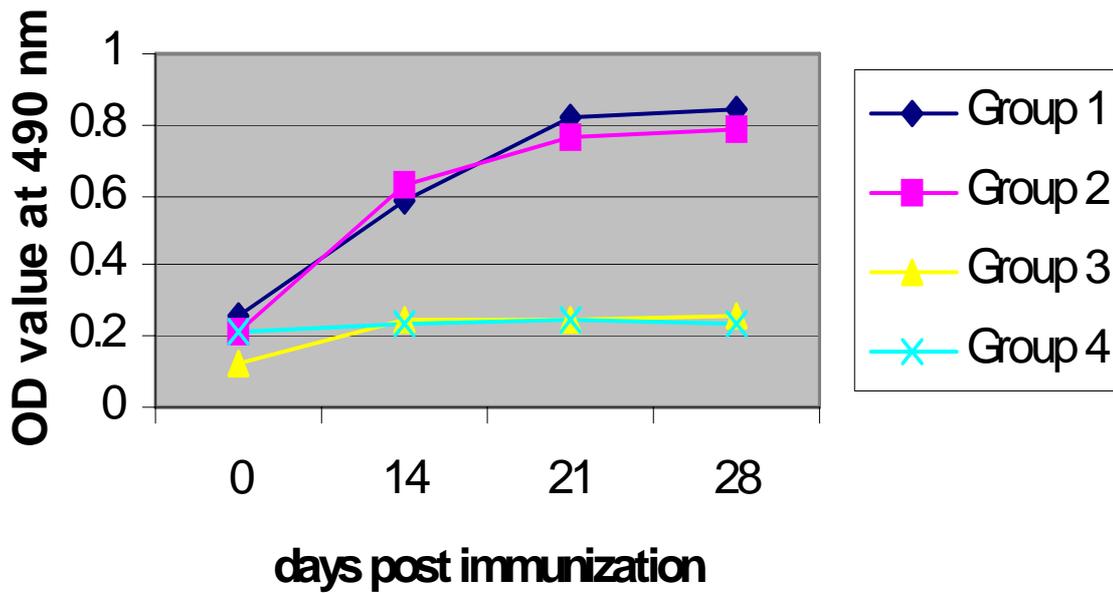


Fig.7. Absorbance values at 490 nm of post-immunization dog sera using ELISA . Group I: pTarget.cav2hex alone, Group 2: pTarget.cav2hex + pTarget.IL-2can; Group 3: pTargetT vector alone , Group 4:Healthy control

