Biotechnology International, Vol 2, No.2, June 2009, 46-59.

Development of a replicon based DNA vaccine encoding canine distemper H gene

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Summary: Among the six proteins of canine distemper namely H, N, P, M.L and F, the highest antigenic variation is is found in the H protein, whereas F and P proteins are affected to a much lower extent. In this study, replicase based DNA vaccine was tried and we found there is rise in both the CD4+ and CD8+ cells of the immunized dogs, the increase in CD8+ cells is more than that in CD4+ cells. The relative quantification of the template strands for the cytokines IFNY, IL2 and IL4 increased over healthy group. Overall, pSinCMV.cdvh rplasmid can be used as a potent vaccine against canine distemper in dogs which increases cellular as well as humoral immune response.

Key words: DNA vaccine, replicase, immunity

Introduction

Canine distemper is one of the infectious diseases of dogs caused by canine distemper virus (CDV) which mostly affects 2 to 6 months old pups. The mortality is between 30 to 80% and the recovery from the disease depends on the immune status of the animal (Fenner *et al*., 1987). Many killed and live modified vaccines against CDV are in use (Bolt *et al*., 1997). Where on one side killed vaccines have been associated with low or no humoral immunity at all (Montali *et al*., 1993; Williams *et al*., 1996), maternal antibodies usually interfere (Pare *et al*., 1999) with live modified vaccines and reduces its efficacy. Maternal antibodies against canine distemper virus have a half life of around 11 days (Pare *et al*., 1999). But these antibodies have been reported to interfere with vaccination with modified live virus vaccines till 18 weeks of age (Pare *et al*., 1999). Replicase based vectors are superior over other conventional vectors in terms of its lower requirement of dose of immunization (Xiong *et al*., 1989; Hariharan *et al*., 1998, Berglund *et al*., 1998, Leitner et al., 2000), the mechanism of breaking tolerance against self antigen, the power of inducing apoptosis (Leitner *et al*., 2003) so that transient but robust expression of antigen is achieved in short period without taking risk of integration into host chromosomes (Jolly, 1994; Miller *et al*., 1993, Samulski *et al*., 1989).

Materials and Methods

rplasmid: The replicase based pSinCMV.cdh was used.

Real time PCR primers:

1.	Canine IL-2 RT forward	461 bp	23	CTC ACA GTA ACC TCA ACT CCT GC
2.	Canine IL-2 RT reverse		24	TTC TGT AAT GGT TGC TGT CTC GTC
3.	Canine IFN gamma RT forward	281 bp	22	TCG GAC GGT GGG TCT CTT TTC G
4.	Canine IFN gamma RT reverse		25	CAC TTT GAT GAG TTC ATT TAT CGC C
5.	Canine IL-4 RT forward	317 bp	23	TAA AGG GTC TCA CCT CCC AAC TG

6. Canine IL-4 RT reverse		24	TAG AAC AGG TCT TGT TTG CCA TGC
7. Beta actin gene forward	540 bp	21	GTGGGCCGCTCTAGCCACCAA
8. Beta actin gene reverse		27	TCTTTGATGTCACGCACGCACGATTTC

Cell Culture: MDCK cell line and BHK21 cell line were obtained from National Centre for Cell Science (NCCS), Pune and maintained in DMEM (Gibco, NY) supplemented with 50 µg/ml gentamicin (Amresco, USA)) and 10% new born calf serum (Gibco, NY).

Virus: Canine distemper virus (Lederle strain) was available in the laboratory.

Immunisation of dogs: One month age old pups were used and one week before the start of immunization trial, they were treated with antihelminthics (Albendazole) and then tested for seronegativity against canine distemper virus.

