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Development of foot-and-mouth disease virus (FMDV) serotype O virus-like-particles (VLPs) vaccine and evaluation of its potency

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ABSTRACT

Foot-and-mouth disease (FMD) is an economically significant viral disease that rampage dairy and other livestock industries in many countries. The disease is being controlled by the use of an inactivated vaccine. However, a recombinant marker vaccine, which avoids the use of live virus, may be an option for the unambiguous differentiation of infected animals from vaccinated animals. A recombinant baculovirus clone containing P1-2A-3C coding sequences of foot-and-mouth disease virus (FMDV) serotype O_1 Manisa was generated. The FMDV structural proteins along with the 3C protease were expressed in Sf9 cells and the generation of virus like particles (VLP) was studied. The recombinant protein was formulated as vaccine using an oil adjuvant, ISA 206 and potency of the vaccine was tested in cattle. The vaccine had a potency value (PD_{50}) of 5.01 and most of the vaccinated animals exhibited neutralizing antibody titers after two immunizations.

1. Introduction

Foot and mouth disease (FMD) is a vesicular disease of cloven hoofed animals, including domesticated ruminants and pigs. FMD is etiologically linked to foot and mouth disease virus (FMDV) that belongs to *Picornaviridae* family. FMDV genome contains a single open reading frame (ORF), which encodes a precursor polyprotein and the precursor protein is cleaved by viral coded proteases into individual mature proteins. One of the virus coded proteases, 3C aids in the cleavage of viral structural proteins and enables the assembly of FMDV capsid in the infected cells. Each capsid subunit is formed by the assembly of a single copy of the structural proteins VP0, VP1 and VP3 that are coded by the P1 region of the viral genome with VP0 being the precursor protein for VP2 and VP4. VP1, VP3 and VP0 spontaneously form the 5S protomer, which subsequently assembles into a 12S pentameric subunit. The pentamers finally assemble into viral capsid (Belsham, 1993; Grubman et al., 1995).

Currently available inactivated FMD vaccine preparations may contain both the viral structural and non-structural proteins, which complicate the differentiation of infected animal from vaccinated animals. Therefore, the FMD free countries do not prefer the use of inactivated vaccines and adopt the policy of culling (Grubman et al., 2010). Additionally, the insufficient inactivation of the virus during vaccine manufacturing process and the virus escape from the vaccine manufacturing facility has been a major concern in using inactivated vaccines (Doel, 2003). Countries that adopt 'stamping out' policy to contain the spread of the disease may consider prophylactic vaccination strategy owing to socio-economic reasons if an efficacious recombinant marker vaccine is made available.

Development of recombinant vaccines and virus like particle (VLP) based vaccines have been attempted using various expression platforms (Balamurugan et al., 2005; Li et al., 2006). However, many of these attempts failed because of the inability of such recombinant vaccines to protect cattle against FMDV challenge. On the contrary, the VLP based FMD vaccine produced in silk worm and a replication deficient human adenovirus vector based vaccine were two of the successful recombinant vaccine candidates in protecting the target species (cattle or swine) against FMDV challenge (Pacheco et al., 2005; Moraes et al., 2002; Li et al., 2008, 2011). The adenoviral vectors may not be a suitable candidate in circumstances that require multiple immunizations (Thacker et al., 2009). Expression of proteins in silk worms on a commercial scale has not been evaluated so far and insect cell lines such as Sf9 are preferred over the insects as a production platform. The studies using insect cell lines to produce FMDV VLPs have been reported with various degrees of success and the development of neutralizing antibodies in guinea pigs was evaluated in some of the studies (Cao et al., 2009; Lewis et al., 1991).

In the present study, an insect cell codon optimized gene of capsid protein precursor (P1-2A) along with 3C protease of FMDV O_1 Manisa were expressed in Sf9 cells and the potency of the vaccine

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formulation was evaluated by a homologous cattle challenge experiment.

