Self Replicating Gene Vaccine Carrying P1-2A Gene of FMDV Serotype O and its Effects on the Immune Responses of Cattle

G. Nagarajan · P. Ravikumar · C. Ashok Kumar ·
G. R. Reddy · H. J. Dechamma · V. V. S. Suryanarayana

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Abstract DNA vaccines are considered as alternatives to live attenuated ones for those diseases like foot-and-mouth disease (FMD) where the production and application of live vaccines have been found unsuccessful. However, stability of DNA and the quantity of antigen expressed are the major limitation with naked DNA vaccines. To address these issues self replicating gene vaccine construct was made for foot-and-mouth disease virus (FMDV) type ‘O’ and studied. The vector for vaccine construct, designated as pSinCMVVac carried CMV promoter and Poly(A) signal sequences at 5′ and 3′ end of Sindbis replicase gene respectively. Gene for structural protein precursor (P1-2A) of FMDV serotype ‘O’ was inserted into pSinCMVVac under subgenomic promoter. 5′UTR (untranslated region) of FMDV was introduced upstream of P1-2A to enhance the level of expression of cloned gene. Functionality of the vaccine construct was confirmed in vitro and in vivo. The self-replicating gene vaccine construct was tested in cattle in comparison with naked DNA vaccine carrying P1-2A and 3CD (pUP3CD). Humoral immune response by ELISA and SNT and cellular response by lymphoproliferation assay using MTT were studied. The default approach of using self replicating gene vaccine in high dose and multiple injection in cattle as followed in our studies might

result in immunosuppression as this was observed in our subsequent experiments in guinea pigs. Hence based on dose response studies, vaccine strategy needs to be decided. However, the approach of using Sindbis polymerase gene and UTR in FMDV vaccine is the first report and shows future scope of developing such vaccines.

Keywords FMD · Self replicating RNA vaccine · Sindbis replicase gene · Immune response · Cattle

Introduction

Foot-and-mouth disease (FMD) is one of the most dreadful diseases of cloven-footed animals. The disease spreads extremely fast among susceptible animals such as cattle, pigs and sheep [5, 9, 25]. The disease is enzootic in Asia (including the Middle East), Africa, Eastern Europe and some parts of South America. The 2001 outbreak of FMD in many parts of the Europe, particularly in UK have warranted the rethinking on the range of disease control methods that are currently being considered in the event of such unexpected incursions.

Thus, emergency vaccination, vaccination to live policies and antiviral approaches are encouraged by the OIE and reports from government sponsored boards of inquiry. FMDV, the causative virus of the disease belongs to the *Aphthovirus* genus of the family *Picornaviridae*. Like other viruses of the family, it is a non-enveloped virus. The virion is composed of 60 capsomers made of four viral proteins, VP1-4. The genome is of 8.5 kb long ssRNA of positive sense. The genome carries single open reading frame resulting in translation of a large polypeptide, which undergoes proteolytic cleavage to form mature viral proteins [14].

Present Address:

G. Nagarajan
National Research Center on Camel, Post Bag No 7, Jorbeer,
Bikaner 334001, Rajasthan, India

P. Ravikumar · C. Ashok Kumar · G. R. Reddy ·
H. J. Dechamma · V. V. S. Suryanarayana (✉)
Molecular Virology Laboratory, Indian Veterinary Research
Institute, Bangalore Campus, Hebbal, Bangalore 560 024,
Karnataka, India
e-mail: veluvarthy@yahoo.com

Regular vaccination has been done in endemic countries to control the disease. However, a few of the infected animals upon infection become carriers and act as reservoirs, which pose a serious problem for FMD eradication. Uncontrolled movement of animals in these countries also leads to the persistence of the virus in the environment. Presence of FMDV in any part of the globe is a threat to the rest of the world. Hence, elimination of carrier status and maintenance of herd immunity in endemic countries is as important as protecting the animals against the disease in FMD free countries. This can only be achieved by regular mass vaccination with efficacious vaccine that can not only mount protective immune response but also cure carrier animals.

