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A single dose of naked DNA expressing E2 protein is capable of providing full protection against CSFV

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Summary: Domestic pigs immunized with DNA vaccine expressing complete protective antigen E2 of Classical swine fever virus (CSFV) conferred total protection against a viral challenge. So, the present study was envisaged to evaluate in vivo immunogenecity of rplasmid called pVAX1.csfv.E2 to fulfill the clear need of efficient and cost effective vaccine against CSFV. Its *in vitro* expression was evaluated by IFAT and IPT revealed good results, provoked an anticipation of its better expression *in vivo.* Pigs of 8 weeks old age were immunized through IM route in order to evaluate the immune response on 28th day post vaccination induced by 100 µg and 200 µg dose of pVAX1.csfv.E2, followed by challenge with 10^5 ID50 of virulent CSFV resulting total protection. Comparative analysis of immune response among 100 µg and 200 µg dose of the said plasmid revealed similar protective neutralizing antibody titer (1:40), while control group showed lower (1:5) non protective SN titer. Stimulation Index (SI), mean % $CD4^+$ and $CD8^+$ level was found higher in both vaccinated groups as compare to healthy control animals with statistically insignificant difference among 100 µg and 200 µg dose of above plasmid, as an indication of T-cell response in the blood of vaccinated pigs suggested the role of cell mediated immunity in protection apart from neutralizing antibodies in serum. Therefore, this study demonstrates that DNA vaccination by single dose of 100 µg pVAX1.csfv.E2 can provide protective immunity 28 days post vaccination by intramuscular route.

Key words: Classical Swine Fever Virus, DNA vaccine, E 2 gene

Introduction

Classical swine fever (CSF), also known as hog cholera, is a highly contagious, multisystemic and hemorrhagic viral disease, included in the list of diseases notifiable to the OIE (www.oie.int), distributed almost world-wide and is considered the most economically important but vaccine-preventable disease of swine in areas of intensive pig farming. Natural host of classical swine fever virus are members of the family *Suidae*, which include domestic pigs and wild boars (Depner *et al*., 1995 ; Laddomada, 2000). A considerable problem is the survival CSF virus in wild pig population, which is considered to be the potential source of the infection.

CSFV is spherical shaped enveloped particlevof about 40-60nm in diameter with a single stranded RNA genome of aboutv 12.3kb with positive polarity, classified as *Pestivirus* with in the family *Flaviviridae*. Genomic RNA possess single ORF of more than 10, 0000 bases which encode about 4000 amino acids. Initially a polyprotein is formed which is further cleaved co-translationally and post-tanslationally by the host and viral genome encoded proteases to yield four structural (C protein, Erns, E1 and E2) and seven non structural proteins. E^{rns}, E1 and E2 are the viral envelope glycoprotein. After infection with CSFV, antibodies are raised against the structural glycoproteins E2 and E^{rns} and non structural protein NS3 (Paton *et al*., 1991; Terpstra, 1988). Antibodies against Erns and NS3 have only low neutralizing capacity or none at all (Rumenapf *et al*., 1991; Konig *et al*., 1995).

E2 glycoprotein contains most of the known humoral and cell mediated protective determinants of CSFV (Ceppi *et al*., 2005), highly immunogenic against which most of the neutralizing antibodies are induced and the only one capable of conferring protection against CSFV challenge (Rumenapf *et al*.,1991; Hulst *et al*., 1993). Thus E2 has become the main candidate for the

development of DNA vaccine against CSFV and therefore work was undertaken for evaluating the immune response against the recombinant DNA construct (pVAX1.csfv.E2) containing complete E2 gene of lapinised Indian vaccinec strain (Accession no.EU857642) in pVAX1 vector.

Materials and Methods

Construction of Recombinant plasmid (pVAX1.csfv.E2): A recombinant plasmid with full length E2 gene of classical swine fever virus (CSFV) isolated from Indian lapinised vaccine strain (Accession no. EU857642) was used. The size of E2 gene is approx. 1.2 kb along with ORF(1.092kb), signal sequence, Kozak translation initiation sequence and an initiation codon (ATG) for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990)at its 5'end, which is present between *EcoRV*and *XbaI* restriction enzyme sites in pVAX1 mammalian expression vector.