2. Materials and methods

2.1. Cells, virus and animals

Spodoptera frugiperda (Sf9) cells (Ingenasa, Spain) were grown using Grace's insect cell medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 27 °C. Baby hamster kidney (BHK) and bovine thyroid (BTY) primary cells were obtained from the tissue culture laboratory of R&D centre, Indian Immunologicals Limited (IIL), Hyderabad. Polyclonal sera were raised in rabbits using *Escherichia coli* expressed VP1 or VP2 proteins of O_1 Manisa. The FMDV VP1 specific mAbs (O12E7 and O3E7) were produced by immunizing mice with FMDV serotype O antigen and these mAbs detected O_1 Manisa virus in ELISA and immuno-fluorescence assay. O_1 Manisa cattle challenge virus was prepared and titrated in sero-negative cattle by standard methods as described previously (Nagendrakumar et al., 2011). Sero-negative (for FMDV capsid proteins and non-structural proteins) cross bred male cattle of 10–12 months of age were used in the animal experiment. Immune rabbit and guinea pig sera against O_1 Manisa virus antigen were used in antigen ELISA.

2.2. Generation of recombinant baculovirus clone containing FMDV P1-2A-3C expression cassette

The nucleotide sequence coding for entire P12A and 3C regions of FMDV strain O_1 Manisa were codon optimized for insect cell expression and the gene was synthesized as a single ORF construct (Geneart, Germany). The synthetic gene construct was cloned into baculovirus transfer vector pBacPak 8 between *EcoRI* and *Not*I restriction enzyme sites, downstream of the polyhedrin promoter. A recombinant baculovirus was generated by co-transfecting Sf9 cells with 500 ng of transfer vector DNA, containing the FMDV P1-2A-3C expression cassette, and 100 ng of BacMagic 2 baculoviral DNA (Novagen, USA) using Genejuice transfection reagent (Novagen, USA). The cell culture supernatant containing baculovirus was collected on day 6 post-transfection, and the recombinant baculovirus clones were identified after three rounds of plaque purification in Sf9 cells. The plaque-purified recombinant baculovirus clones were used to infect Sf9 cells and the cell lysate was subjected to FMDV antigen ELISA (Hamblin et al., 1984) to identify the positive clones.

2.3. Fluorescent antibody test using FMDV VP1 specific monoclonal antibodies

Sf9 cell monolayer was infected with the recombinant baculovirus clone at a multiplicity of infection (moi) of 2, and the cells were fixed 3 days post-infection using ice-cold 70% acetone. The fixed cells were incubated with FMDV VP1 specific monoclonal antibodies (O12E7 and O3E7) for 45 min at 37 °C in a humid chamber. The cells were washed thrice with phosphate-buffered saline (PBS); stained with rabbit anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma, USA) and observed under a fluorescence microscope (Olympus, Japan). The Sf9 cell monolayer infected with wild type baculovirus was also processed in an identical manner to rule out background fluorescence.

