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SELF REPLICATING DNA VACCINE WITH POLYVALENT C-TERMINAL IMMUNE REACTIVE PROTEIN GENE OF FOOT AND MOUTH DISEASE VIRUS, CONSTRUCTED FOR HUMORAL RESPONSE, ELICITS A HIGHER IMMUNE RESPONSE IN GUINEA PIGS AND PROTECTS AGAINST CHALLENGE VIRUSES.

Pranaya Pradhan, Reddy G. R., Suryanarayana V. V. S*, Sarika Sasi and Dechamma, HJ

FMD Research Laboratory, Indian Veterinary Research Institute, Bengaluru, Karnataka-560024, India.

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***Corresponding Author**
Suryanarayana V. V. S
FMD Research laboratory, Indian Veterinary Research Institute, Bengaluru, Karnataka-560024, India.

ABSTRACT

Foot and mouth disease of cloven footed animals is of great concern to the world because, of its devastating effect in the livestock industry. Regular mass vaccination with the conventional inactivated virus is a pragmatic approach to control FMD in endemic countries. However these vaccines have several drawbacks which include short duration of immune response, possible risk of virus dissemination during vaccine preparation and the vaccine is ineffective against carrier animals. In view of these several newer approaches are under investigation to develop alternative vaccines. One of this is the self replicating DNA/RNA vaccine using alpha virus replicase gene to augment the expression of the cloned immunogen and to elicit γ- interferon response. However the earlier work, based on this approach showed little success because of the lack of efficient presentation of the expressed antigen. Here we report the production of Self Replicating DNA/RNA vaccine that can elicit both humoral and cellular response in the vaccinated animals. Multiple epitope gene (990 bp) corresponding to the C-terminal major immunogenic region of VP1 of 3 serotypes (Indian vaccine strains), O, A, and Asia 1, linked in contiguous was cloned into the vector and the expression of the same was confirmed in BHK cell monolayers. The vaccine was evaluated in guinea pigs both for neutralizing anti-body response and protection against challenge with the three serotypes and found to be more efficacious. This approach, which is first of its kind may pave the way for development of potential vaccines against FMD.

KEYWORDS: DNA vaccine, Foot and Mouth Disease (FMD), Sindbis virus Replicase, Immunization, self replication.

1. INTRODUCTION

Foot and Mouth Disease (FMD) is one of the most feared diseases of the livestock and it is of great concern to the world due to high economic impact. FMD outbreaks do occur both in domestic and wild cloven-hoof animals. The productivity losses include reduction in milk yield and draught power in adult animals and death in young animals due to myocarditis. FMD is endemic in parts of Asia, Africa, the Middle East and South America (Parida S et al., 2006). In endemic countries, vaccination is the only approach to control FMD. The identified vaccine strain is propagated in BHK-21 cell line, concentrated and chemically inactivated virus is used as a vaccine. Although the inactivated virus vaccine has been shown to be effective, there are reports of disease outbreak in vaccinated animals due to the incomplete inactivation of virus (Rivas AL et al., 2006). Moreover large-scale production of virus for vaccine preparation in fermenters sometimes may result in the escape of the live virus from production laboratories (Taboga O et al., 1997; Rodriguez LL and Grubman MJ, 2009). Besides, safety concern conventional cell culture vaccines have other drawbacks of low thermal stability and need for expensive inputs. These drawbacks prompted the researchers to develop alternative vaccines using antigens expressed in various host systems like E. Coli (Suryanarayana VVS et al., 1999), yeast [Balamurugan V et al., 2003; Balamurugan V et al., 2007), plant (Rice J et al., 2005; Sunil Kumar GB et al., 2007) or naked DNA constructs [Bae JY et al., 2009). However these approaches were not successful either due to induction of low levels of cytokine response needed for virus clearance [Parida S et al., 2006; McVicar JW et al., 1976] or poor antibody response needed for virus neutralization (Goodbourn S et al., 2000; Brown F, 1995; JoAnn CCB et al., 1997; Pfaff E et al., 1982). Hence the attention has been directed to develop vaccines that elicit both these immunological responses which may be achieved by using high antigen payload and/or directing the delivered vaccines to replicate like an attenuated virus in the host. This may be achieved by inserting the genes in viral vectors or under the control of virus replicase gene. Best examples of the vaccines that have been successfully tried are Adenovirus and Sindbis virus vector based vaccines (Hariharan MJ et al., 1998; Saxena S et al., 2008; Ying H et al., 1999; Xiong C et al., 1989; Herweijer H et al., 1995). However the environmental safety and inactivation of the replicon by the antibodies against vector backbone are major concerns in the case of viral vector vaccines. Hence the replicase based vaccine may be safe for routine application. Though the initial

work with Alpha virus replicase based self-replicating RNA vaccines for FMD was not successful (Nagarajan G, 2004), still there is a scope for further development to emerge as viable alternatives to conventional vaccines.