Large scale preparation of ultra pure plasmid DNA: The starter culture of *E.coli* cells harboring pVAX1.csfv.E2 was diluted with 1: 500 ratio into 6 litre of LB medium containing kanamycin (50 µg/ml) and grown overnight at 37o C in fermenter.Simultaneously, 1.2 litre of LB medium was equally dispensed into eight conical flasks of 500 ml capacity, added kanamycin @ 50 µg/ml and finally starter culture of *E.coli* cells possessing only pVAX1 vector was inoculated in 1:500 ratio under laminar hood. All these eight flasks were placed in orbital shaker at 170 rpm for 16-18 h.Later both, recombinant plasmid and vector were isolated separately in large quantity by using the Silica gel technique (Rai et al., 2009).

Antibody against pVAX1.csfv.E2: Primary polyclonal antibody against the CSFV E2 gene was raised in mouse by hyper immunization of five mice with pVAX1.csfv.E2 plasmid. 50 µg of plasmid DNA was injected intramuscularly in tibialis anterior region of hind leg (lateral region of thigh muscles) of each mouse once a week and repeated for four weeks consecutively. Mice were bled through inner canthus of eyes with a capillary and serum was prepared (Kumar, 2008).

Expression of pVAX1.csfv.E2 in BHK 21 cells: The 1 µg ultra pure plasmid DNA (***pVAX1.csfv.E2)*** was transfected to BHK-21 cells, obtained from National Centre for Cell Science (NCCS, Pune), using Lipfectamine^{TM} 2000 transfection Reagent (Invitrogen, USA following the manufacturer's instructions.As control for the assay, cell monolayers were transfected with empty vector (pVAX1). After 96 h of transfection, cells were fixed with 80% chilled acetone and expression of E2 was analyzed by Indirect fluorescent antibody test (IFAT) and Immunoperoxidase test (IPT). Cells were incubated with Mouse primary anti CSFV hyperimmune sera in1:50 dilution for 2 h.

For IFAT, after incubation, all wells were washed with 1X PBS and incubated with 1:50 dilution of FITC conjugated secondary goat anti-mouse antibodies (Banglore Genei). After 2 h of incubation at room temperature wells were washed with 1X PBS, mounted with 50% glycerol in PBS. The plate was examined under fluorescent microscope (Nikon) and photographed.

For IPT, HRPO conjugated secondary rabbit anti-mouse antibody (1:50 dilution) was added to wells and incubated for 1 h at room temperature. The cells were again washed with PBS thrice and incubated with 3, 3'-diaminobenzidine (DAB 1mg/ml in PBS with 1 µl/ml H2O2) for 5 min at room temperature. After the development of color, cells were washed with PBS, dried in air and observed under microscope and photographed.

Immune response studies

The *in vivo* experimental setup was designed with eight weeks old piglets, which were purchased from a local pig owner, where there is no tradition of vaccination and free from classical swine fever. One week before the start of immunization trial, all piglets were treated with antihelminthics (Albendazole). These piglets were then tested for seronegativity against classical swine fever.Piglets of subgroup A1 and A2 were injected intramuscularly with 100 µg and 200 µg r-plasmid (pVAX1.csfv.E2) respectively in nuclease free water, subgroup B1 and B2 were injected intramuscularly 100 µg and 200 µg vector alone(pVAX1) respectively also in nuclease free water, while group C were kept as healthy control.

Serum neutralization test (SNT) using neutralizing peroxidase- linked assay (NPLA): The neutralizing antibodies in the blood were measured through NPLA, modified by Terpstra *et al.*, (1984).