2.4. Expression of P1-2A-3C in Sf9 cells and assembly of VLPs

The Sf9 cells were grown in a shake flask at 27 °C and 110 rpm. The cells, at a density of 1×10^6 cells/ml, were infected with the recombinant baculovirus at a moi of 2. The infected cells were observed daily for cytopathic effect (CPE) and the cells were harvested after 5 days, when the CPE was almost 100%. The harvested cells were pelleted by centrifugation at 3500*g* for 10 min, washed thrice using PBS and were reconstituted with 25 mM sodium bicarbonate (pH 7.0). The cell suspension was kept over ice for four hours following which they were centrifuged at 10,000*g* for 15 min. The supernatant was collected in a separate tube and the high molecular weight proteins were precipitated using 25% ammonium sulphate. The precipitated proteins were resuspended in sterile PBS and dialysed extensively for three days against PBS with periodic buffer changes to remove ammonium sulphate. The dialysed solution was centrifuged at 10,000*g* for 15 min to remove any aggregates. The supernatant was loaded onto a gel filtration column packed with 2% cross linked agarose beads (Agarose Beads Technology, Spain). The purification was performed following the procedure described by Buck et al. (2006). The eluted fractions from the agarose bead column were analysed using FMDV specific antigen ELISA (Hamblin et al., 1984) and the fractions that exhibited higher ELISA activity were pooled. The total protein content of the preparation was determined using bicinchoninic acid (BCA) reagent (Sigma, USA). The pooled fractions were resolved in a 12% SDS–PAGE and transferred onto a Polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The immuno-blot was probed either using VP1 or VP2 specific rabbit polyclonal sera at 1:500 dilution in PBS. Protein A/G HRPO conjugate (Pierce, USA) was used as secondary antibody and the blot was developed using 3,3′-Diaminobenzidine (Sigma–aldrich, USA) and H_2O_2 (Sigma–aldrich, USA). The VLPs were further characterized by transmission electron microscopy (TEM). The fractions with high ELISA activity were used for immunization experiments.

2.5. VLP quantification

The pooled fractions from the agarose bead column chromatography was subjected to an immunocapture ELISA (IC-ELISA) to quantify the VLPs. Briefly, maxisorp ELISA plates (Nunc, Denmark) were coated with conformation specific O3E7 mAb (1 in 4000 dilution) in carbonate bicarbonate buffer (pH 9.6) and incubated for over-night at +4 °C. The ELISA plates were washed thrice using PBS containing 0.05% tween 20 (PBST). Free surface in the wells were blocked by adding 2% normal rabbit serum and 3% normal bovine serum in PBST and incubated at 37 °C for one hour. The plates were washed again and the P1-2A-3C antigen was added in 2-fold serial dilutions. Similarly, the purified and known quantity of O_1 Manisa 146S antigen (standard protein) was added in 2-fold serial dilutions starting from 2 μg/ml. The ELISA plates were incubated at 37 °C for one hour and washed with PBST. The bound antigens were detected using biotin labeled conformational specific mAb O3E7 (1 in 4000 dilution) and streptavidin HRPO conjugate (1in 8000 dilution; Sigma–aldrich, USA). The plates were read on a multi-channel spectrophotometer (Versamax, Molecular device, USA) at 492 nm (*A*492). The OD_{492} value from the sucrose density gradient purified 146S antigen was used to draw the standard curve. The concentration of the prepared VLP antigen was determined using the standard curve and expressed as μg/ml.

2.6. Vaccines, vaccination and experimental design

Twenty cross-bred male cattle of 10–12 months of age were housed separately in three groups of six-vaccinated-animals and one group of two-unvaccinated-controls within the isolation units at Indian Immunologicals Limited, Hyderabad. Recombinant vaccine containing partially-purified FMDV VLP antigen preparation with total protein content of 75 μg per dose (~2.5 μg of VLP as measured by the IC-ELISA) was formulated as a water-in-oil-in-water

(W/O/W) emulsion with Montanide ISA 206 (Seppic, France). Based on the TEM picture and immuno-blot results, it was evident that the recombinant antigen preparation contained VLPs, individual structural proteins, intermediate processed P1-2A proteins and insect cell host proteins. The potency test in cattle was conducted as per Indian pharmacopoeia (IP, 2010) with slight modification. Briefly, three groups of six cattle each were vaccinated with 75 μg of total protein in 2 ml volume (full dose vaccine), 15 μg of total protein in 0.4 ml volume (1/5th of vaccine dose) and 3 μg of total protein in 0.08 ml volume (1/25th of vaccine dose) of O_1 Manisa recombinant vaccine. A second dose was administered after 21 days interval. All the vaccinated and unvaccinated controls were challenged intra-dermo-lingually at two sites on 28 days post vaccination (dpv) with 10^4 cattle infective dose 50% of O_1 Manisa cattle challenge virus. The rectal temperature and FMD specific clinical signs were recorded for 8 days post challenge.