Foot and Mouth virus (FMDV) is an RNA virus which belongs to the genus Aphthovirus of the family Piconaviridae (Rodrigo MJ and Dopazo J, 1995). The virus has positive sense single stranded RNA genome encapsulated by four structural proteins, VP1-VP4 encoded by P1 poly-protein gene (Domingo E et al., 2002). Among the four capsid proteins VP1 has been shown to be the major immunogen that elicit a neutralizing antibody response in vaccinated animals (Makoff AJ et al., 1982). However the animals vaccinated with VP1 alone could not withstand the challenge virus indicating the response was not robust and for protection both humoral and cellular responses were necessary (Balamurugan V et al., 2005; Wong H et al., 2000). Moreover the studies have shown that the amount of subunit protein needed for achieving measurable quantity of humoral response was very high as compared to the whole virus. Self-replicating DNA vaccines are designed using Sindbis polymerase gene to drive the expression of antigen specific genes for high antigen production (Hariharan MJ et al., 1998). However most of the replicase based vectors reported to date are efficient in eliciting cellular response (Saxena S et al 2008). Here we report the studies on the immune response in guinea pigs of a self-replicating DNA vaccine construct with the polyvalent immunogen gene of FMDV developed using a vector that has been designed in our laboratory for humoral response.

2. MATERIAL AND METHODS

2.1 Construction of Self Replicating vaccine for humoral response.

Multiple epitope gene (MEG) constructs carrying nucleotide sequences of C-terminal half of the FMDV-1D gene (330×3) of three Indian vaccine strains namely ‘O’R2/75 (Madras), ‘A’Mukteswar, and ‘Asia1’IND 63/72, linked contiguously is available in the FMD Research Laboratory [Nagarajan G et al., 2008; Nagarajan G, 2004]. Construction of Sindbis virus polymerase gene-based Self Replicating DNA vaccine vector for the humoral response (pVac self-rep) that helps in secreting and anchoring of the expressed immunogen using pVac backbone has been described elsewhere (Patent filed for the vector pVac Self Rep; Reference no. Regd. 160/DEL/2011). In the MCS at Mlu 1 site of the vector, the linked MEG was introduced, and the final vaccine construct has been designated as pVac Self Rep 990 (Fig.1).

2.2Functionality studies on the vaccine construct

In order to test the functionality of the vaccine construct, the plasmid DNA was transfected to BHK-21 cells using lipofectamineTM2000 (Invitrogen, USA) as per the Manufacturer's protocol. Briefly, BHK 21 cells ($0.5x10^5$/ml) were seeded in 6 well cell culture cluster (Nunc, USA) in the presence of 10% FBS and antibiotics and allowed to grow to 90% confluence in an incubator with 5% CO_2. Pre incubated DNA (5µg) and LipofectamineTM2000 (12.5 µl) was used for transfection to the washed monolayer cells. After 4-6 hour of incubation in CO_2 incubator complexes was removed and fresh DMEM/F12 Ham maintenance medium containing 2% serum added and incubated further at 37^0C with 5% CO_2 for 24hr.The cells were lysed by repeated freeze-thaw cycles, the lysate was centrifuged at 5000g for 10 min and the supernatant was collected. The proteins (200 µg) in the supernatants were analyzed by 12.5% sodium dodecylsulphate-poly acrylamide gel electrophoresis (SDS-PAGE) and Western blotting as per (Towbin H et al., 1979). Presence of FMDV specific proteins in the lysate supernatants was detected by Western Blot analysis as per standard procedures with modifications. The separated proteins from the gel were transferred onto a nitrocellulose (NC) membrane using TransBlot-SD electrophoretic transfer cell (Bio-Rad) according to Manufacturer's instructions. Transfer of proteins was done at 15V for 45 min and at the end of the transfer, the membrane was washed once with PBST and blocked with PBST containing 3% defatted milk powder (blocking buffer) at 37^0C for 1hr to saturate the leftover sites. The presence of virus specific proteins was detected with anti FMDV (A22) guinea pig serum, rabbit anti-guinea pig HRPO conjugate (Bangalore Genei) and ortho-dianisidine-dihydrochloride (ODD) substrate (Bayry J et al., 1999).