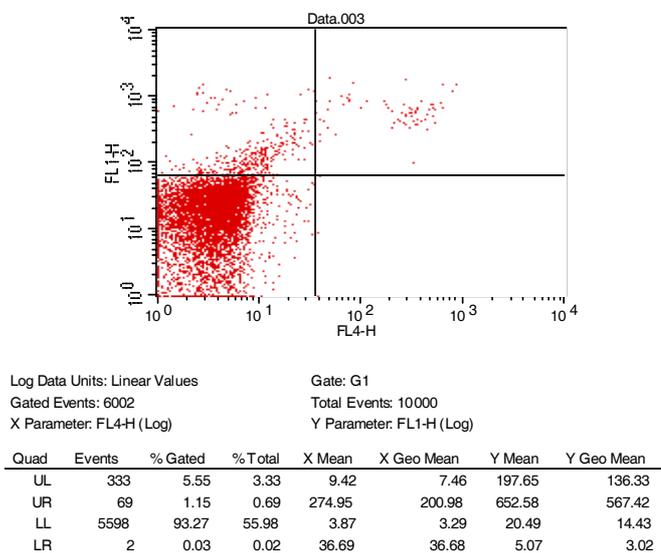


Fig81 FACS analysis of lymphocytes from the healthy dogs

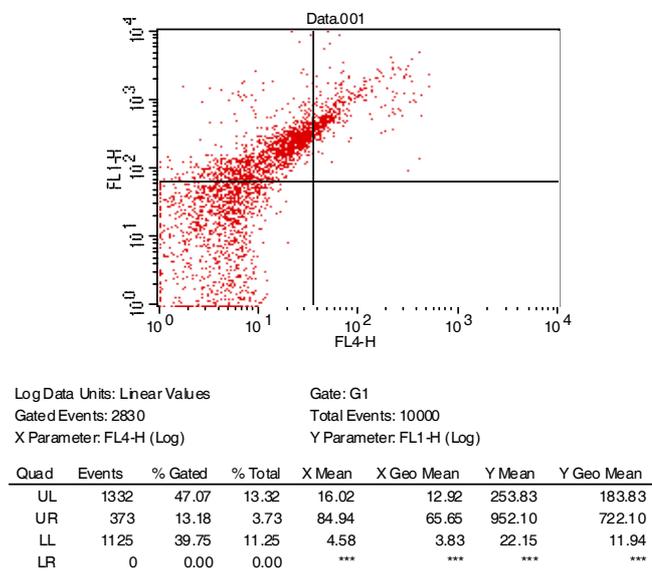


Fig92 FACS analysis of lymphocytes from the vaccinated dogs

FACS analysis of control and vaccinated dogs for CD8+ count from PBMCs. The CD3+ is plotted on X- axis and CD8+ is on Y axis.

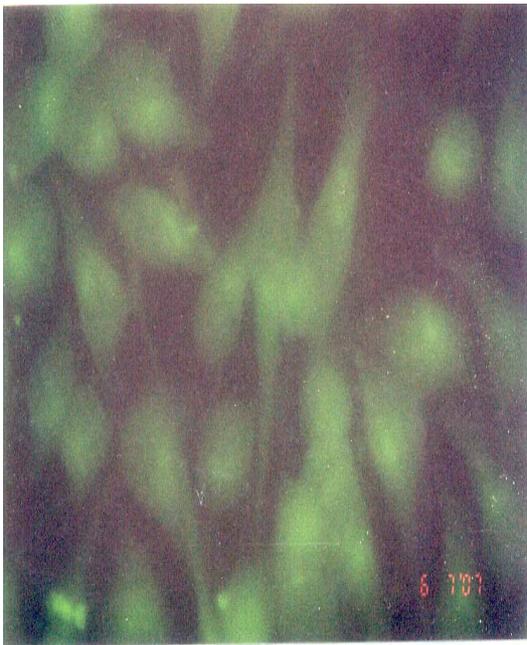


Fig.10. pTargetT.cav2hex transfected MDCK cells showing fluorescence in FAT.

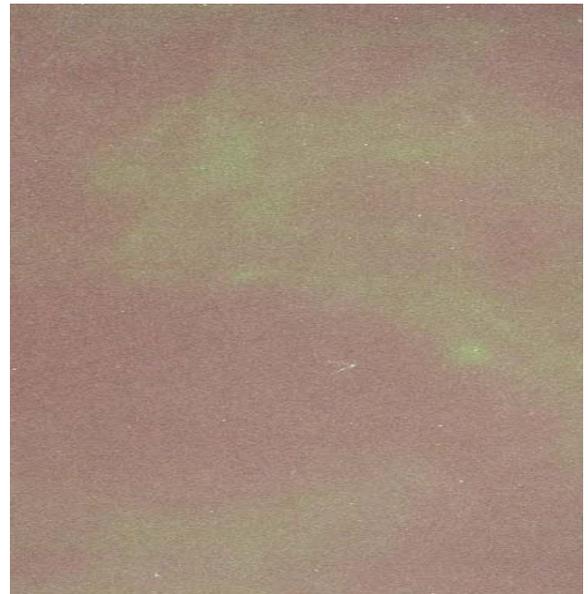


Fig.11. Mock transfected MDCK cells showing no fluorescence in FAT.