Lymphocyte proliferation assay: Whole blood was collected as per the method mentioned in section (2.5.2) in EDTA coated vial. 4 ml of lymphocyte separation medium (LSM 1077, PAA) was taken in a 15 ml conical tube. Equal volume of whole blood sample was carefully layered over the LSM. The tubes were centrifuged at 1800 rpm for 30 min at 4°C. Lymphocytes were collected from the plasma-LSM interface and washed twice with PBS at 1200 rpm for 10 min and finally the cell pellet was resuspended in 2 ml of RPMI-1640 growth medium (Sigma) without having phenol red and supplemented with 10% FCS. The cell count and viability was determined by Trypan-blue dye exclusion method (Freshney, 2006).The blastogenic response of lymphocytes was assessed by MTT colorimetry method as described by Mosmann (1983).

Estimation of CD4+ and CD8+ T cells by FACS: The whole blood was collected from each piglet in EDTA coated vial separately. 10 µl of mouse anti pig CD4 : FITC and 10 µl of mouse anti pig : RPE (AbD serotec) were mixed to the 100 µl of blood in dark room, mixed well and incubated at 37°C for 30 min. Then 1.4 ml of freshly prepared AbD serotec erythrolysis buffer was added to the mix, slightly vortexed and incubated at 37°C for 7 min in dark. After incubation, centrifugation was done at 2500 rpm for 5 min. Lysis buffer was decanted out and washed the cell pellet twice in PBS, before making final resuspension in 500 µl of PBS to get ready for acquiring data by flow cytometry (BD Biosciences). Stained cells were quantified in BD FACSCalibur by forward site scatter and FITC and RPE fluorescence. In each sample 10,000 cells were recorded. Forward site scatter gates were set on lymphocytes fraction to exclude dead cells.

CSFV challenge: All the pigs were challenged subcutaneously with 1 ml of 10^5 ID_{50} of challenge CSFV after collecting blood and sera on 28^{th} day post vaccination.

Body temperatures were monitored daily and healthy control piglets that were severely affected (assessed by high temperature, constipation followed by diarrhea, staggering gait, flushing of skin) were euthanized on 11^{th} day after challenge. Surviving piglets with or without mild clinical symptoms were sacrificed on 16^{th} day and then subjected to an exhaustive necropsy for evaluating pathological signs.

Results

Expression of recombinant plasmid: The expression of r-plasmid (pVAX1.csfv.E2) was checked by Indirect fluorescent antibody test (IFAT),revealed good amount of fluorescence as observed in BHK-21 cells transfected with r-plasmid pVAX1.csfv.E2 (Fig. 1), while there was no fluorescence in mock transfected cells (Fig. 2).

Similar expression determined by Immunoperoxidase test (IPT) in BHK-21 transfected cells revealed intense brown color staining as an indication of the expression of E2 protein (Fig. 3), while healthy cell control did not show any color change (Fig.4).

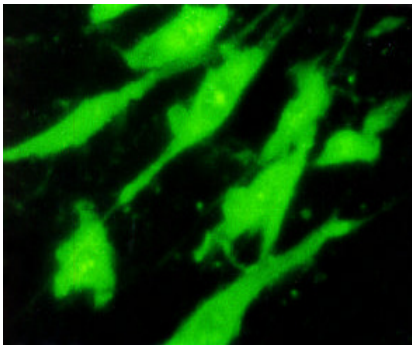

Fig. 1.BHK-21 cells transfected with recombinant plasmid pVAX1.csfv.E2 showing fluorescence in IFAT, X 400

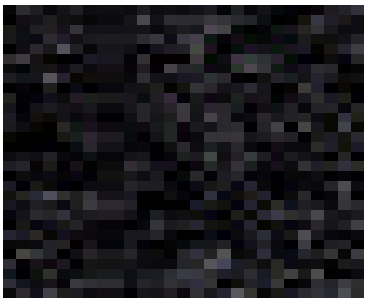

Fig.2. BHK-21 cells mock transfect showing no fluorescence in IFAT, X 400

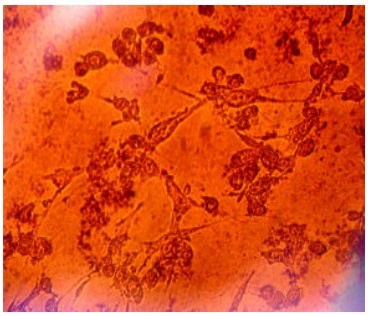

Fig.3. BHK-21 cells transfected with recombinant plasmid pVAX1.csfv.E2 showing intense brown color development in IPT, X 400