2.3Immunological Evaluation of vaccine constructs in Guinea Pigs

2.3.1 Dose optimization.

The optimal guinea pig dose of pVac self rep DNA vaccine needed for immune response was fixed by conducting initial dose response studies. Adult guinea pigs (25 no) of both sexes with average body weight of 400 to 500 g were divided into 5 groups of 5 animals each. The animals were injected intradermal with DNA vaccine constructs at the back of the earlobe with 10ng, 100ng, 1µg and 10µg/dose. The animals were bled on day 28 post vaccination (dpv), sera were separated, heat inactivated at 56^0C for 30min and stored at -20^0C for ELISA and serum neutralization titration.

2.3.1.1 Detection of antibody response by ELISA

ELISA for antibody detection was performed as per the method of (Abu Elzein, E.M.E., and Crowther JR, 1978] with modification. Ninety-six well flat bottom immunoplates (F96 MAXISORP, Nunc USA) were coated with convalescent cattle serum against FMDV 'O' at a predetermined dilution of 1:2000 in carbonate-bicarbonate buffer pH 9.0 (coating buffer) in an incubator at 37^0C under humid condition. The plates were washed with PBST and the leftover sites were blocked with 100 µl/well blocking buffer (PBST containing 5% defatted milk powder) at 37^0C in an incubator for one hour. Affinity purified E. coli expressed polyvalent protein carrying the amino acid sequences corresponding to C-terminal half of VP1 of the three Indian vaccine viruses was added to the wells (0.1µg/well in blocking buffer) and incubated. After washing the wells were charged with serial doubling dilutions of sera (1:20 to 1:160 in blocking 37^0C) from vaccinated guinea-pigs and incubated. Later the plates were washed, loaded with 37^0C of diluted (1:30,000in blocking 37^0C) anti-guinea pig goat antibody HRP conjugate (Sigma, USA)and incubated at 37^0C for 1 hour. The unbound conjugate was washed, 100 ul/well of ortho-phenylenediaminodihydrochloride (OPD) (0.02% in acetate buffer containing 0.4% H_2O_2) was added and incubated at room temperature for colour development. The reaction was stopped after 20 min with the addition (100 ul/well) of1M H2SO4. A492 of the colour development was measured in an ELISA reader.

2.3.1.2 Serum Neutralization Test (SNT) (*α*-SNT method)

Heat inactivated test sera (50µl) of guinea pigs vaccinated with different doses of DNA were added to 50µl Dulbecco's MEM in duplicate wells of 96-Well Flat Bottom Tissue Culture Treated-Polystyrene plates (Nunc USA). Pre titrated FMDV 'O' diluted in Dulbecco's MEM to contain 10, 100 and 1000 TCID50 per 50 ul was added to the wells.

The plates were gently agitated to mix the contents. Appropriate controls like test serum, cell control and virus control were included in the assay. The plates were incubated at 370C in a humidified chamber with 5% CO_2 tension for 1 hour. Then 50 ul of cell suspension was added to each well, the plates were agitated gently for even distribution of the cells and returned to the incubator for 48 hr. Later the plates were observed under a microscope and the highest virus concentration at which the serum showed the inhibition of the virus growth by 50% was observed and the neutralization index was calculated as per standard method.

2.3.2 Vaccine efficacy studies in guinea pigs

2.3.2.1 Vaccination of guinea pigs with different vaccine

After confirming the optimal dose needed for a guinea pig vaccine trial was conducted to study the efficacy. Sixty four guinea pigs of both the sexes with average body weight of 250 g were divided into 4 groups of 16 animals each. The animal groups were identified by the vaccine constructs i.e. pVac Self Rep 990 (Group I), pVac 990 (Group II), Conventional oil adjuvant vaccine (Group III) and pVac Self Rep vector control (Group IV). In the case of pVac Self Rep 990 (Group I) and pVac 990 (Group II), and pVac Self vector (Group III) the animals was injected intradermally in the back of the ear with 0.125 umol/dose (1 µg of Self Rep construct in 50ul PBS) of endotoxin free DNA constructs, whereas Group IV animals received 250 µl/dose (1/10th of bovine dose) of conventional polyvalent oil adjuvanted vaccine through intramuscular route. Booster injections were given 21 days after the first injection with the same concentrations of the vaccines.

2.3.2.2 Evaluation of humoral response in vaccinated guinea pigs by ELISA and SNT

The animals were bled on 0, 14, 28 and 42 dpv, the sera were collected and tested for humoral antibody response by sandwich ELISA against 'O' as described earlier. Further the sera were also evaluated for neutralizing antibody titres against FMDV 'O' using 100 TCID50 viruses as per standard protocol (Reed LJ and Muench HA, 1938).