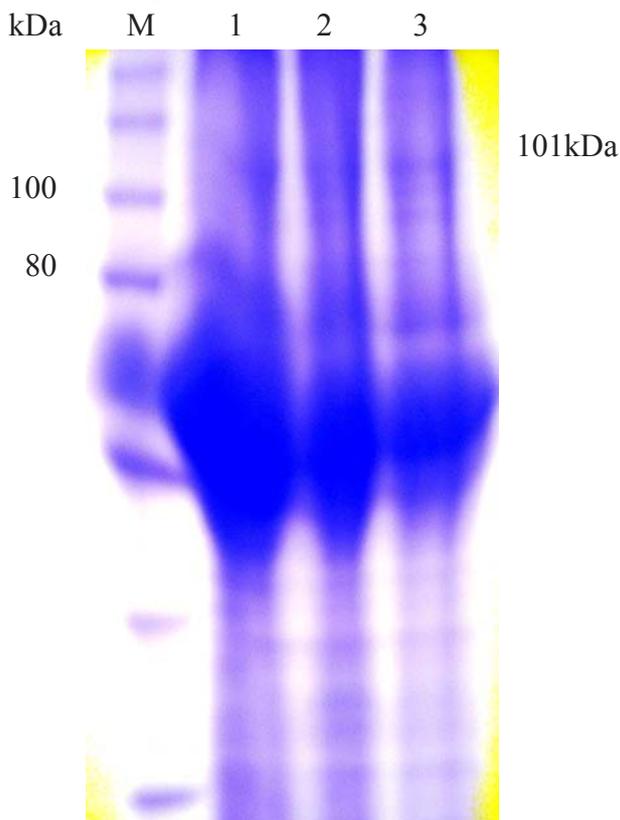


Fig.12. SDS-PAGE analysis of pTargetT.cav2hex transfected MDCK cells showing expressed protein. Lane M, Protein MW marker; 1, Control non-transfected cells; 3, pTargetT.cav2hex transfected showing 101kDa protein



Fig.13. Western blot showing expressed protein to be cav2hex in lane 3.

## **Discussion**

Infectious laryngotracheitis (ILT) caused by CAV-2 is highly contagious diseases of dogs and other members of canid family like foxes. Both the diseases are recorded worldwide. Vaccination is the most effective method to control and prevent ILT in dogs. The live modified vaccines containing CAV2 results in continued persistence of virus in respiratory tract. It causes respiratory distress (Willis, 2000). The killed inactivated virus vaccines do not produce lesions in dogs but must be given frequently to provide protection equal to modified live vaccines. Thus, a continuous pressure was felt to develop next generation of vaccines to control and eradicate these diseases.

The recent advances in genetic engineering and molecular biology have made rapid strides especially in the area of vaccine development. A considerable effort has been directed to develop safe and effective vaccines. These efforts led to the development of the simple and potentially powerful technology of DNA vaccination. 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). The DNA vaccine technology is used to circumvent the problems of conventional vaccines like handling of pathogenic organisms, reversion to virulence and requirement of cold chain etc in addition to being highly immunogenic and economic.

Though a lot of work has been done to develop DNA vaccines against different diseases, no literature can be cited for canine adenoviruses. Canine adenoviruses have a double stranded DNA genome with a non-enveloped, icosahedral nucleocapsid which consists of hexons, pentons and fibre proteins. Among which the hexon is major capsid protein and accounts for more than 83% of capsid proteins. 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 CAV2 was amplified using a single set of primers by Taq DNA polymerase. The PCR product was gel purified

Clones with cav2hex in right orientation were designated as pTarget.cav2hex. The selected clones were sequenced using T7 sequencing primers. These sequences were analyzed with other available hexon gene sequences of adenoviridae family for homology by BLAST (data not presented). 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, since the molecular weight deduced by DNASTAR is 101.1 kDa for cav2hex. 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.cav2hex showed a SN titre of 32 and 128 after first and second immunization respectively. All the immunized pups developed significant level of ELISA antibody titre after single immunization whereas the control dogs remained serologically negative. The ELISA antibody in sera collected after second immunization had high titres of 1024 as compared to single immunization titre of 512. 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 mediated immune response. Stimulation index data showed that rplasmids pTarget.cav2hex (SI = 1.2) 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 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.cav2hex. 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 cytokines (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 immunogenicity of hexon gene as shown by all the tests, and especially by SNT and FACS analysis. It demonstrates that the recombinant plasmids can be used as DNA vaccines against CAV-2 diseases in canines