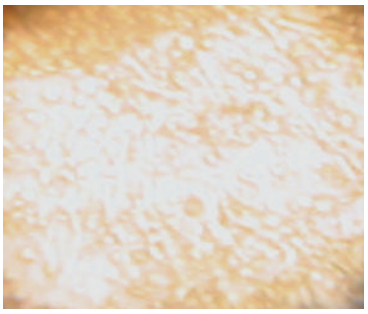

Fig.4. BHK-21 cells mock transfected showing no staining in IPT, X 400

Neutralizing peroxidase linked Serum neutralization test

Serum neutralization test through NPLA performed on serum of pigs on 28th day post immunization showed that level of neutralizing antibody titer was 40, when pVAX1.csfv.E2 was inoculated intramuscularly in group no. 1 and 2 in 100 µg and 200 µg respectively. The groups maintained as vector control and healthy control showed antibody titer less than five against CSFV (Table 1).

Table 1 : Neutralizing antibody titer of pigs on 28th day post immunization

Sl. No.	Groups		No. of piglets	Serum antibody titer (SN) on 28th day post immunization
1	Vaccinated pVAX1.csfv.E2	(100 µg)	3	40
2	Vaccinated pVAX1.csfv.E2	(200 µg)	3	40
3	Vector control pVAX1	(100 µg)	2	< 5
4	Vector control pVAX1	(200 µg)	2	< 5
5	Healthy control		2	< 5

* SN titer was calculated as reciprocal of highest dilution of serum able to neutralize 50% of CSFV in culture replicates.

Lymphoproliferative assay on PBMCs of immunized pigs: Pigs immunized with 100 µg and 200 µg of pVAX1.csfv.E2 shown stimulation index (SI) of 1.11 and 1.21 at 28 days of immunization respectively with cell culture adapted CSFV. Unstimulated samples from each group maintained maximum absorbance (A_{550}) near about same 0.7. The SI (with virus) showed an increasing trend from control groups to both vaccinated groups with significantly higher in group no.2. The results obtained are presented in the table 3.2and Fig.12.

Table 2 : Antigen specific response of lymphocytes at 28th day post immunization as assessed by MTT colorimetric assay

S. No.	Groups	Stimulation indices (Mean±SE)	
		With ConA	With virus
1	pVAX1.csfv.E2 (100 µg)	1.50±0.032[a]	1.11±.014[ab]
2	pVAX1.csfv.E2 (200 µg)	1.21±0.016[b]	1.21±0.073[a]
3	Vector control pVAX1 (100 µg)	1.09±0.036[b]	1.07±0.03[ab]
4	Vector control pVAX1 (200 µg)	1.19±0.035[b]	1.05±0.025[ab]
5	Healthy control	1.17±0.025[b]	1.02±0.040[b]