2.7. Sample collection and processing

Blood samples were collected on 0, 7, 14 and 21 dpv and on 0 (28 dpv), 5, 8 and 18 days post challenge (dpc) to evaluate antibodies against structural and non-structural proteins. Sterilized cotton swabs (Copan Flock Technologies, Italy) were used to collect nasal secretions on days 0, 3, 5, 8 and 18 dpc for virus isolation (Vi) and quantitative real-time RT-PCR (qRT-PCR). The oro-pharyngeal fluid (OPF) were collected from the upper oesophagus and pharynx using a small probang sampling cup on 0, 8 and 18 dpc for qRT-PCR and Vi.

2.8. Virus isolation

Nasal secretions and probang samples were examined for the presence of live virus using bovine thyroid (BTY) primary cells (Snowdon, 1966). BTY tubes were inoculated with 250 μl of the sample (five tubes per sample) and incubated in a stationary position for 30 min at 37 °C. BTY cells were then gently washed with 0.04 M phosphate buffer containing antibiotics. The washed cells were added with 2 ml of virus maintenance medium and incubated at 37 °C on roller drums. The cells were examined for CPE at periodic time intervals of 24, 48 and 72 h post inoculation. BTY cell culture supernatants with no signs of CPE after 72 h were pooled and repassaged once. The presence or absence of FMDV in culture supernatant was also verified by antigen ELISA (Hamblin et al., 1984).

2.9. Virus neutralizing antibody test (VNT)

Virus neutralization test was performed for the sera in flat-bottomed tissue culture grade micro titre plates (Nunclon™, Denmark) as described previously (Golding et al., 1976). Antibody titres were expressed as the reciprocal of the highest dilution of serum in the serum/virus mixture which neutralized an estimated 100 $TCID_{50}$ of virus at the 50% end-point (Karber, 1931). Cell culture adapted FMDV O_1 Manisa was used in the experiment.

2.10. Non-structural protein antibodies

Antibodies to FMDV non-structural protein (NSP) 3ABC were tested using PrioCHECK® FMDV NS kit (Prionics Lelystad B.V., The Netherlands; Sorensen et al., 1998) following the procedure recommended by the manufacturer. Samples were considered positive if the percentage of inhibition was $\geqslant 50$.

2.11. Quantitative real-time RT-PCR assay for detection of viral RNA

The viral RNA in nasal secretions and probang samples were quantified by qRT-PCR (Shaw et al., 2007). The total nucleic acid was extracted from liquid samples with MagNApure LC total nucleic acid isolation kit (Roche Diagnostics, Germany) in an automated nucleic acid robotic workstation (MagNApure LC, Roche Diagnostics, Germany). For the generation of standard curves, a FMDV RNA standard was synthesized *in vitro* from a plasmid containing a 79 base pair insert of the internal ribosomal entry site (IRES) of a type O FMDV (kindly provided by Dr. Donald P. King, Institute for Animal Health, UK) using a MEGAScript™ T7 kit (Ambion, USA) as described previously (Quan et al., 2004). The realtime PCR was performed in an IQ®5 Multicolor Real-time PCR detection system (BioRad, USA). The results from all samples were analyzed using Bio-Rad iQ®5 optical system software and CT values were assigned to each reaction (Reid et al., 2002). Viral RNA was quantified using a standard curve derived from the standard RNA preparation of various copy numbers (10^8–10^1).

2.12. Statistical analyses

The results of VNT was analyzed by one way ANOVA, and qRT-PCR from the nasal secretions and probang samples were analyzed by student '*t*' test.

3. Results

3.1. Generation of recombinant baculovirus and expression of P1-2A-3C in insect cells

Recombinant baculovirus clone containing FMDV P1-2A-3C expression cassette was created by co-transfecting baculovirus DNA and the transfer vector plasmid having the P1-2A-3C coding sequence. Supernatant from the transfected cells were subjected to plaque purification and the recombinant clones were identified by performing FMDV antigen ELISA. The clone with the highest OD_{492} value in the antigen ELISA was selected for further characterization. The Sf9 cells infected with the FMDV P1-2A-3C baculovirus clone produced specific fluorescence when stained with mAbs, O12E7 and O3E7 (Fig. 1a).