Groups	First vaccination	Day	Booster	No. of dogs	Injectible dose
Healthy control	NSS (I/M)	0 day	30th day	4	0.5 ml
pSinCMV vector	4 µg (I/M)	0 day	30th day	4	0.5 ml
pSinCMV.cdvh	2 µg (I/M)	0 day	30th day	4	0.5 ml
pSinCMV.cdvh	4 µg (I/M)	0 day	30th day	4	0.5 ml

Collection of blood and serum samples

Blood samples were collected from each dog with a 24 gauge sterile needle and 5 ml syringes at 0 day, 30 days of primary vaccination and 30 days of booster immunization. For processing under uncoagulated condition, blood was collected in heparin (200 units/ml of blood) containing vials, while for serum collection blood was collected in sterilized vials without any anticoagulant, kept slanted overnight at 4°C and centrifuged at 2,500 rpm for 10 minutes. Serum was collected without disturbing the clot.

Serum neutralization test: Serum collected from pups was heat inactivated at 56°C for 30 minutes. A two fold serial dilution of dog serum was made in DMEM without serum and 0.05 ml of each dilution was dispensed in 96 well plates. 0.05 ml of 100TCID50 virus titer was added to each well. The serum-virus mixture was incubated at 37°C for 2h and 100 µl of MDCK cell suspension ($2X10^5$ cells/ml) in culture media was added to each well. Neutralizing antibody titer was calculated as per the standard protocol.

Lymphocyte stimulation test: Lymphocytes were dispensed in 96 well flat bottom tissue culture plate with 2X 105 cells per well in triplicate. Cells were treated with Concavalin A @ 50µg/well and with CD virus (10 µl of 100 TCID50). A negative control was also maintained which contained neither virus nor ConA. The plate was incubated at 37°C in a humidified CO2 incubator for next 72 hours. Then 25 µl of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma (5 mg/ml)] was added to each well. Plates were incubated for another 4 h. Then 100 µl of 0.04 N HCl in 2-propanol was added to each well. Mean optical density (OD) was determined as absorbance at 550nm (A550) on a

microplate ELISA reader. Blastogenic response for the assay was expressed as stimulation index (SI), calculated by dividing the mean absorbance of stimulated group by mean absorbance of unstimulated group (Keck and Bodine 2006; Haddad et al., 1994).

Quantitative assay of cytokines by Real Time PCR: PBMC was collected from 3 ml of freshly obtained pooled blood for each group. Total RNA was isolated from PBMCs using TRIzol reagent (Invitrogen), following the supplier's protocol and quantified by spectrophotometry at 260 nm. cDNA was synthesized using MMLV reverse transcriptase as per the protocol given in the kit. cDNA from different group was brought near equal concentration by normal PCR amplification with beta actin. Genes for cytokines IFN gamma, IL2 and IL4 were amplified using specific primers, Brilliant SYBR® Green QPCR master mix (Stratagene, USA) and Mx3000P spectroflourometric thermal cycler operated by MxProTM QPCR software. A 25 µl of reaction volume was prepared with following components. Beta actin gene was kept as house keeping gene to compare relative abundance of cytokines. NTC (Non template control) was always kept to check any non specific amplification: template cDNA 1 µl, forward primer 10 pmol, reverse primer 10 pmol, 2X SYBR® Green QPCR master mix 12.5µl, nuclease free water to 25 µl. After 40 cycles of amplification threshold cycle (Ct) value was obtained for each cytokines and internal control gene beta actin (reference gene). These Ct values were used to determine the relative abundance of different cytokines among different groups. The following formula was used to calculate the ratio of different cytokines mRNA in immunized animals to healthy control animals (Pfaffl 2001).

$$\text{Ratio} = \{(\text{E target})^{\Delta Ct \text{ target (control - treated)}}\} / \{(\text{E ref})^{\Delta Ct \text{ ref (control - treated)}}\}$$

Estimation of $CD4^+$ and $CD8^+$ T cells by FACS: The whole blood was collected from each dog and pooled among groups. Ten microlitre of anti-canine CD3: FITC/CD4: RPE/CD8: Alexa fluor®647 cocktail (Serotec, England) was diluted in 50 µl of 1X PBS. 50 µl of pooled blood was added to the diluted cocktail and vortexed. The mixture was incubated at room temperature for 30 minutes andcentrifuged at 5000 rpm for 5 minutes. The aspirate was thrown away and pellet was washed twice with 1X PBS at 5000 rpm for 5 minutes. The pellet was redissolved in 1 ml of 1 X NH_4Cl, incubated at room temperature for 10 minutes and centrifuged at 5000 rpm for 5 minutes. Lysis buffer was decanted out and pellet was dissolved in 0.5 ml of 1X PBS for use in FACS (BD Biosciences).