2.3.2.3 Protective response of the polyvalent vaccine against the challenge viruses

On 48 dpv the vaccinated guinea pigs from each group (12 from each group) were divided into 3 sub-groups of 4 each. The animals in the sub-groups were challenged separately with 100GPID50of FMDV serotype 'O' 'Asia 1' and 'A' by injecting a live virus into the tunnels made in the plantar region of the footpad. Development of the lesions was recorded at every 24hour consecutively for four days. Absence of secondary lesions (lesions on un-injected footpad) was considered as protection.

3. RESULTS

3.1 Expression of the cloned poly-protein gene in BHK 21 Cells.

Expression of the MEG in the vaccine constructs pVac Self Rep 990 and pVac990 under the control of eEF1 promoter was studied in BHK21 cells after transfection. Because, these vectors have a secretary and anchoring signals, the expressed antigenic proteins are expected to be secreted out and anchored in the cell membrane. The proteins in the lysate supernatant were subjected for SDS PAGE (Fig. 2A) followed by immune-detection (Fig.2B) using anti

FMDV A22serumand conjugate. As shown in Fig 2A several protein bands of various sizes were visible in poly-acrylamide gels which are of equal intensity in all the lanes corresponding to cell control, lysate proteins of pVac 990 and pVac Self Rep 990 transfected cells (Fig.2A, lane 1,2and3 respectively), however, when the proteins were immune-detected, bands around 12 kDa could be seen in the lysates of BHK cells transfected with pVac-990 and pVac Self Rep 990 at 24 her (lane 2&3 respectively) but not in the case of cell control (lane 1), indicating the cloned gene was expressed in the transfected cells.

3.2 Immune response studies in guinea pigs

3.2.1 The optimum dose needed for guinea pig vaccination

3.2.1.1 Detection of dose-wise antibody response by ELISA

Since, alpha virus polymerase based self-replicating DNA vectors cause apoptosis and may lead to immune suppression, we felt it was necessary to fix the optimum dose before conducting vaccine efficacy studies in experimental animals. The sera from pVac Self Rep 990 DNA-vaccinated guinea pigs were evaluated by sandwich ELISA using E. coli expressed multiple epitope protein as antigen which was captured by the antibodies in convalescent serum. As shown in the Fig.3, the sera from animals vaccinated with a 1µg dose of pVac Self Rep 990 have higher absorbance values as compared to other doses. The average A492nmvalue in the ELISA of 4 guinea pigs of this group was around 0.18 at 1:20. Though the animals vaccinated with 10ug dose, also showed an ELISA reading of 0.18, upon further dilution (1:40 and 1:80) there was a decrease in the A492nm values in this case indicating the animals vaccinated with 1.0µg of pVac Self Rep 990 have high antibody titres as compared to 10µg, 100ng and 10 ng dose.

3.2.1.2 Detection of dose-wise virus neutralizing antibody response (Alpha method of SNT)

As shown in the Fig.4, the highest virus concentration at which 50% of the monolayers were protected by the sera from vaccinated guinea pigs were studied by alpha method and it was 3.0 (log TCID50) in the case of animals vaccinated with 100 ng and 1µg/dose. However the neutralization efficiency was 2.5 and 2.0 (log TCID50) in the case of animals vaccinated with 10 ng and 10 µg/dose respectively, confirming the optimum dose for guinea pig, as per virus neutralization titres, is 100 ng to 1.0µg. Based on the results of SNT and ELISA the vaccine dose fixed for guinea pig protection studies was 1.0µg.

3.2.2 Self Rep DNA- vaccine efficacy studies in guinea pigs

3.2.2.1 Humoral antibody titres in vaccinated guinea pigs

Guinea pigs (in 4 groups) were vaccinated with various vaccines and antibody responses were studied by ELISA (Fig. 5) and SNT (Fig. 6). As shown in the Fig. 6, the antibody titres in the case of animals vaccinated with pVac990 were 2.00, 1.4 at 14th and 28thdpv respectively, while in the case of pVac Self Rep 990 the titres were 2.1 and 1.6respectively. The oil adjuvanted vaccine group showed antibody titers of 0.9 and 1.4 respectively. This indicates that the antibody response against pVac Self Rep990 was higher when compared to conventional vaccines. However, after the booster injection, the ELISA titres came down in the case of self- replicating vaccine and pVac 990 vaccine while, the titres have gone up in the case of conventional vaccine group. However, in the case of pVac the booster injection resulted in an increase in the titre at 42 dpv (1.95). The titers of oil adjuvanted conventional vaccine and pVac self rep 990 were similar ie 1.62 at 42 dpv which shows that booster injection has a negative effect in the case of Self Rep DNA vaccines. Interestingly quick and high immune response was noticed even at 14 dpv in the case of pVac and pVac Self Rep vaccines.