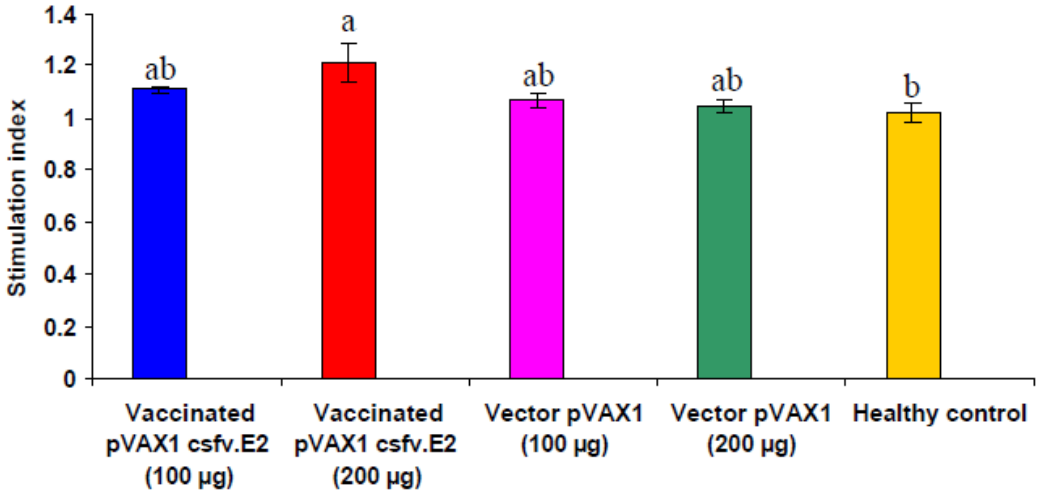

Fig. 5. ***In vitro*** **lymphoproliferative responses of pigs on 28th day post immunization. Each column represents mean±SE of animals in each group. Mean values with common superscript do not differ significantly at P<0.05 row wise**

CD4+ and CD8+ T-cells estimation by FACS: Mean % CD4+ level was found significantly higher in both vaccinated groups as compared to healthy control. Although statistically not significant, mean % CD8+ level showed a progressive trend from control groups to vaccinated groups and that was exhibited in reduction of the ratio of mean % CD4+ to CD8+ T cells, revealed a slight tilt of enhancement of immunity towards cellular side (Table 3.3, Fig. 6)

Table 3 : Flow cytometry analysis of $CD4^+$ and $CD8^+$T cells at 28 days post immunization

S. No	Groups	Total no. of piglets	Mean % $CD4^+$ ±SE	Mean % $CD8^+$ ±SE	Mean % $CD4^+$/ $CD8^+$ ±SE
1	pVAX1.csfv.E2 (100 µg)	3	24.18 ± 0.140[a]	18.83 ± 1.06[a]	1.284
2	pVAX1.csfv.E2 (200 µg)	3	25.02 ± 1.57[a]	20.30 ±2.84[a]	1.232
3	Vector control pVAX1 (100 µg)	2	18.62 ± 0.325[b]	15.84 ± 2.05[a]	1.175
4	Vector control pVAX1 (200 µg)	2	20.49 ± 2.27[b]	16.88 ± 1.40[a]	1.213
5	Healthy control	2	16.1 ± 1.37[b]	16.39 ± 1.08[a]	0.982

* Mean values with a common superscript donot differ significantly at P<0.05 row wise.