Results

Immunological response of pSinCMV.CDVH

Virus neutralization test performed on serum of dogs indicated that when pSinCMV.cdvh was used in a dose of 2 µg the antibody titer was found 8 and 32 at 30 days of immunization and 30 days of booster immunization respectively, while the dogs immunized with 4 µg of pSinCMV.cdvh gave a titer of 128 both at 30 days of immunization and 30 days of booster immunization. The groups maintained as vector control and healthy control did not show any antibody titer against CDV(T**able 1)**.

Table 1. SN antibody titer of dogs after immunization

Groups	SN antibody titer* at 30 days of immunization	SN antibody titer* at 30 days of booster
pSINCMV.cdvh (2 µg)	8	32
pSINCMV.cdvh (4 µg)	128	128
pSINCMV (4 µg)	0	0
Healthy	0	0

*Titers have been shown as reciprocal of maximum dilution of serum inhibiting the 100TCID50 CDV specific cytopathic effects

MTT assay showed that dogs immunized with 2 µg of pSinCMV.cdvh showed a stimulation index of 1.35 and 1.76 at 30 days of immunization and at 30 days of booster immunization respectively. The dogs immunized with 4 µg of pSinCMV.cdvh showed higher stimulation index of 2.54 and 2.57 at 30 days of immunization and 30 days of booster immunization respectively. Positive controls stimulated with mitogen ConA gave a maximum absorbance (A_{550}) of around 0.6 in all the groups. Unstimulated samples from each group maintained near about same 0.2 (**table 2, 3** and **Fig. 21-26**).

Table 2. Mean absorbance and stimulation index at 30 days of immunization

	With ConA	With virus	Without ConA	SI
pSinCMV.cdvh (2 µg)	0.598	0.307	0.228	1.35
pSinCMV.cdvh (4 µg)	0.631	0.508	0.200	2.54
pSinCMV (Vector control)	0.616	0.226	0.200	1.13
Healthy control	0.611	0.168	0.156	1.10

Table 3. Mean absorbance and stimulation index at 30 days post booster immunization

	With ConA	With virus	Without ConA	SI
pSinCMV.cdvh (2 µg)	0.592	0.441	0.251	1.76
pSinCMV.cdvh (4 µg)	0.601	0.631	0.246	2.57
pSinCMV (Vector control)	0.634	0.230	0.201	1.14
Healthy control	0.655	0.150	0.140	1.07

The FACS analysis of pooled blood collected from animals of each group revealed tilt of enhancement of immunity towards cellular side. Although there is rise in both the $CD4^+$ and $CD8^+$ cells of the immunized dogs, the increase in $CD8^+$ cells

is more than that in $CD4^+$ cells. The % count of $CD4^+$ cells and $CD8^+$ cells and the ratio of CD4 to $CD8^+$ cells are given in the **table 4, 5** and **Fig.1.**

Table 4. $CD4^+$ and $CD8^+$ T cells after 30 days of immunization

Groups	% $CD4^+$ T cells	% $CD8^+$ T cells	Ratio of CD4+/ $CD8^+$ T cells
pSinCMV.cdvh (2 µg)	1.14	1.14	1.0
pSinCMV.cdvh (4 µg)	5.87	1.27	0.877
pSinCMV(4 µg)	0.43	0.32	1.344
Healthy	0.14	0.13	1.077