Immunization with antigen-encoding plasmid as DNA vaccines are being used to induce both humoral and cell mediated immune responses against a number of infectious diseases including FMD [32]. Numerous investigations have demonstrated the ability of DNA immunization to induce protective immune response against certain diseases. However, the results were less encouraging in the case of HSV infection wherein the level of immunity induced is not complete [15, 24]. Moreover, the use of DNA vaccines to elicit mucosal immune responses remains inconsistent [18]. This may be due to the amount of antigen expressed in response to DNA vaccination may be sub-optimal. Since, FMD viral antigens are known to be weak immunogens, the self replicating gene vaccines containing alpha viral replicase gene may be the best alternative to naked DNA vaccines for FMDV.

Alpha viruses are single stranded positive sense RNA viruses with an envelope structure. The genomes of three members of the alpha virus family, semliki forest virus (SFV) [22], Sindbis virus (SIN) [37] and Venezuelan equine encephalitis virus (VEE) [10] have been thoroughly studied. Among the three types of vectors that has been developed using alpha virus genome, DNA vector in which only polymerase gene along with subgenomic promoter cloned under the control of a strong eukaryotic promoter has found its application in gene vaccines. The gene of interest is cloned under subgenomic promoter and the expression is driven by eukaryotic promoter introduced upstream of polymerase gene. Initial transcription of DNA leads to the self-amplification of RNA (replicon), which allows direct use of plasmid DNA for transfection and expression [3, 13]. If the gene of interest carries immunogenic epitopes, the construct becomes self replicating vaccine [23].

A few examples of these vaccines include the constructs carrying NS3 (p80) protein of Bovine Viral Diarrhoea virus [27], gp41 of Human Immunodeficiency Virus-1 [16], Influenza virus Nucleoprotein [39], Influenza virus Haemagglutinin [4], β -galactosidase for cancer [38], Hepatitis B surface antigen [12] and Herpes Simplex Virus-1 glycoprotein B [17].

Conventional FMD vaccines can deliver the antigenic mass, however they are poor in providing long lasting immunity due to the poor elicitation of cell mediated immune (CMI) response, which is also necessary for abolishing carrier status. On the other hand, naked DNA vaccines can elicit cell mediated response, but they fail to deliver desired antigenic mass, thereby limiting humoral immune response. These limitations prompted us to develop self replicating gene vaccine for FMD which may deliver high antigenic mass and study its immune response in homologous host.

Materials and Methods

Virus

FMDV type 'O' (V) (IndR2/75) grown in BHK21 clone 13 cells was used as a source of standard RNA template for RT-PCR.

Vaccine Constructs

pUP 'O' (pcDNA vector carrying 5'UTR plus P1-2A of FMDV serotype 'O') pUP 3CD (pcDNA vector carrying 5'UTR plus P1-2A and 3CD of FMDV serotype 'O') available in Molecular Virology Lab, IVRI, Bangalore, India were used in this study.

Serum

Serum raised against purified and inactivated 146S particles of type 'O' in rabbits and against the inactivated tissue culture virus of the same in cattle was used in this study.

Host System

E. coli host DH5 α and BHK21 clone 13 cells were used for gene cloning and expression studies, respectively.

Construction of pSinCMVVac

Commercially available pSinHis A (Invitrogen, USA) containing Sindbis virus replicase gene was used as a source of polymerase gene for this study. CMV and T7 promoter sequences were retrieved from a commercial eukaryotic expression vector, the ends of approximately 1 kb sized fragment were blunt ended using T4 DNA polymerase and cloned at blunt ended *SacI* site of the pSinHis A vector. Bovine growth hormone (BGH) poly (A) signal sequence including the flanking sequence of around 220 bp was amplified from the pcDNA vector (as a template) using forward primer poly A Sig(L)(*ApaI*) 5'd

GCC GCT CGA GTC TAG AGG GCC CGT TTA AAC–OH 3' and reverse primer poly A Sig(R)(*NotI*) 5d CCC CTC GAGC GG CCG CTT AAT TAA ATG CAA TTT CCT CAT TTT ATT AGG AA–OH 3'. The reaction mix for amplification of the above consisted of 10 ng (2 µl) of the purified DNA template, 20 pm of each primer, 25 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 100 µM each of the four dNTPs and 1–2.5 units of Taq DNA polymerase in a total volume of 50 µl. The mix was subjected to 40 cycles of amplification in thermal cycler after initial denaturation of the template at 94°C for 3 min. Each cycle consisted of a denaturation phase of 30 s at 94°C, followed by annealing at 60°C for 30 s and primer extension at 72°C also for 30 s. The amplified product was purified from the 2% preparative agarose gel.