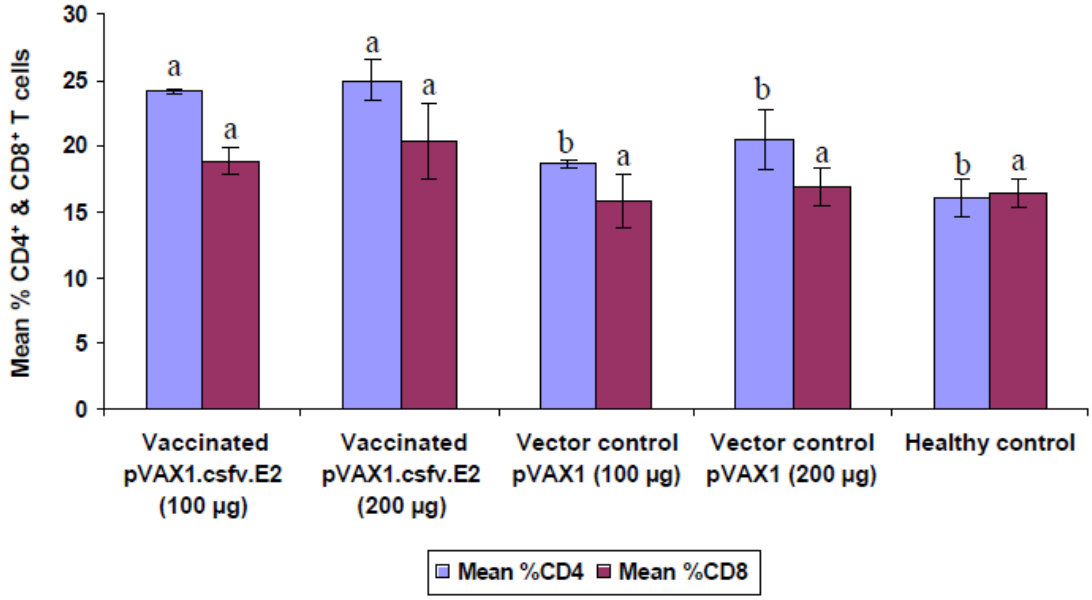

Fig 6. Mean % $CD4^+$ and % $CD8^+$ count comparison among different groups on 28th day post immunization. Bars and error bars represent mean values and standard error respectively. Mean values with common superscript do not differ significantly at P<0.05 Total protection against CSFV is attributed to the induction of both neutralizing antibodies and cell mediated response

Twenty eight days after immunization with pVAX1.csfv.E2, all six pigs belonging to group no.1 and 2 completely resisted the challenge of 10^5 ID_{50} virulent CSFV, as determined by the observation of clinical signs, necropsy findings. Pig no. 1 and 5 showed slight rise of temperature on 9th and 10^{th} day respectively (> 104°F with full recovery with in 24 hours)

without exhibiting other clinical symptoms as shown by pigs of control groups and no pathological lesions typical of classical swine fever were noticed in post mortem examination of animals of both the vaccinated group, on 16th day post challenge.

The six pigs of vector control and healthy control group showed severe symptoms of disease such as peak temperature on 7-8 days post challenge with a rise from 5-6th day, diarrhea followed by constipation and conjunctivitis was observed between 10-12 days post challenge. Two challenged pigs of healthy control group sacrificed on 11th day showed congestion in their mesenteric lymph nodes. Out of the four remaining animals of vector control group, two exhibited weaving gait from 14th day and one pig showed erythematous lesion on its limbs. All the four pigs of vector control group showed infarction at the margin of spleen and hemorrhagic lymph nodes typical of classical swine fever in necropsy findings on 16th day post challenge.

Table 4: Protection test of pigs challenged with 10 ID_{50} of CSFV

Sl. No.	Group	Piglet No.	Peak temperature (^{o}F)	Total no. of piglets	No. of piglets showing clinical symptoms	% protection
1.	pVAX1.csfv.E2 (100 µg)	1	105.2*	3	0	100
		2	103.8			
		3	103.3			
2.	pVAX1.csfv.E2 (200 µg)	4	103.8	3	0	100
		5	104.5*			
		6	103.9			
3.	Vector pVAX1 (100 µg)	7	104.8*	2	2	0
		8	105.9*			
4.	Vector pVAX1 (200 µg)	9	105.9*	2	2	0
		10	106.8*			
5.	Healthy control	11	107.3*	2	2	0
		12	106.9*			