Table 5. $CD4^+$ and $CD8^+$ T cells after 30 days of booster immunization

Groups	% $CD4^+$ T cells	% $CD8^+$ T cells	Ratio of $CD4^+$/ $CD8^+$ T cells
pSinCMV.cdvh (2 µg)	3.46	7.67	0.446
pSinCMV.cdvh (4 µg)	8.16	9.56	0.854
pSinCMV(4 µg)	0.60	0.43	1.395
Healthy	0.16	0.17	0.941

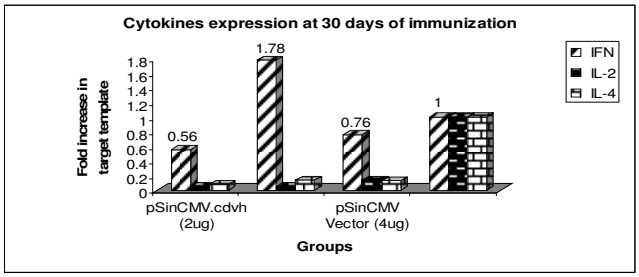
The relative quantification of the template strands for the cytokines IFNγ, IL2 and IL4 was done by threshold cycle value (Ct value) of amplification curve. Ct value for IFN gamma, IL2 and IL4 for the sample taken at 30 days of immunization was 22.82, 27.92, 28.65 for dogs vaccinated with 2 µg of pSinCMV.cdvh, 24.52, 28.06, and 29.45 for dogs vaccinated with 4 µg of pSinCMV.cdvh, 22.45, 28.72, and 31.03 for dogs vaccinated with 4 µg of vector pSinCMV; 29.95, 28.95 and 31.16 for healthy dogs respectively. Ct value at 30 days of booster immunization for the cytokines IFN gamma and IL2 was 16.90, and 28.61 for dogs immunized with 2 µg of pSinCMV.cdvh; 22.15 and 28.48 for dogs immunized with 4 µg of pSinCMV.cdvh; 23.93 and 28.91 for dogs immunized with 4 µg of pSinCMV vector and 24.21 and 29.05 for healthy group respectively. To normalize the values, gene for beta actin (constitutive genes) was also amplified for each sample and the respective Ct values of beta actin for dogs vaccinated with 2 ìg of pSinCMV.cdvh was 15.57 and 18.53 at 30 days of immunization and 30 days of booster immunization respectively. The same for the dogs immunized with 4 µg of vaccine pSinCMV.cdvh was 15.61 and 18.71 at 30 days of immunization and 30 days of booster immunization. The fold increase in expression of different cytokines mRNA in vaccinated animals and vector injected animals was calculated with respect to healthy control. The fold increase was normalized against the value of internal control reference gene beta actin. Summarily, at 30 days of immunization with 2 µg of pSinCMv.cdvh, increase in IFN gamma is .56 times that of healthy group, while IL2 and IL4 dint show any increase, instead they have shown a decreasing trend. The dogs immunized with 4 µg of pSinCMV.cdvh at 30 days of immunization have shown appreciable amount (1.78 times) of increase in IFN gamma expression. Again at this dose also there has not been much increase in

IL2 and IL4. Vector group exhibits higher level of expression than the group immunized with 2 µg of pSinCMV.cdvh. 30 days after booster immunization there has been obvious increase in the expression of IFN gamma and IL2 but the increase in IFN gamma is as high as 22.64 times (**table 6** and **Fig. 49-52 below**).