The amplicon was cloned at *ApaI*–*NotI* sites of the pSinHis A vector. The resultant plasmid containing CMV promoter along with T7 promoter sequences and polyadenylation signal sequence at respective 5' and 3' ends of Sindbis virus replicase gene was named as pSinCMVVac.

Construction of Self-Replicating Gene Vaccine for FMDV Serotype 'O'

The gene cassette consisting of UTR followed by structural protein precursor P1-2A of FMDV type 'O' cloned in pCDNA (pUP 'O') was available in Molecular Virology Laboratory, IVRI, Bangalore. The pUP 'O' was digested with *XbaI* enzyme to get the gene cassette of UTR-P1-2A of FMDV 'O' serotype and the resultant fragment of 2.8 kb was blunt ended with T4 DNA polymerase and cloned at *StuI* site of pSinCMVVac vector. Subsequently, the resultant recombinant plasmid was used as a self replicating gene vaccine of FMDV serotype 'O' in cattle after in vitro evaluation for expression.

Coupled Transcription and Translation

Since the cloned structural protein gene of FMDV 'O' serotype was under the control of both CMV and T7 promoters, the intactness of the insert was studied in vitro by coupled transcription and translation (Promega) (TnT) using rabbit reticulocyte lysate system in the presence of ³⁵S methionine. The radioactive bands were detected by fluorography as described by Suryanarayana et al. [35] and the molecular weight of the radioactive bands were compared with the marker proteins.

BHK 21 Cell Transfection and Western Blotting

About 1.5 µg of endotoxin free plasmid DNAs were mixed with 4.5 µl of Gene Juice (Novagen) diluted to 100 µl with serum free Eagle's MEM and incubated for 15 min. at

room temperature. Then the mixture was layered drop by drop on BHK21 cell monolayer with 80% confluence along with 3 ml of growth medium without serum in each well of six well tissue culture plates. Vector DNA control and cell controls were included in the plate. After incubating the plates at 37°C with 5% CO₂ tension for 4 h, the medium was decanted, the cell monolayer was washed with fresh PBS (pH 7.2) and the cells were pooled in TE buffer (pH 8.0) with 1 mM PMSF. After subjecting the cells to freezing and thawing couple of times, the proteins from the lysate were analyzed in 10% SDS-PAGE stained with Commassie brilliant blue (CBB) in methanol:acetic acid:water (45:45:10) and destained in the same solvent solution as per the protocol described in the method [19]. Duplicate PAGE gel run in the similar way was used for immunoblotting as per standard protocol [31]. The protein in the gels was transferred to a Polyvinylidene Dihydrofluoride (PVDF) membrane using Trans Blot-SD electrophoretic transfer cell (BioRad) according to manufacturer's instructions. After completing the electro-transfer using transfer buffer (50 mM Tris HCl, pH 7.5, 19 mM glycine and 20% methanol) at the voltage of 15 V for 50 min, the membrane was blocked with blocking buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 5% skimmed milk powder and 0.05% Tween 20) at 37°C for 1 h. Then the membrane was transferred to a container containing 1:1000 diluted convalescent cattle sera in blocking buffer and incubated at 37°C for 1 h with gentle agitation. The membrane was washed three times and then incubated in 1:1000 diluted rabbit anti-bovine HRPO conjugate (Bangalore Genei) in Tris-buffered saline (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.01% Tween 20) buffer at 37°C for 1 h. Three more similar washings of the membrane followed this to eliminate unbound conjugate. Thereafter the membrane was transferred to substrate solution [0.02% (w/v) O-Dianisidine Dihydrochloride in 50 mM sodium acetate (pH 5), 10 mM EDTA, 0.1% Triton X-100 and 0.02% Hydrogen peroxide] and incubated in dark for brown colour development (about 20 min). The reaction was stopped by washing the membrane with excess of distilled water and the result was documented.