The serum neutralization assay of the 35 dpv sera with 100 TCID50 of 'O' virus, which was conducted before the challenge, showed the average titres ranged 1.5 and 1.8 in the case of pVac 990 and pVac Self Rep 990 respectively while it was 1.5 in the case of conventional vaccine.

3.2.2.2 Protection in vaccinated guinea pigs against challenge viruses.

As seen in the table the guinea pigs vaccinated with self-replicating DNA vaccine showed high protective response at 42 dpv against Asia 1 (4/4) and A (4/4) as compared to 2/4 and 0/4 respectively with DNA vaccine prepared with pVac vector backbone carrying eIF promoter and secretory signal. However, the response against 'O'was better in the case of pVac990 (3/3) as compared to pVac Self Rep990 which was 50% (2/4) in this case. There was no protection when vector alone was used. The conventional polyvalent vaccine showed 50% protection in the case of 'O' and 75% protection in the case of 'A' and Asia 1 under the conditions used.

Table. Protective immune response of pVac Self Rep 990 vaccine against challenge viruses in guinea pigs

Vaccinated Groups	Secondary Lesions		
	'O' Virus	Asia I Virus.	A_{22} Virus
pVac990	-	+	+
	-	+	+
	-	-	+
	-	-	+
Animals Protected	4/4	2/4	0/4
pVac Self Rep990	-	-	-
	+	-	-
	+	-	-
	-	-	-
Animals Protected	2/4	4/4	4/4
pVac Self Rep Vector	+	+	+
	+	+	+
	+	+	+
	+	-	+
Animals Protected	0/4	1/4	0/4
Conventional Vaccine	-	+	+
	+	-	-
	+	-	-
	-	-	-
Animals Protected	2/4	3/4	3/4
Control	+	+	+
	+	+	+
	+	+	+
	+	+	+
Animals Protected	0/4	0/4	0/4

Presence (+) or absence (-) of secondary lesions in individual guinea pigs after challenging with 100 $GPID_{50}$ of guinea pig adopted FMDV O, A, or Asia 1.

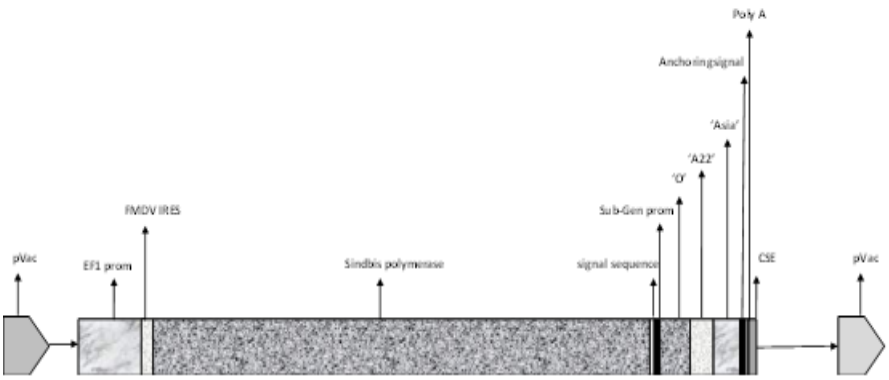

Fig. 1 Schematic representation of the pVac Self Rep990 vaccine constructs (not as per scale) The Vaccine constructs of 13051bp was made using pVac vectors (In Vivogen) as

a backbone. The Rh eEF1 promoter was used to drive the expression of the cloned genes. FMDV IRES replaces the IRES of the Sindbis virus replicase gene (7.5 kbp). The sub-genomic promoter which is recognized for RNA amplification is upstream of the IL2 secretory signal. MEG and PLAP-anchoring signal were inserted downstream of secretory signal. Conserved Sequence Element (CSE) was inserted downstream of Poly (A).

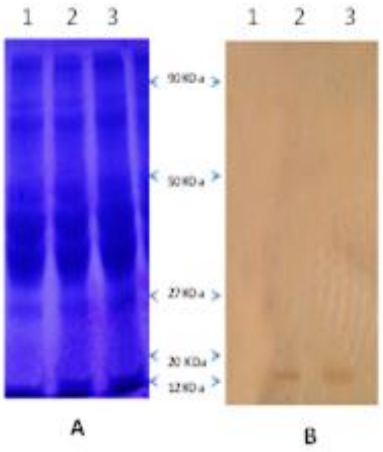

Fig. 2. Detection of expressed proteins in pVac Self rep 990 transfectd BHK 21 cells by SDS PAGE and immunoblot analysis.

A. lane1: cell control; **lane2, 3:** lysate proteins from pVac 990 and pVac Self Rep 990 transfected cells respectively. **B**. Immunoblot of the transferred proteins as above.