Ct values at 30 days of immunization					Ct values at 30 days of booster immunization			
Groups	**IFN Gamma**	**IL2**	**Il4**	**Beta actin**	**IFN**	**IL2**	**IL4**	**Beta actin**
pSinCMV.cdvh (2 ug)	22.82	27.92	28.65	15.57	16.9	28.61		18.53
pSinCMV.cdvh (4 ug)	24.52	28.06	29.45	15.61	22.15	28.48		18.71
pSinCMV (4 ug)	25.45	28.72	31.03	17.87	23.93	28.91		19.31
Healthy control	25.95	28.95	31.16	17.88	24.21	29.05		21.33
Delta Ct								
pSinCMV.cdvh (2 ug)	3.13	1.03	2.51	2.3	7.3	0.44		2.8
pSinCMV.cdvh (4 ug)	1.43	0.86	1.71	2.27	2	0.57		2.62
pSinCMV (4 ug)	0.5	0.23	0.13	0.01	0.3	0.14		2.02
Healthy control								
Fold increase								
pSinCMV.cdvh (2 ug)	8.75	2.04	5.67	4.92	157.59	1.36		6.96
pSinCMV.cdvh (4 ug)	2.69	1.82	3.27	4.82	4	1.48		6.25
pSinCMV (4 ug)	1.41	1.17	1.09	1.01	1.23	1.1		4.06
Ratio with Ref gene								
pSinCMV.cdvh (2 ug)	0.56	0.05	0.08		0.65	0.24		
pSinCMV.cdvh (4 ug)	1.78	0.05	0.14		22.64	0.2		
pSinCMV (4 ug)	0.76	0.14	0.13		1.76	0.27		

Table. Real Time PCR data of delta Ct and fold increase

	Fold increase in Cytokines at 30 days of immunization			Fold increase in cytokines at 30 dyas post booster vaccination	
Groups	**IFN**	**IL-2**	**IL-4**	**IL-2**	**IFN**
pSinCMV.cdvh (2ug)	0.56	0.05	0.08	0.24	0.65
pSinCMV.cdvh (4ug)	1.78	0.05	0.14	0.2	22.64
pSinCMV Vector (4ug)	0.76	0.14	0.13	0.27	1.76
Healthy control	1	1	1	1	1



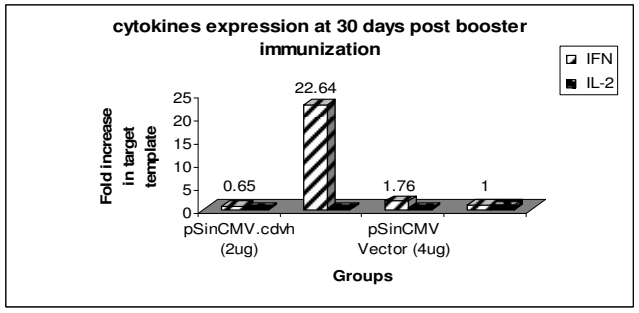


Fig. Fold increase in expression of cytokines in vaccinated animals with respect to healthy control

Discussion

Canine distemper is one of the infectious diseases of dogs caused by canine distemper virus (CDV) which mostly affects 2 to 6 months old pups. The mortality is between 30 to 80% and the recovery from the disease depends on the immune status of the animal (Fenner *et al*., 1987). Many killed and live modified vaccines against CDV are in use (Bolt *et al*., 1997). Where on one side killed vaccines have been associated with low or no humoral immunity at all (Montali *et al*., 1993; Williams *et al*., 1996), maternal antibodies usually interfere (Pare *et al*., 1999) with live modified vaccines and reduces its efficacy. Maternal antibodies against canine distemper virus have a half life of around 11 days (Pare *et al*., 1999). But these antibodies have been reported to interfere with vaccination with modified live virus vaccines till 18 weeks of age (Pare *et al*., 1999). Modified live virus vaccines have been suspected to retain residual virulence (Appel and Harris, 1988; Durchfeld *et al*., 1990; Carpenter *et al*., 1976, 1998). DNA vaccines were attempted to achieve a balance between strength of immunity conferred and the extent of safety (Cirone *et al*., 2004; Dahl *et al*., 2004; Polack *et al*., 2000).