Experimental Animals and Vaccine Trial studies

Twenty-one healthy calves (free of antibodies to FMD virus serotype 'O') of either sex and of 6–8 months old were divided into five groups; three groups comprise five animals each and two control groups of three animals each. The animals were housed, fed and managed under identical conditions. As shown in the Table 1, the animal groups were identified by the vaccine constructs and were immunized.

Table 1 Humoral and cellular immune responses of various vaccine constructs in cattle

Group and vaccine constructs inoculated	ELISA antibody titres ^A (mean $\pm$ SD)		SNT titres ^B (mean $\pm$ SD)		Stimulation Index (SI) ^C (mean $\pm$ SD)
	Prevac	72DPV	Prevac	72DPV	
I pSinCMVVac vector	<1.398 (0/3)	1.398 $\pm$ 0.00 ^a (3)	<0.903 (0/3)	0.903 $\pm$ 0.00 ^a (3)	1.173 $\pm$ 0.01 ^a (3)
II pSinCMVVac 'O' P1-2A	<1.398 (0/5)	1.759 $\pm$ 0.25 ^b (5)	<0.903 (0/5)	1.564 $\pm$ 0.33 ^b (5)	1.212 $\pm$ 0.02 ^b (5)
III pcDNA 3.1 + vector control	<1.398 (0/3)	1.398 $\pm$ 0.00 ^a (3)	<0.903 (0/3)	0.903 $\pm$ 0.00 ^a (3)	1.227 $\pm$ 0.01 ^b (3)
IV pUP3CD	<1.398 (0/5)	1.699 $\pm$ 0.21 ^b (5)	<0.903 (0/5)	1.445 $\pm$ 0.54 ^b (3)	1.250 $\pm$ 0.01 ^c (3)
V Conventional virus vaccine	<1.398 (0/5)	2.301 $\pm$ 0.00 ^c (5)	<0.903 (0/5)	2.107 $\pm$ 0.00 ^c (5)	

Means with same superscripts do not differ significantly ($P < 0.05$)

Method: One way analysis of variance and DMRT analysis, SPSS14.0

^A The log of serum dilution (1:25) at A492 nm values

^B The log of serum dilution at which 50% of BHK 21 cell monolayer had shown cytopathic effect

^C The ratio of OD of stimulated lymphocytes to OD of unstimulated lymphocytes in MTT assay

Each animal was inoculated four times with 500 μ g/dose of endotoxin free DNA construct on day 0, 21, 42 and 63. The animals were bled on day 0 prior to first inoculation and 9 days after the third booster (72 days post vaccination) for culturing of lymphocytes for lymphoproliferation assay. The sera collected were assayed for humoral immune response by ELISA and SNT.

Sandwich ELISA

Ninety six well-F 96 Maxisorp Nunc Immunoplates were coated with rabbit anti 146S antibodies at a dilution of 1:7000 in 0.1 M carbonate/bicarbonate buffer, pH 9.6 and incubated at 4°C overnight. Then the plates were washed with washing buffer three times and the left over sites were blocked with blocking buffer (phosphate buffered saline, 0.05% v/v Tween 20 and 5% w/v skimmed milk powder) at 37°C for 1 h. After washing the plates as described before, FMDV type 'O' antigen diluted to a concentration of 2 μ g/ml in blocking buffer was added and incubated at 37°C for 1 h. Subsequently the plates were incubated with two fold serially diluted test sera (1:25 to 1:200) from immunized cattle in duplicate wells. After 1 h of incubation followed by washing, rabbit anti-bovine IgG-peroxidase conjugate (Bangalore Genei) at 1:1000 dilution in blocking buffer was added to the plates and incubated for 1 h at 37°C. Antigen-antibody reaction was detected by the addition of substrate, Orthophenyl Diamine (OPD, 0.05%) and 0.01% hydrogen peroxide in phosphate-citrate buffer (pH 5.0). The reddish brown colour developed upon incubation in dark for 30 min. was terminated with 1 M H₂SO₄ and absorbance values were measured at 492 nm in an ELISA reader.