3.2. FMDV VLP production, characterization and quantification

The baculovirus clone was scaled up to a titer of $>10^8$ pfu/ml as determined by plaque assay. The Sf9 cells in suspension were infected with the recombinant baculovirus clone. Infected cells were harvested 5 days post infection and the VLPs were partially purified using 2% cross linked agarose beads. The partially purified preparation contained 30 μg/ml of FMD VLP as determined by IC ELISA ($R^2 = 0.9959$). TEM analysis of the VLP preparation revealed capsid particles similar to the size of FMDV (25 ± 5 nm) suggestive of VLPs (Fig. 1b). The preparation was also resolved in a 12% SDS–PAGE, transferred onto PVDF membrane and probed either using FMDV-type-O VP1 or VP2 specific polyclonal sera. The protein bands corresponding to the sizes of the individual structural proteins (VP1 and VP2) and intermediate proteins (VP1 + VP3 and VP0) could be identified in the immuno-blot, indicating the processing of the P1-2A poly-protein by protease 3C into their cleavage products. The protein profile using VP1 polyclonal sera was similar to those obtained using FMDV-type-O (O_1 Manisa) virus concentrate (Fig. 2).

3.3. Neutralizing antibody response

Four vaccinated cattle from full dose group (#5798, #5859, #5944 and #6073), two vaccinated cattle from 1/5 dose group (#6175 and #6064) showed serum neutralizing antibody response (SN_{50}) of $>10^{1.2}$ on the day of challenge. Both the unvaccinated