Among the six proteins of canine distemper namely H, N, P, M.L and F, the highest antigenic variation is found in the H protein, whereas F and P proteins are affected to a much lower extent (Blixenkrone-Moller *et al*., 1993). As the H

protein's primary function is cell receptor binding, its characteristic is therefore most closely related to the effective infectivity and host specificity of the virus (Gassen *et al*., 2000). The protective efficacy of anti H monoclonal antibodies is higher than that of anti F mAbs (Hirayama *et al*., 1991). We have used CDVH gene of Lederle strain to induce immunity against canine distemper. On BLAST the sequence was found to bear 96-98 % homology with other genomic and CDVH gene complete CDS.

The reported intramuscular dose of DNA vaccines is 100 to 300 µg per animal. We cloned the H gene in replicase based mammalian expression vector pSinCMV, a fourth generation DNA vaccine and see its immunological response in dogs. Replicase based vectors are superior over other conventional vectors in terms of its lower requirement of dose of immunization (Xiong *et al*., 1989; Hariharan *et al*., 1998, Berglund *et al*., 1998, Leitner et al., 2000), the mechanism of breaking tolerance against self antigen, the power of inducing apoptosis (Leitner *et al*., 2003) so that transient but robust expression of antigen is achieved in short period without taking risk of integration into host chromosomes (Jolly, 1994; Miller *et al*., 1993, Samulski *et al*., 1989), a broad host range, and infection of non-dividing cells (Strauss *et al*., 1994). pSinCMV is an alpha virus (Sindbis virus) based plasmid vector which serves as negative strand once it enters into the host cell. After entering into the nucleus it is transcribed by host RNA polymerase enzyme from CMV promoter into a full length RNA transcript. This full length transcript then acts as positive sense alpha virus which in turn is translated in cytoplasm to form replicase protein. This protein serves as RNA dependent RNA polymerase enzyme and forms negative sense RNA from positive sense transcript. From this negative sense strand full length as well as smaller fragments from subgenomic promoters are transcribed which in-turn is translated into proteins. Since the cloned insert is downstream to the subgenomic promoter, the translated protein represents our target protein. The subgenomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it.

In comparative studies of conventional (non-replicating) plasmid DNA vectors and alphavirus DNA-based replicon vectors, the latter generally induces stronger immune responses and at a significantly lower DNA concentrations than does conventional vectors (Berglund *et al*., 1998 Hariharan *et al*., 1998) It has been shown that innate antiviral pathways implicated in the molecular mechanisms of innate antiviral immunity (double stranded RNA recognition and interferon action) are also one of the mechanism underlying the superior efficacy of replicase based DNA vaccines (Leitner *et al*., 2003). By various experiments it is now beyond doubt that apoptosis has direct relation with immunostimulatory action. Genes for pro-apoptotic molecules have been co-delivered with DNA vaccines to specifically induce apoptosis in transfected cells. Apoptosis inducing alpha virus replicase based RNA and DNA constructs have been used to deliver antigens of interest. These naked nucleic acid vaccines owe their enhanced immunogenicity not to increased antigen production, but to the requisite production of double stranded RNA species, which results in the quantitative induction of apoptosis and innate immunity (Leitner *et al*., 2000; Ying *et al*., 1999). Induced apoptosis is one of the mechanisms for the enhanced immunogenicity of replicase based DNA vaccines apart from the large amount of antigen production under its subgenomic promoter (Leitner *et al*., 2004).

Serological testing of wild dogs immunized with inactivated oil emulsion CDV vaccine using SNT (Cirone *et al*., 2004) showed antibody titer of 1:40 to 1:320. Van de Bildt *et al*., (2002) reported that neutralizing antibody levels less than 1:20 are not protective against CDV whereas titers more than 1: 20 may confer protection.We found that pSinCMV.cdvh induces very meagre neutralizing antibodies (titer 8) at 30 days of primary vaccination when given in dose of 2 µg. But a titer of 32 was found after booster immunization. When 4µg pSinCMV.cdvh was given, neutralizing antibody titer rose to 1: 128 at 30 days post primary immunization which remained constant even after 30 days post booster immunization. This showed that pSinCMV.cdvh is capable of inducing immunity at both the doses 2 µg and 4 µg but 2 µg dose needs a booster. Dogs exhibiting titers of CDV neutralizing antibodies of <1:100, 50% neutralizing dose (ND50) are considered to be susceptible and titers of maternal antibodies of >1:20 may interfere with vaccination success in pups (Appel *et al*., 1984; Chappius, 1995). The titer achieved after booster immunization with 4 µg dose is par 1:100 and this gives us confidence that our construct may induce protective antibodies.