Serum Neutralization Test (SNT)

Complement inactivated test sera samples from immunized cattle were diluted serially from 1:2 to 1:32 in Eagle's

MEM in Corning 96 well flat bottom Tissue culture treated polystyrene plates and added to wells in triplicate (50 μ l/well). Previously titrated type 'O' virus diluted in Eagle's MEM containing 100TCID₅₀/50 μ l was added to each well at the rate of 50 μ l/well. Controls with respect to test sera, challenge virus, cell control and virus titration were included. The plates were incubated at 37°C in a humidified chamber with 5% CO₂ tension for 1 h. Then BHK21 cell suspension in Eagle's MEM was added at the rate of 50 μ l/well and plates were reincubated for 48 h. The 50% serum neutralization end point was calculated by the method of Reed and Muench [28].

MTT Assay

Whole blood samples collected in EDTA coated vacutainer tubes were diluted with equal volume of sterile PBS (pH 7.2) and centrifuged at 1700 rpm for 30 min at room temperature. The separated buffy coat was layered on 2 ml cushion of Histopaque-1077 and centrifuged at 1500 rpm for 30 min at room temperature. The separated lymphocytes at the interface between the lower Histopaque-1077 and the upper plasma were washed three times with sterile PBS and suspended in 0.5 ml of RPMI 1640 medium with 10% foetal calf serum. After taking the cell count in a haemocytometer, the cell density was adjusted to 2×10^6 cells/ml. Lymphocyte stimulation test was performed in Corning 96 well flat bottom tissue culture treated polystyrene plates as per standard procedure. The lymphocytes were stimulated with BEI-inactivated FMDV serotype 'O'. Unstimulated and Con A stimulated lymphocytes were included as negative and positive controls respectively. Triplicate cultures were setup for each treatment. After incubating the plates at 37°C in a humidified CO₂ incubator for 96 h, an amount equivalent to 10% of the culture volume of MTT (5 μ g/ml) was added to each well. After incubating the cells at 37°C in 5% CO₂ for 4 h, MTT

solubilization solution (Sigma) equal to the volume of culture medium was added to each well to dissolve the resultant formazan crystals and A570 nm was measured spectrophotometrically. Background absorbance of multi-well plates was measured at 690 nm and subtracted from 570 nm readings. The ratio of OD of stimulated cultures to OD of unstimulated cultures was calculated as Stimulation Index (SI).

Statistical Analysis

One-way analysis of variance and DMRT analysis from the statistical package SPSS14.0 were employed for the analysis of the data obtained from this study.

Results

Vaccine Vector Construction

A Fragment of around 1.0 kb carrying CMV along with T7 promoter sequences released from the pcDNA3.1+ vector was ligated into *SacI* digested, blunt ended pSinHis A vector. The transformants were screened for the presence of plasmid with insert in positive orientation by restriction analysis. Since there is a single *KpnI* site in the vector and single *KpnI* site in the insert, a release of 3.2 kb fragment upon digestion of the recombinant plasmid with *KpnI* enzyme confirmed the presence of insert in right orientation (Fig. 1, lane 3).

Poly (A) signal of bovine growth hormone gene from the pcDNA3.1+ vector was amplified using the forward primer specific to pcDNA 3.1+ vector and reverse primer

Fig. 1 Agarose gel electrophoresis of *KpnI* digested pSinCMV vector. Lane 1 1.0 kb DNA ladder; lane 2 pSinCMV vector control digestion; lane 3 *KpnI* digested pSinCMV plasmid showing a release of 3.2 kb representing CMV in correct orientation

Fig. 2 Agarose gel electrophoresis of *ApaI* and *NotI* digested recombinant pSinCMV plasmid DNA. Lane 1 100 bp ladder; lane 2 pSinCMV vector control digestion; lane 3 *ApaI* and *NotI* digested pSinCMV showing a release of 220 bp poly (A) signal fragment

specific to pSinHis A vector and an amplicon of size about 220 bp was cloned into *ApaI* and *NotI* sites of pSinHis A vector. Presence of the cloned fragment was confirmed by sequential digestion of the plasmid DNA (Fig. 2, lane 3). The resulting recombinant plasmid having CMV promoter sequences and polyadenylation signal sequences at respective 5' and 3' ends of Sindbis virus replicase gene has been designated as pSinCMVVac.