*** Peak temperature was 104° F**

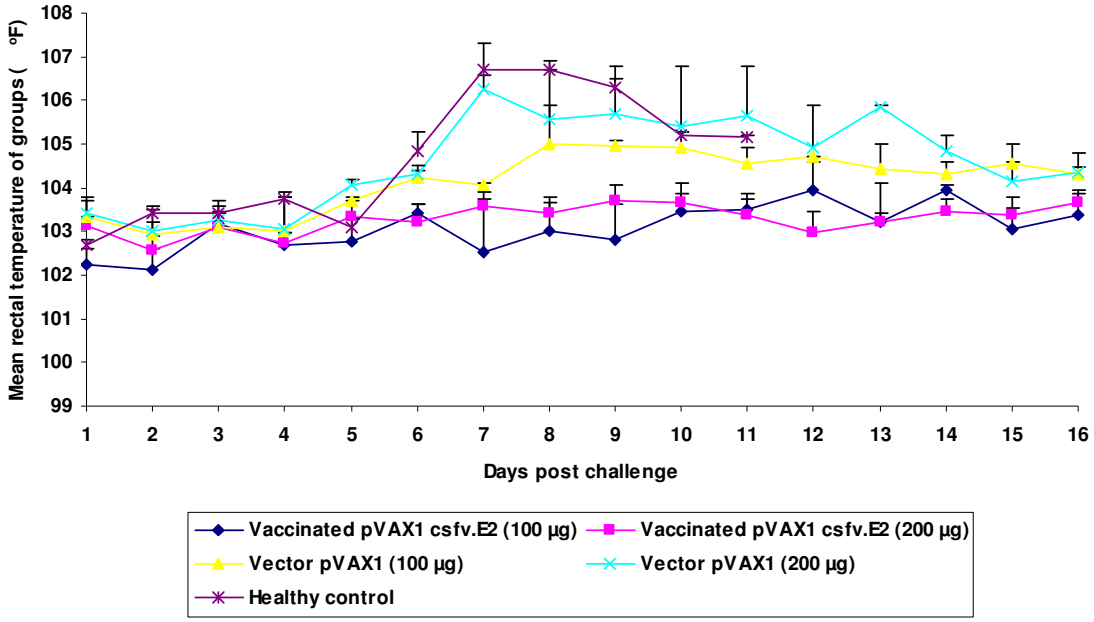

Fig. 7: **Daily recorded mean rectal temperature of pigs of different groups. Challenged with 100 ID_{50} of virulent $CSFV_0$ 28 days post-immunization. Control pigs were sacrificed on 11th day due to severe disease. The limit of body temperature is indicated by dotted line**

Discussion

. The control policy for CSF depends on the incidence and prevalence of the infection in the domestic and wild pig populations, respectively. In countries with CSF endemic in domestic pigs, it is common practice to vaccinate against the disease, there by avoiding serious losses. E2 is the major envelope glycoprotein (55 kDa) exposed on the outer surface of virion and represents an important target for induction of immune response during infection. Thus, E2 has become main candidate for the development of DNA vaccine against CSFV. So, the present study was undertaken to evaluate *in vivo* immunogenecity of rplasmid called pVAX1.csfv.E2 to fulfill the demand of efficient and cost effective vaccine against CSFV.

Confirmation of E2 gene expression by *in vitro* expression analysis is essential to use recombinant construct as DNA vaccine. The expression of pVax1.csfv.E2 was confirmed by transfection in BHK-21 cells. After 96 hours of transfection, the expression of E2 protein was successfully demonstrated by IFAT and IPT (**Fig 1-4**).

The immunological response induced by pVax1.csfv.E2 in two different doses of 100 µg and 200 µg respectively, was studied in pigs of 8 weeks old because Terpstra and Wensvoort (1987) drew attention to a phenomenon that maternal antibodies suppress CSFV infection or induction of neutralizing antibodies following vaccination before 7 weeks of age. The higher the maternal antibody titers at vaccination, the stronger the inhibition of the development of vaccinal immunity (Lai et al., 1980 ; Aynaud, 1988 : Vandeputte et al., 2001).

An easier but indirect method of estimating the efficacy of a vaccine could be measuring the titer of neutralizing antibodies in the blood (Terpstra and Kroese, 1996) as we measured it through NPLA, modified by Terpstra et al, (1984). The neutralising peroxidase linked assay (NPLA) is the gold standard for the detection of antibodies to CSF virus (Drew, OIE, 2008).