Humoral immunity does not totally explain resistance to CD (Appel and Summers 1999; Greene *et al*., 2001). There is considerable evidence to the importance of the CMI in the clearance of Morbillivirus infections (Good and Zak 1956; vanBinnendijk *et al*., 1990). We measured the proliferative capacity of PBMCs which were sensitized with CDVH protein through immunization with pSinCMV.cdvh. The vaccinated groups show clear increase in stimulation index after stimulation with CDV over vector and healthy control. A dose of 2 µg with pSinCMV.cdvh triggers a stimulation index of 1.35 at 30 days of immunization. After booster immunization there is clear increase in SI to 1.76. With 4 µg dose of immunization, the SI obtained is much higher (2.54) than that obtained with 2 µg dose. The booster with 4 µg dose seems not relevant, as it fails to increase SI any further. This reflects that a 4µg dose is better than 2µg dose as it is capable of inducing immunity in single immunization itself and there seems no need of booster.

DNA vaccines are particularly useful in situation where cytotoxic T cell mediated immune responses are essential for protective immunity. It mimics the live replicating agents because of endogenous production of cell associated antigens. And their association with MHC class I molecules often results in CTL responses. Sometimes when live vaccines are contraindicated such as in immunologically compromised or suppressed hosts, DNA vaccines are particularly useful.

DNA vaccine selectively induces Th1 cells whereas protein vaccines induce Th2 or mixed Th1/Th2 responses (Patricia *et al*., 2000). In this study FACS data of ratio between CD4 and CD8 cells can still be utilized for determining the proportion of these cells with respect to each other. Replicase based vaccines have been reported to give a strong CD8+ T cell response in the effector phase (Leitner *et al*., 2003). At 30 days of immunization with either 2 µg or 4 µg of dose there is a clear decrease in the ratio of CD4 to CD8 cells over the vector alone immunized and healthy control groups. The 4µg dose seems to be better stimulator of CD8 positive cells than 2 µg immunization dose. After booster immunization in 2µg dose group we see that the CD4 to CD8 cells ratio has further gone down to 0.446, while with 4 µg does there is only a slight decrease from 0.877 to 0.854.We can

conclude that the absolute induction of CD8 cells is higher in 2 µg dose group after booster. However in single immunization, 4µg does is superior over 2µg dose in inducing more of CD8 cells than CD4 cells. A lower ratio of CD4 to CD8 cells in immunized shows that there is higher CMI induction in animals than humoral immunity. It has been reported that in peracute infection of CDV the humoral immunity against H protein is almost nil, while in persistently infected and convalescent dogs, humoral immune response against H protein starts appearing at the earliest of 2-3 weeks but still the titer is very less (Norbby *et al*., 1986). On vaccination with pSinCMV.cdvh, the $CD8^+$ cells as well as cytokines signatory to CMI are found to increase. The increase in the count of $CD8^+$ T cells is well supported by the simultaneous increase in IFN gamma measured by Real Time PCR.