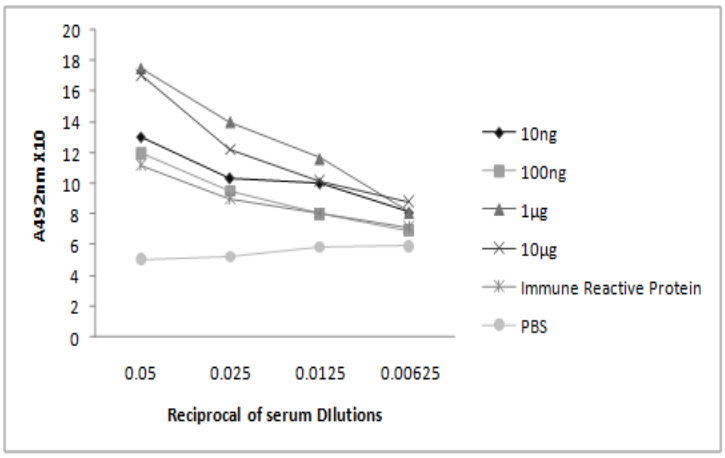

Fig.3: Dose response studies for humoral antibodies in guinea pigs vaccinated with different concentrations of pVac Self Rep 990 vaccine construct

The antibody levels in the sera of vaccinated guinea pigs were studied by Sandwich ELISA. The graph shows the average A492nm of the colorful reactions vs reciprocal of the dilutions

of sera at various dilutions from guinea pigs vaccinated with DNA vaccines. At 10 ng (♦), 100 ng (■), 1µg (▲) and 10µg (×) of Self rep 990 DNA respectively. Immune Reactive protein (□) and mock vaccinated with PBS (●).

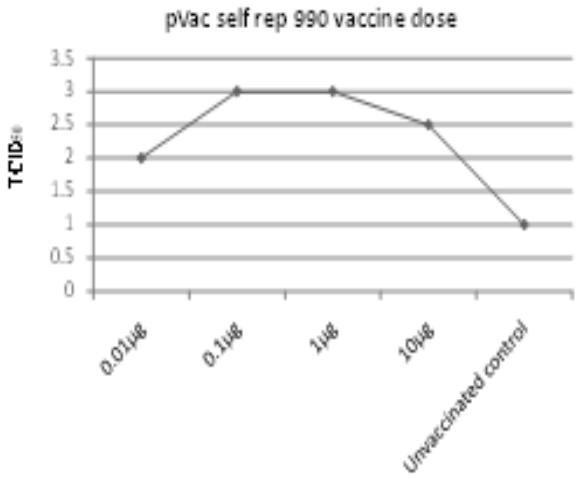

Fig. 4. Virus Neutralizing antibody response in guinea pigs vaccinated with different concentrations of pVac Selfrep 990 vaccine construct.

The average values of the virus dilution (log TCID 50) at which the sera showed 50% CPE in BHK cell molnolayers were plotted against the concentration of the vaccine used for injecting guinea pigs.

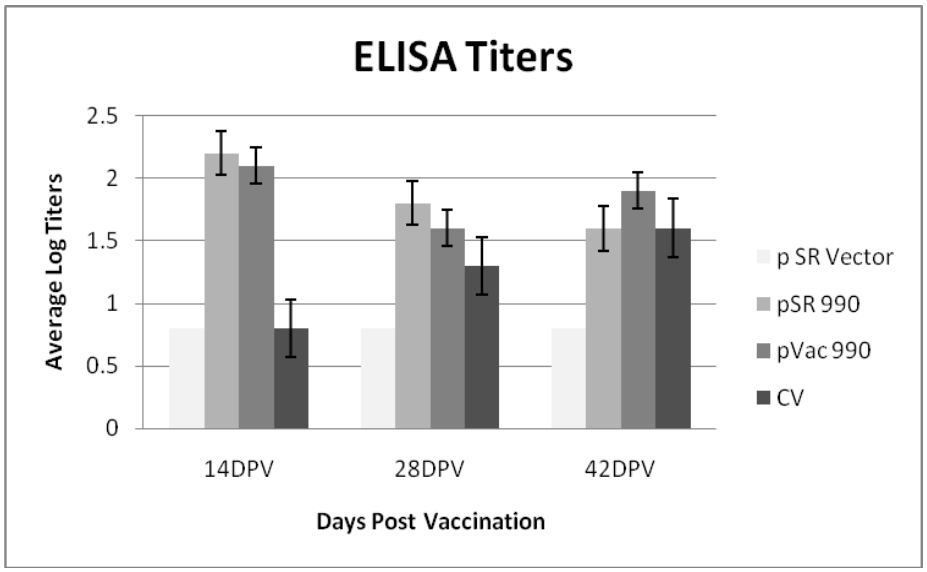

Fig. 5 Humoral antibody titres against 'O' in Pvac Selfrep 990 DNA-vaccinated guinea pigs at different time points as detected by double sandwich ELISA.