Cloning of UTR-P1-2A in pSinCMVVac

A fragment of 2.8 kb representing IRES-P1-2A of type 'O' was released from the plasmid (pUP 'O') with *XbaI*, blunt ended and cloned at *StuI* site of pSinCMVVac. Plasmid DNAs were purified from individual clones and subjected to PCR amplification using forward vector specific primer (subgenomic promoter (SGP) 5'd GCA TCT CTA CGG TGG TCC TAA ATA-OH 3' and reverse gene specific primer (2AR Eco RI-5' d GCG GAA TTC GC GAA GGG CCC AG GG TT GGA CTC-OH 3'). Amplification of 3 kb DNA confirmed the presence and right orientation of the insert (Fig. 3, lane 4). The presence of the insert in self replicating gene vaccine construct (pSin FMDVac) was further confirmed by *SphI* and *ApaI* digestion of the plasmid DNA that resulted in the release of 2.8 kb fragment (Fig. 3, lane 3).

Functional Analysis of Self Replicating Gene Vaccine Cassette

Since 5' end of P1-2A carries T7 promoter sequences in addition to CMV promoter, the DNA vaccine construct was subjected to coupled transcription-translation reaction using TNT kit (Promega) in the presence of ^{35}S methionine

Fig. 3 Agarose gel electrophoresis of *SphI* and *ApaI* digested recombinant pSinCMV Vac IRES-PI-2A plasmid DNA. Lane 1 1.0 kb DNA ladder; lane 2 pSinCMV Vac vector control digestion; lane 3 *SphI* and *ApaI* digested pSinCMV Vac IRES-PI-2A showing a release of 2.8 kb PI-2A gene fragment; lane 4 amplification of 3.0 kb PCR product shows the right orientation of IRES-PI-2A in pSinCMV Vac

Fig. 4 Western blot analysis of cell lysate of BHK 21 cells transfected with DNA vaccine constructs along with cell lysate of untransfected BHK 21 cells. Lane 1 Lysate from cells transfected with pSinCMV Vac IRES-PI-2A; lane 2 lysate from cells transfected with pUP; lane 3 lysate from cells transfected with pSinCMV Vac; lane 4 lysate from untransfected cells; lane 5 standard prestained protein molecular weight marker

and the products were analyzed by SDS-PAGE and fluorography. Functionality of the cassette in terms independent translation of the target gene under IRES to a full length protein (83 kDa) in vitro was first confirmed (figure not shown). Then the vaccine construct with CMV promoter upstream was tested in vivo for expression BHK21 cells. The SDS-PAGE separated proteins were subjected for immunodetection using antibodies raised against capsid protein VP1 (Fig. 4). A single and intense colour reaction was seen with the protein of size 83 kDa on the blot (Fig. 4, lane 2) that corresponded to the positive control where pcDNA carrying UTR-P1-2A was used for transfection (lane 1). No colour reaction was seen in the case of mock transfected cell lysate (Fig. 4, lanes 3, 4).

Humoral Immune Response in Cattle Vaccinated with Vaccine Constructs

Cattle were vaccinated with the purified vaccine construct and the serum antibody titres were evaluated. As shown in the Table 1, the sera from the animals inoculated with self replicating gene vaccine construct (Group II) and conventional gene vaccine construct (Group IV) showed significant increase in antibody titre by ELISA as compared to their respective control groups (Group I and Group III). However, the sera from the animals vaccinated with conventional virus vaccine (Group V) exhibited significantly higher antibody titres in ELISA. On the other hand, serum neutralization titres of the animals inoculated with self replicating gene vaccine construct (Group II) and conventional gene vaccine construct (Group IV) were significantly higher as compared to their respective control groups (Group I and Group III). Similarly the serum neutralization titres of the animals vaccinated with conventional virus vaccine (Group V) were higher than those of animals vaccinated with gene vaccines.