It has been demonstrated that neutralizing antibody titer below 1:32 using live attenuated vaccine 'C' strain, do not provide protection against lethal challenge of CSFV and from an epidemiological point of view was found inadequate (Terpstra and Wensvoort, 1988). The neutralizing antibody titer of vaccinated pigs with 100μg or 200μg of pVAX1.csfv.E2 remained at the same level (1: 40) from 28th day post vaccination till moment of challenge. This observation shows that of pVAX1.csfv.E2 is capable of inducing same degree of humoral immunity at both the doses of 100μg and 200μg. To determine, whether the vaccine is effective in protecting pigs against natural infection of CSFV, vaccination-challenge experiments are commonly carried out.

Pigs vaccinated with 100 μg or 200 μg of pVAX1.csfv.E2 showing SN

titer 1:40, were protected, while pigs of vector control and healthy control group showed SN titer <5, remained unprotected against challenge with CSFV as assessed by clinical symptoms and necropsy findings. It is consistent with previous findings by Bouma et al., (1999) who described a correlation between the presence of serum antibody titers and the protection against mortality after challenge. Similar findings have been reported by Terpstra and Wensvoort (1988) with live attenuated vaccine 'C' strain. After single immunization with 200 μg of DNA containing E2 gene of CSFV, Andrew et al., (2000) observed antibodies by week 3 and continued to rise over the next 10 days. We observed pre-challenge titer 1:40 in both the vaccinated groups on 28th day post immunization, that was found protective after challenge.

A single immunization with DNA can induce both antibody and CTL

response in several model systems. As a general rule animal developing neutralizing antibodies will be protected against subsequent challenge (Terpstra and Wensvoort,1988 : Suradhat *et al*., 2001), but protection after vaccination in the absence of neutralizing antibodies also has been observed (Ganges *et al*.,2005). Ganges *et al*. E2 expressing the complete E2 protein resulted in no detectable antibodies, yet conferred full protection against a severe viral challenge. When compared with Ganges's study, we found that in our study antibodies were induced by pVAX1.csfv.E2 on 28th day post vaccination may be attributed to low DNA dose (100 μg and 200 μg) in single immunization schedule, while on the other hand Ganges's inoculated higher DNA dose (400 μg at 14 days interval in 3 doses), detected antibodies 17 days post the last immunization which is shorter than those we analyzed (on 28thday post vaccination), different immunization/ challenge schedule as well as E2 genes were isolated from different strains in each case. CSFV E2 gene used in Ganges's study was isolated from Margarita strain present in pcDNA3.1 vector, while in this study E2 gene isolated from Indian lapinized vaccine strain (Accession no. EU857642) was present in pVAX1 vector which is specifically designed for DNA vaccine as approved by FDA and entrusted for better gene expression in mammalian cells with a minimum possibility of chromosomal integration.

The proliferative capacity of PBMCs which were already sensitized with CSFV E2 protein through immunization with pVAX1.csfv.E2 was measured and found that stimulation index (with CSFV) showed an increasing trend from control groups to vaccinated group 100 μg to 200 μg dose of the same plasmid, with significantly higher in group no.2 as compare to healthy control, which could be correlated with protection through cellular immunity. The flow cytometry permits counting of different subpopulations of lymphocyte CD4+ and CD8+ T-cells , which reveals the immune status of animal. Pig lymphocytes having some unusual properties such as CD4+ to CD8+ T-cell ratio is lower than other species. This ratio is slightly higher but less than one (<1) in young pigs. This ratio is found normally 0.6 in conventionally reared adult pigs, only occur in pathological conditions in humans (Lunney and Pescovitz, 1987). The mean % CD4+ level on 28th day post vaccination was found significantly higher, while mean % CD8+ level showed a progressive trend although statistically not significant, from control animals to vaccinated animals

with pVAX1.csfv.E2, 100 µg and 200 µg dose separately (Table 3.3). DNA vaccine increases CD8+ T-cell level and it was evident by the reduction in the ratio of mean % CD4+ to CD8+ T-cells, revealing tilt of enhancement of immunity towards cellular side. Higher S.I. was shown by the protected animals also suggesting an involvement of cellular immunity in protection

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