Antibody response in sera from vaccinated guinea pigs were evaluated by ELISA using *E. coli* expressed polyvalent immunoreactive protein as antigen. The booster injection was given

at 21 dpv. The average of the log of the highest serum dilution of guinea pigs in each group at which the A492 nm were higher than unvaccinated controls were taken as titres (shown as an insert).

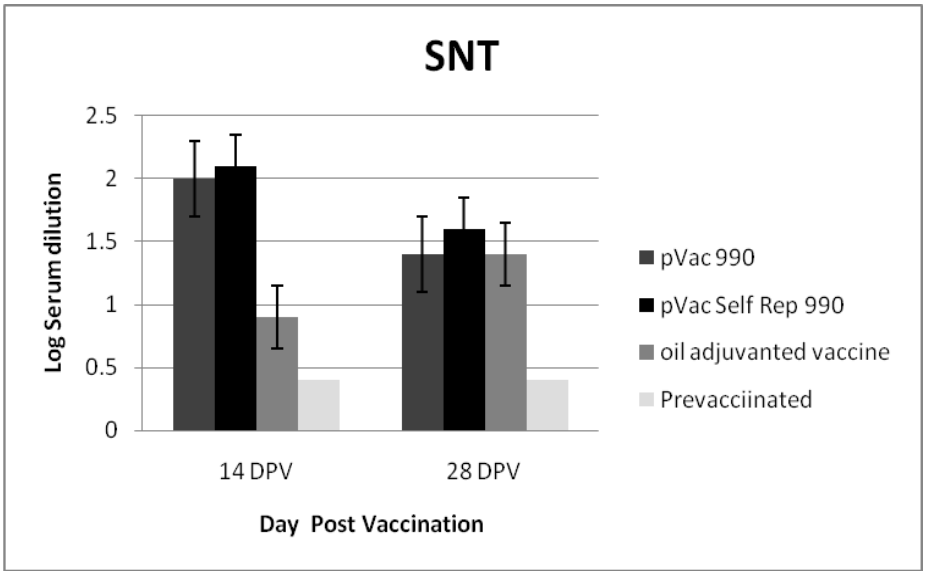

Fig.6. Serum neutralizing antibody response against FMDV 'O' in vaccinated guinea pigs.

The serum collected at day 14 and 28 dpv from different groups of vaccinated guinea-pig were subjected for serum neutralization assay against FMDV 'O' using 100 TCID50 virus. The log 10 serum dilution at which 50% of the monolayers did not show CPE was taken as the titre. The average of the titres was plotted against the dpv in vaccinated guinea pig group (shown as an insert)

4. DISCUSSION

Conventional cell culture based vaccines, though potent, have several limitations like poor thermal stability, risk of handling live virus for vaccine preparation and high cost of the vaccine. Therefore the production of alternate potent molecular vaccines that can replace the conventional inactivated virus vaccines has been identified as the present-day requirement to control FMD in endemic countries. Both protein and DNA based vaccines have been tried, however, they could not prove their worth as alternatives to conventional formulations, because, either they fail to elicit desired qualitatively good immune response due to a poor presentation of the epitopes, as in the case of sub-units or fail to elicit desired levels of humoral response, though the cellular responses are good, as in the case of DNA vaccines. The extensive research conducted on FMD Vaccinology clearly support the necessity of

having high neutralizing antibody along with cell mediated immune response in the vaccinated animals for protection and long duration of immunity, the latter being helpful in viral clearance in the case of carrier animals.

Inactivated virus vaccine elicits high neutralizing antibody response and protects vaccinated animals against challenge virus though short lived. Also the studies showed (Cox SJ et al., 2010) that high antigen payload is directly proportional to duration of immunity. Hence it is imperative that for a better protective immune response, even in the case of molecular vaccines, the vaccine constructs should facilitate the high antigen expression in the host cell and the expressed antigen should be available for the antigen

presenting cells outside. This may be achieved by getting the antigen over-expressed and the expressed protein is secreted and anchored in the membrane so that the macrophages can see and process the protein for antibody response. We constructed self replicating DNA/RNA vaccine with necessary signals that allow the antigen to be secreted out and anchored on the surface.