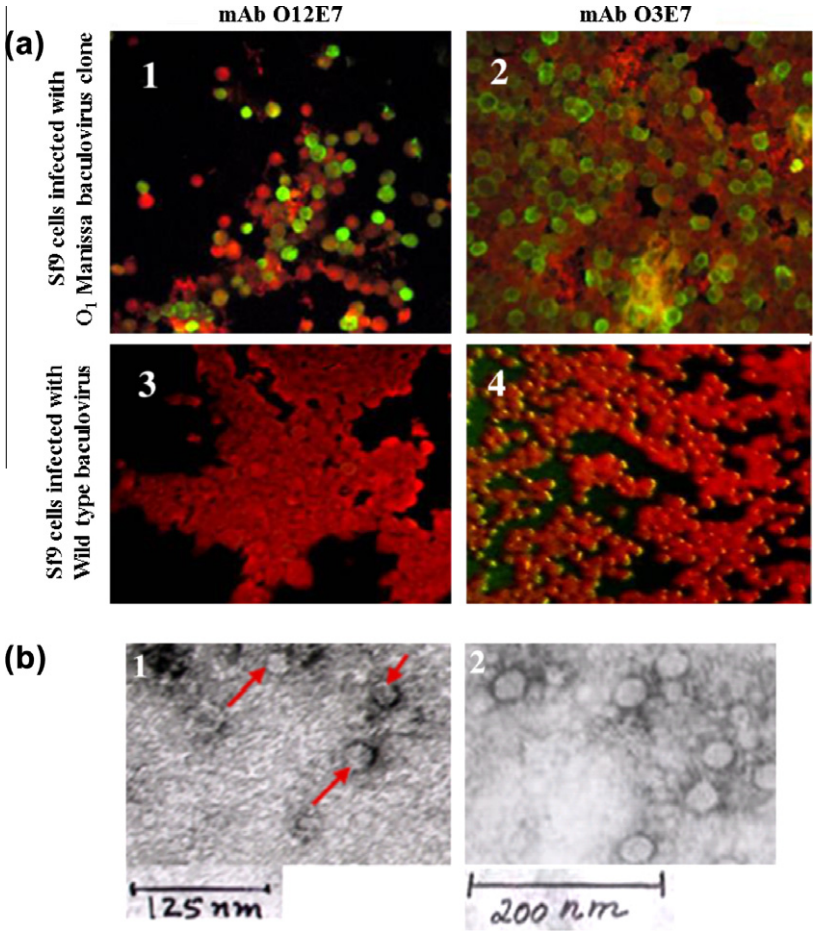

Fig. 1. (a) Immuno-fluorescence test using FMDV VP1 specific mAbs. Panel 1 and 2 are Sf9 cells infected with recombinant P1-2A-3C baculovirus clone and detected using FMDV serotype O specific monoclonal antibodies O12E7 and O3E7, respectively. Panel 3 and 4 contains Sf9 cells infected with wild type baculovirus and developed using mAb O12E7 and O3E7, respectively. (b) Electron micrograph (TEM picture) for recombinant FMDV VLPs. The VLPs are shown highlighted using arrow heads. Panel 1: O_1 Manisa VLPs; Panel 2: O_1 Manisa virus.

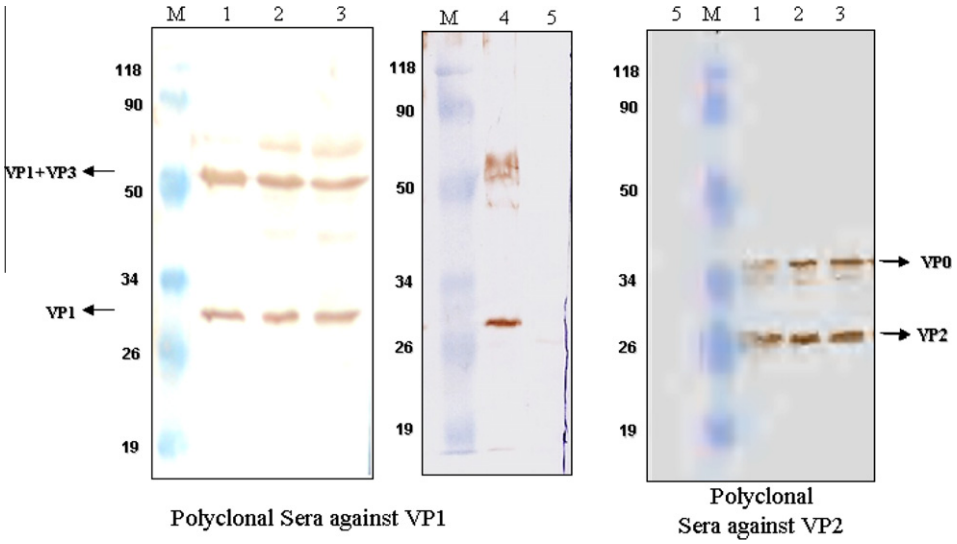

Fig. 2. Immuno-blots probed using FMDV capsid protein (VP1 or VP2) specific rabbit sera. Three different batches of partially purified P1-2A-3C preparations were tested and O_1 Manisa virus concentrate was used for comparison. The presence of individual capsid proteins and its intermediary forms confirms that the polyprotein P12A was processed by the protease 3C. 1–3: Sf9 cells infected with P1-2A-3C baculovirus clone (three different batches); 4: O_1 Manisa virus concentrate; 5: Sf9 cells infected with wild type baculovirus.

cattle did not show any neutralizing antibody response on the day of challenge. Neutralizing antibody titres on 28 dpv averaged to 1.210 (95% CI; 0.610–1.985), 0.760 (95% CI; 0.610–1.062) and 0.940 (95% CI; 0.610–1.482) $\log_{10}SN_{50}$ for groups full dose, 1/5