Cell Mediated Immune Response in Vaccinated Animals

Since gene vaccines are known to elicit high cellular immune response in addition to humoral response the vaccinated animals were studied for CMI response. As shown in the Table 1, the stimulation indices obtained from MTT assay applied to lymphocytes collected from the animals vaccinated with self replicating gene vaccine construct (Group II) and conventional gene vaccine construct (Group IV) were significantly higher as compared to their respective control groups (Group I and Group III). However, the values of stimulation indices from the self replicating gene vaccine construct (Group II) did not differ significantly with that of the Group III.

Discussion

DNA vaccination strategy has emerged as a powerful approach in those diseases where attenuation is not possible. However the major limitation in the DNA vaccination is poor stability of the DNA and/or the low level of antigen expression. Hence in those cases where the antigen concentration plays major role for protective response like FMD, an alternative strategy to improve the antigen expression is needed. Amplification of transcribed RNA corresponding to the antigen of interest using RNA dependent RNA polymerase of Alpha virus may improve the antigen production. The RNA under the control of sub-genomic promoter is expected to amplify several hundreds

of folds thereby increasing the antigen production. Keeping this in view we have used Sindbis virus RNA replicase gene for the construction of vaccine. Strong eukaryotic promoter i.e. CMV promoter was used to drive the nuclear transcription of the cassette. The methodology followed for the development of the self-replicating gene vaccine for FMDV type 'O' and the immune response of the resulting vaccine in homologous host is discussed below.

Plasmid carrying replicase gene and subgenomic promoter sequences of Sindbis virus obtained commercially (Invitrogen, USA) was cloned downstream of CMV promoter which has broad mammalian tissue specificity. This strategy was earlier used for producing self replicating gene vaccines for Herpes Simplex virus infection [17], Hepatitis B infection [12] and Bovine Viral Diarrhoea infection [27].

In the case of FMDV we have selected P1 polyprotein gene encoding VP4–VP2–VP3–VP1 of serotype 'O' and inserted downstream of sub-genomic promoter. Since P1 is a precursor of the structural proteins, all the major epitopes VP1 and other minor epitopes of other capsid proteins are present. Several studies have been conducted on the use of complete P1 gene either as a DNA vaccine [32] or for expressing in heterologous system to use as protein vaccines [1, 21]. To enhance the translation efficiency of the target gene we included 5'UTR containing internal ribosome entry site (IRES) of FMDV Asia 1 upstream of the P1-2A coding gene. FMDV IRES has been shown to be highly efficient in driving cap independent translation [2, 36]. The P1-2A along with IRES at 5' end was retrieved from the plasmid pUP 'O' (pcDNA vector carrying IRES-P1-2A 'O') and inserted at *StuI* site of pSinCMVVac. The expression of the inserted gene with authenticated translation initiation site followed by 28 amino acids from L protease as a fusion was confirmed both in vivo by coupled transcription and translation and in vivo in BHK₂₁ cells by immunoblot assay. Similar approach was made in the case of naked DNA vaccine constructed in our lab [26].

It was earlier believed that 2A acts as a protease and helps spontaneous cleavage between P1-2A and P2 [6, 30]. However, the recent studies showed that there is a ribosome skipping between 2A and 2B junction, which results in the production of P1 polyprotein of 80 kDa [11, 33]. Along with 28 aa of L-protease the size of the fusion protein is 83 kDa which was the observed molecular weight. However, we do not have any data on the proteins corresponding to replicase gene and processing activity of the polyprotein produced from the replicase gene. Nevertheless the immunoblot showed the presence of FMDV specific protein in the blot by reacting with antiserum.

It is expected that the protein production due to self replication of RNA to be higher. However our observation revealed that there is only marginal increase in the protein

expression as compared to translation from naked DNA construct. This may be due to the lower transfection efficiency of the DNA due to the size difference or death of the transfected cells due to apoptosis or both. The studies conducted previously [17, 38] showed that the expression due to self replicating RNA was only slightly more than 200% in terms of amount of antigen produced by a conventional CMV promoter DNA.