We found that the increase in IFN gamma is higher in vector group than in vaccinated group with 2µg pSinCMV.cdvh. We suspect that 2µg dose is not adequate to boost immunity. The vector given in dose of 4 µg is perhaps inducing IFN gamma through dsRNA intermediate (Tanaka *et al*., 1998).Overall we find an increase in IFN gamma level. Under natural condition of infection in early distemper CNS lesions pro-inflamatory cytokines (IL6, IL8, IL12 and TNF alpha) have been found to increase while there is down regulation of anti-inflammatory cytokines (IL2 and IFN gamma) (Markus et al., 2002).Gamma interferon classically associated with cellular immunity, plays important role in antibody-mediated protection against infection in mice (Parent *et al*., 2006). At 30 days of immunization with 4 µg pSinCMV.cdvh we find IFN gamma expression is 1.78 times more than in healthy animals. IL2 is a growth factor for antigen stimulated T lymphocytes and is responsible for T cell clonal expansion after antigen recognition. IL2 is produced by CD4 T cells and in lesser amounts by CD8 T cells. In our study we have seen that increase in CD4 cells are relatively less in comparison to $CD8^+$ T cells. This finding is supported by Real Time PCR estimation of IL2 and IFN gamma molecules from the immunized animals. We see that IL4 expression is slightly less than IFN gamma. IL4 is the signature cytokine of the Th2 cells and hence enhances humoral arm of immunity. These are secreted from $CD4^+$ T cells.

We found that after primary vaccination, there is only minimal increase in CD4+ cells but the same has increased after booster vaccination. This is in confirmation with the findings of many other workers with H DNA (Feltquate *et al*., 1997) that after a single immunization with H DNA a booster immunization is needed to get high titre of antibodies.We found that after booster immunization $CD8^+$ cells have increased many folds than the respective increase in $CD4^+$ cells, which is indicative of tilt towards cell mediated immunity. Although, our study doesn't reflect whether this dose is protective or not but as has been found in the study of Paticia *et al*. (2000), despite of its limited ability to induce humoral immunity the DNA vaccine could protect against challenge. Our data suggests that the booster is needed perhaps to maintain the persistency of immunity. We found increase in IFN gamma level in vaccinated dogs, which is in consistency with the findings of Ljungberg *et al*. (2007) who found increase in the level of cytokines IFN gamma and IL2 after immunization with Venezuelan encephalitis virus replicon-based plasmid vaccine using 1µg dose intramuscular.

Although we kept the dose very minimal upto a maximum of 4µg, it is of special notice that our vector size is itself large being above 10 kb. Recent experiments utilizing unmethylated CpG motifs indicate that increased amounts of DNA not specifically coding for antigen may have a nonspecific immunostimulatory activity (Krieg *et al*., 1995). pSinCMV.cdvh being above 10 kb, may have some non specific immunostimulatory activity. We could not compare the relative increase in IFN gamma and IL 2/IL 5 response so as to have a better picture of replicase induced enhancement of immune response. CMV A promoters are favoured over regulated or endogenous eukaryotic promoters (Montgomery *et al.*, 1993, 1994). Sixt *et al*. (1998) have used plasmid vaccine against CDV which utilizes CMV promoter. In pSinCMV.cdvh two types of promoters are used. One is CMV promoter making the vaccine better over other vaccines.Using replicase based DNA vaccines such as ours pSinCMV.cdvh, there is an upburst of antigen within short period of time. This makes it a successful candidate vaccine against CDV even in the presence of high maternal antibody titer. Since, most intereference in recombinant DNA vaccine is from circulating antibodies acquired transplacentally or transcolostrally, the intramuscular route of injection overcomes maternal interference to much extent.The cells transfected with replicase based vaccine had undergone apoptosis while there was no such change in healthy control. This prompts us to expect that *in vivo* our vaccine might be inducing apoptosis which is expected from replicase based vaccines.

Reports are controversial over the mechanism of higher immune response of Sindbis virus based vaccines. Some says it is due to higher amount of antigen expressed from the very strong subgenomic promoter (Leitner *et al*., 2000) while, some reports say it is due to double stranded RNA intermediate induced activation of antiviral innate immunity (Derrick *et al*., 2005). We found a relatively high increase in the level of IFN gamma and $CD8^+$ T cells in immunized dogs which are in consistency with the findings of Derrick *et al.* (2005).

Acknowledgement

Authors thank Director, IVRI Izatnagar for providing facilities to carry out this work.

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