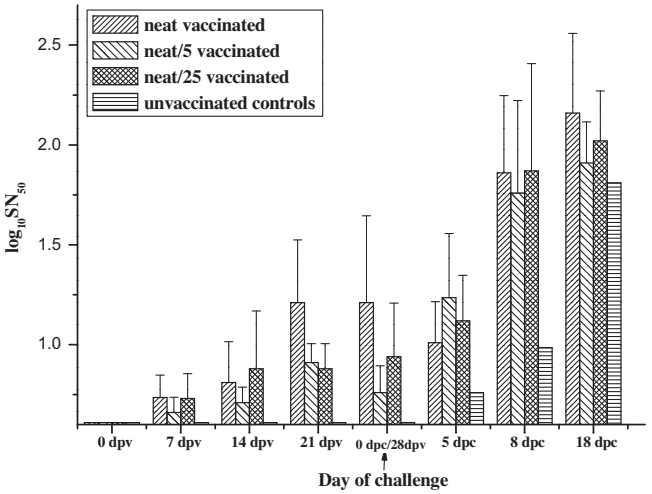

Fig. 3. FMDV specific neutralizing antibody response in groups of animals immunized with VLP vaccine. The antibody titers were monitored from 0 dpv to 18 dpc. Error bars indicate standard deviation. The neutralizing antibody titers were evident from 7 dpv in vaccinated animals. However, the mean antibody titers were higher in the groups of animals immunized with full dose vaccine. None of the unvaccinated animals showed sero-conversion before challenge.

and 1/25 dose, respectively. The full dose vaccinated group showed significantly higher (one way-*ANOVA*, $p < 0.05$) antibody response on 28 dpv (Fig. 3).

3.4. Protection from challenge

Both the unvaccinated control cattle developed generalized disease with visible vesicles on all four feet by day 2. Three out of six cattle each vaccinated with full dose, 1/5 dose recombinant FMDV vaccine and three out of five in 1/25 dose were protected against the development of vesicles following challenge with FMDV O_1 Manisa virus (Table 1) as one cattle from 1/25 dose group died before challenge. All the unprotected animals of the vaccinated groups showed pyrexia ($\geqslant$39.5 °C) 4 to 7 days post challenge whereas the unvaccinated control animals showed pyrexia at 2 to 3 days post challenge (Table 1). Based on the cattle challenge experiment, the 50% protective dose (PD_{50}) value for the FMDV P1-2A-3C vaccine was 5.01.

3.5. Virus isolation and quantitation of FMDV RNA from nasal and probang samples

The results of virus isolation and viral RNA quantification from nasal secretions are summarised in Table 2. Significantly high mean viral RNA ($p < 0.05$) was found in the nasal swabs of unvaccinated animals, 1/5 and 1/25 dose groups of vaccinated animals compared to the full dose vaccinated animals on 8 and 18 dpc. Virus was isolated from the nasal swabs collected on 3 and 5 dpc from both (#3582 and #3586) the unvaccinated control animals (Table 2). However, virus could be isolated from only one animal each from full dose group (#5871) and 1/25 dose group (#6059) at 5 dpc.

The results of virus isolation and viral RNA quantification from probang samples are summarised in Table 3. The mean viral RNA copy numbers were significantly higher ($p < 0.05$) from OP fluid collected in unvaccinated cattle compared to the cattle in vaccinated groups at 18 dpc. Virus was isolated from two (# 5871 and #6073) out of six cattle of full dose group, five (#6166, #6119, #6057, #6115 and #6169) out of six cattle of 1/5 dose group and two (#6059 and #6113) out of five cattle of 1/25 dose group at 8 dpc. Virus was isolated at 8 and 18 dpc from both unvaccinated control animals (#3582 and #3586; Table 3).