By default, as per the previous protocols we have used very high amount of candidate vaccine, i.e. 100 µg/dose. Over and above we have used repeated injections up to 4. Though this was done to avoid the in dose responses studies in cattle, our subsequent studies in guinea pigs using different antigen gene construct clearly showed that there was a negative effect of high dose. This may be due to immunosuppressive activity of the high concentration of immunogen expressed in the animal. Our recent studies with guinea pigs showed that single dose of Self Rep DNA vaccine construct as low as 1/10th concentration of naked DNA (1 µg/dose) gave high immune response and protected animals against challenge virus, while at normal dose (10 µg) showed severe infection (more than control animals). Thus the results in the present studies in cattle need to be considered in that perspective. More over as a default four injections were given to the animals and the immune response was studied. There is every possibility of the neutralizing antibodies produced in the first injection will be subsequently neutralized by the expressed antigen. This may be the reason that the vaccine trial showed that the animals vaccinated with self-replicating gene vaccine construct mounted marginally increased neutralizing antibody response. Also the high antigen expression or gamma interferon production due to dsRNA production or both might have resulted in the immune suppression. Rolls and his team [29], while studying the replication of vesicular stomatitis virus observed that theoretically it is possible to produce 2×10^5 copies of the antigen by self replicating RNA vaccines within 4 h accounting for 25% of the total cell proteins. However, we observed an increase in the production of P1-2A by approximately two fold in BHK cells (as observed by positive colour reaction in immunoblot). This increase may be due to the increase in the RNA concentration or high efficiency of the translation under IRES at 5' end or both. Ying and his team [38] observed 200% increase in β -galactosidase production in self-replicating RNA construct under SFV replicase as compared to conventional CMV promoter construct without replicase gene. Hence, the observed higher immune response in case of FMDV 'O' self replicating DNA vaccine construct may be due to the virus like replication in vivo.

In the case of conventional DNA vaccines carrying P1-2A-3CD, there was a better and comparable humoral immune response as detected by ELISA which may be

either due to the large number of epitopes present in the construct (P1-2A, 3C, 3D) or formation of empty capsids or high molar concentration (smaller size) of the DNA used for vaccination.

Similarly, self-replicating gene vaccine, like naked DNA vaccine also elicited CMI response as evidenced by stimulation indices obtained in MTT assay. This might be due to the production of interferon molecules in vivo in response to dsRNA formation [20]. Since conventional virus vaccine is known to elicit poor CMI response, we have not evaluated in these studies. In the case of foot-and-mouth disease, the immune response is not only B-cell but also T-cell dependent [5, 7, 8, 34].

The newly introduced naked DNA vaccine technology may help in overcoming several limitations that are observed in conventional vaccines. However, they need co-adjuvantation for better antigen presentation. This may be true in the case of FMD whose antigen is a weak immunogen providing short duration of immune response when used as inactivated protein antigen. In such cases, self replicating DNA/RNA vaccines mimicking virus like replication facilitates the better antigen presentation and better immune response. These vaccines unlike recombinant protein antigen vaccines or naked DNA vaccines may not need all the epitopes of the pathogen, a few selected epitopes of the pathogen when included in the vaccine may suffice. Thus, the future vaccines for FMDV using self replicating gene vaccine technology may include only major immunogenic regions. Since, multiple serotypes are to be controlled by linking the serotype specific epitopes in the vaccine, one may prepare a polyvalent vaccine construct in the form of self replicating gene vaccines. In our studies we have used high DNA concentration (500 µg) per dose and four doses for injecting the animals. This needs further evaluation as one can expect suppression of the immune response at high antigen concentration or gamma interferon production. By reducing DNA concentration and giving single dose one may achieve better response.

Though, the results from preliminary studies are encouraging further work has to be carried out to confirm the usefulness of self-replicating vector vaccine against FMD. These are standardization of dose response in guinea pig model for extrapolating to cattle, inclusion of more experimental animals in vaccine trials, conducting challenge studies in cattle, quantification of RNA produced from self replicating gene vaccine construct in transfected BHK-21 cells and comparison with RNA from transfected DNA vaccine without replicase gene and the last but not the least one has to workout the ideal route for vaccine administration.

If further evaluation of the vaccine is done as above, the self replicating gene vaccine would be an ideal alternative for the conventional vaccine for controlling FMD.

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