4.1 Functionality of the constructed vaccine.

Transcription of the Sindbis virus-polymerase inside the cell was achieved by cloning the gene under the eukaryotic elongation factor promoter (eEIF) and for efficient translation of the polymerase transcript, the 5'UTR of the gene was replaced with the FMDV IRES. The sub-genomic promoter was inserted downstream following which the initiation codon and multiple cloning sites (MCS) were included. The secretory signal and anchoring signals of the original pVac vector were retained. FMDV C-terminal half of VP1 of serotype O, A and Asia 1 were linked in frame (Nagarajan G, 2004) and inserted in the vector at Mlu 1 site. The presence of the cloned fragments in the vector was confirmed by sequence analysis. This construct was made to achieve expression of the cloned FMDV genes as a fusion of N-terminal Interleukin-2 (IL2) secretory signal and C-terminal Placental Alkaline Phosphatase (PLA) anchoring signal in injected cells as these are present upstream and downstream of the polymerase gene respectively under the control of sub-genomic promoter. The high antigen expression is expected to cause apoptosis to the cells and this has been observed in vitro. In this construct, in the junctions of the three linked FMDV genes there are 14 aa 2A sequence which may cause the ribosomal skip (Funston GM et al., 2008)resulting in the secretion of 12 kDaimmune-reactive proteins of FMDV 'O' and 'A' while the protein of 'Asia 1' may still remain anchored in the membrane. Hence in the immunoblot assay the supernatant showed

12 kDa protein which confirms the expression and processing of FMDV proteins. Thus the vector acts as a self replicating DNA/RNA vaccine that facilitates high level expression of cloned genes besides permitting the secretion of the expressed protein. To improve the efficiency of translation of the polymerase specific mRNA we have replaced 5 UTR of Sindbis virus polymerse gene with FMDV IRES. However, we have not studied the efficiency of polymerase production in the transfected cells. The expression of cloned antigen genes under sub-genomic promoter was confirmed by SDS PAGE and Western blotting. We have also confirmed the presence of anchored immune-reactive protein specifically for Asia 1 by immune fluorescence assay using antiserum against recombinant immune-reactive protein (Fig. Not shown). Thus the function of the vector was confirmed. High level of expression and dsRNA production is expected to cause apoptosis in the transfected cells and which was observed.

4.2 Immune responses of the vaccine in guinea pigs.

It is expected that the delivery of high concentration of antigen may lead to immune-suppression. In the case of self-replicating DNA constructs, as reported (Nagarajan G et al., 2011) over expression of the antigen in the cell causes lysis of cells and the resulting effect may have a negative effect on immune response. This may be the reason, in several reports; the researchers have shown poor immune response of the self- replicating DNA vaccine as compared to conventional vaccines (Nagarajan G et al., 2008). In most cases DNA constructs have been used at very high concentration without checking the dose response, which as per our observations, may not show the true picture of efficacy as there may be suppression of immune response at very high concentration. In the present studies a dose of 100 ng to 1 µg has been found optimum and the animals showed better antibody response by ELISA and SNT. Therefore 1 µg was selected as optimum dose for subsequent experiments. The vaccine showed protection against all the serotypes, though with different degrees which may be due to he animal variation or variations in challenge virus. The variation in challenge virus may be a possible reason as similar trend was observed in the case of control animals also.

Earlier studies in our lab (Choudary S et al ., 2008, Jadav SK et al., 2011) showed that cloned VP1 gene of FMDV type A in pVac vector showed protective immune response in guinea pigs against challenge virus, thus indicating that the humoral antibody response is essential for protection against FMDV challenge. However, as per the reported literature (Parida S et al., 2006) the γ interferon (IFN- γ) inhibits FMDV replication and therefore induction of the

same is essential to cure carrier animals. The Self replicating DNA vaccine vector constructed by us carries both Sindbis virus polymerase genes for self replicating the RNA of the cloned gene and secretary and anchoring signals for secreting and anchoring the expressed protein outside the cell. Thus the vaccine is expected to elicit both humoral and cell mediated immune response. Our studies showed that it was enough to use limited epitopes, and still get protective response. However one may insert a full VP1 gene or VP1 genes of all the serotypes that are linked in contiguous without 2A and study the immune response. The reason for poor protective response in the case of 'O' may be due to the animal variation or non availability of the protein to the immune system. Therefore it may be ideal to remove 2A and get unprocessed protein anchored in the membrane for better immune response against all the serotypes of FMDV. Presently we are working in this line. We don't have data to show the localization of 12 Kda proteins of 'O' and 'A. The vaccine needs to be further evaluated in homologous host ie bovine for protective response and duration of immunity. The vaccine efficacy can be further improved by the use of micro particles or nano particles. However this approach may pave the way for future research to develop safe and potent vaccine for FMD.

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