**References**

- Ahi, Y. S. 2006. Cloning of rabies virus glycoprotein gene in bicistronic expression vector for use as a DNA vaccine. MVSc Thesis, Deemed University, IVRI.
- Bounous D. I., Campagnoli R. P. and Brown J. 1992 Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocytes. *Avian Dis.* 36:1022-1027.
- Bourdoiseau G., Bonnefont C., Magnol J. P., Saint-André I., Chabanne L. 1997. Lymphocyte subset abnormalities in canine leishmaniasis. *Vet Immunol Immunopathol* 56: 345-351
- Boyum A. 1976 Isolation of lymphocytes, granulocytes and macrophages. *Scand J Immunol. Suppl* 5:9-15.
- Bu J., Song Y., Rompato G. Burgess D.J., Garmendia A.E. 2003. Co-delivery of IL-2 or liposomes augment the response of mice to a DNA vaccine for pseudorabies virus IE 180. *Comp Immunol Microbiol Infec Dis.* 26: 175-187.
- Bustin S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrino.* 25: 169-193.
- Bustin S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrino.* 29:23-39
- Byrne K., Kim H. W., Chew B. P., Reinhardt G.A. and Hayek M. G. 2000 A standardized gating technique for the generation of flow cytometry data for normal canine and normal feline blood lymphocytes. *Vet Immunol Immunopathol* 73: 167-182
- Chauhan S., Rai A., Rai N., Dighe N., Roy B., Gupta P. K., Divya . 2005. Clonig of synthetic VP2 gene of infectious bursal disease virus and its use as DNA vaccine in chicken. *Current Sci.* 88: 1164-1167.
- Ditchfield J., Macpherson L.W. and Zbitnew A. 1962. Association of a canine adenovirus (Toronto A26/61) with an outbreak of Laryngotracheitis (kennel cough). A preliminary report. *Can Vet J.* 3: 238-247.
- Fan M. W., Bian Z., Peng Z. X., Zhong Y., Chen Z., Peng B. and Jia R. 2002. A DNA vaccine encoding a cell-surface protein antigen of *Streptococcus mutans* protects gnotobiotic rats from caries. *J Dent Res.* 81:784-787.
- Gupta, P. K., Rai, A., Rai, N. Raut A. A. and Chauhan S. 2005. Cloning of canine parvovirus VP2 gene for use as a DNA vaccine in dogs. *Current Sci.* 88: 778-782.
- Gupta, P. K., Rai, A., Rai, N. and Saini, M. 2005. Immunogenicity of a recombinant plasmid DNA vaccine encoding glycoprotein gene of rabies virus CVS in mice and dogs. *J. Immunol. Immunopathol.* 7: 58-61.
- Jianrong L, Xueya L, Yaowei H, Songshu M, Ronghui X, Ruitang D and Lian 2004 .Enhancement of the immunogenicity of DNA vaccine against infectious bursal disease virus by co-delivery with plasmid encoding chicken interleukin 2. *Virology.* 329: 89-100.

- Kodihalli S., Goto H., Kobasa D. L., Krauss S., Kawaoka Y. and Webster R. G. 1999. DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. *J Virol.* 73:2094-2098.
- Koptopoulos G. and Cornwell H.J.C. 1981 Canine adenoviruses: A review. *Vet Bull.* 51: 135-42
- Kumar S. 2006. Cloning of infectious bursal disease virus VP2 gene in bicistronic mammalian expression vector for use as a DNA vaccine. M. V. Sc. thesis. Deemed university IVRI.
- Patial S., Chaturvedi V. K., Rai A., Saini M., Chandra R., Saini Y., Gupta P. K. 2007. Virus neutralizing antibody response in mice and dogs with a bicistronic DNA vaccine encoding rabies virus glycoprotein and canine parvovirus VP2. *Vaccine* (Accepted).
- Rai A., Barua S., Rai A. 2005. Induction of immune response in chicken vaccinated with plasmid DNA encoding fowl adenovirus 4 hexon gene. *J immunol immunopathol.* 7: 58-60.
- Rai, A., Gupta, P.K., Rai, N. 2002. Cloning of rabies virus glycoprotein gene in a mammalian expression vector and immunogenicity of the recombinant plasmid dna. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 23:123-126
- Rai N, Kaushik K. P. Rai A. 2005b. Development of rabies DNA vaccine using a recombinant plasmid. *Acta virol.* 49: 207-210.
- Serezani, C.H., Franco, A.R., Wajc, M., Umada Yokoyama-Yasunaka, J.K., Wunderlich, G., Borges, M.M., Uliana, S.R. 2002. Evaluation of the murine immune response to Leishmania meta 1 antigen delivered as recombinant protein or DNA vaccine. *Vaccine.* 20: 3755–3763
- Tribe G.W. and Wolff D.C. 1973 Protection of dogs against canine hepatitis with Toronto A26/61 virus (Kennel Cough Isolate) hexon antigen. *J small Anim Pract.* 14: 251-255.
- Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., Hawe, L.A., Leander, K.R., Martinez, D., Perry, H.C., Shiver, J.W., Montgomery, D.L. and Liu, M.A. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science.* 259: 1745–1749.
- Wigand R., Keller D. 1982 Virological diagnosis of ocular adenovirus infection *Klin Monatsbl Augenheilkd.* 180: 545-547.
- Willis A.M.. Canine viral infections. 2000. *Vet Clin North Am Small Anim Pract.* 30:1119-1133.