3.6. NSP antibody response

The results of NSP serology are summarized in Table 4. Two vaccinated cattle from full dose group (#6019 and #6073), five cattle from 1/5 dose group (#6047, #6166, #6119, #6057 and #6115) and one cattle in 1/25 dose group (#6064) were positive for NSP antibody response at least once in between 5 and 18 dpc. Both

Table 1
Clinical signs and protection status of the cattle during the challenge experiment. The rectal temperature scores and number of feet having FMDV vesicles until 8 dpc are provided in the table. Potency of the vaccine was determined based on the challenge experiment results using reed and muench calculation (Indian pharmacopoeia, 2010). The potency of the vaccine preparation was found to be 5.01.

Groups	Animal no.	$\log_{10}$ SN_{50} on 0 dpc	Clinical score of rectal temperature on dpc[a]								No. of feet having vesicles on dpc								Percentage protection
			1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	
Recombinant FMDV vaccine – neat	5798	1.210	0	0	0	0	1	2	0	0	0	0	0	0	0	1	3	3	3/6 (50%)
	5871	0.760	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	
	5859	1.510	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	5944	1.510	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	6019	0.610	0	0	0	1	2	2	0	0	0	0	0	0	4	4	4	4	
	6073	1.660	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Recombinant FMDV vaccine – neat/5	6047	0.910	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3/6 (50%)
	6166	0.610	0	0	0	1	2	1	0	0	0	0	0	0	4	4	4	4	
	6119	0.910	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	
	6057	0.610	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	6115	0.760	0	0	0	0	0	2	0	0	0	0	0	2	4	4	4	4	
	6169	0.760	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Recombinant FMDV vaccine – neat/25	6175	1.210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3/5 (60%)[b]
	6059	0.910	0	0	0	0	1	2	0	0	0	0	0	0	4	4	4	4	
	6113	0.760	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	6064	1.210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	6156	0.610	0	0	0	0	0	2	0	0	0	0	0	0	4	4	4	4	
Unvaccinated controls	3582	0.610	0	1	2	0	0	0	0	0	0	4	4	4	4	4	4	4	0/2 (0%)
	3586	0.610	0	1	2	0	0	0	0	0	0	4	4	4	4	4	4	4	

[a] Rectal temperature was scored as follows: $\leqslant$39.5 °C = 0, $\geqslant$39.5 °C to 40.0 °C = 1, $\geqslant$40.0 °C = 2.
[b] One animal in this group died before challenge.

Table 2
The mean FMDV RNA copy numbers and virus isolation results for the nasal swab collected on different dpc. The mean RNA copy numbers of vaccinated groups were compared with control animals and the significantly less mean values were indicated.

Groups	Animal No.	0 dpc		3 dpc		5 dpc		8 dpc		18 dpc	
		VI	qRT-PCR[c]	VI	qRT-PCR[c]	VI	qRT-PCR[c]	VI	qRT-PCR[c]	VI	qRT-PCR[c]
Recombinant FMDV vaccine – neat	5798	–[a]	0.00	–	7.17	–	6.26	–	4.91	–	6.19
	5871	–	0.00	–	5.70	+[b]	5.88	–	4.43	–	0.00
	5859	–	0.00	–	5.73	–	5.16	–	0.00	–	0.00
	5944	–	0.00	–	5.37	–	0.00	–	4.73	–	0.00
	6019	–	0.00	–	7.27	–	5.38	–	0.00	–	0.00
	6073	–	0.00	–	5.52	–	6.05	–	0.00	–	5.61
	mean		0.00		6.13		4.79		2.35**		1.97*
	SD		0.00		0.86		2.38		2.57		3.05
Recombinant FMDV vaccine – neat/5	6047	–	0.00	–	6.49	–	4.67	–	4.74	–	0.00
	6166	–	0.00	–	7.83	–	6.14	–	6.12	–	5.41
	6119	–	0.00	–	8.19	–	5.07	–	5.49	–	0.00
	6057	–	0.00	–	6.56	–	4.98	–	5.21	–	0.00
	6115	–	0.00	–	7.11	–	5.46	–	5.36	–	5.46
	6169	–	0.00	–	7.39	–	0.00	–	4.93	–	5.41
	mean		0.00		7.26		4.39		5.31		2.71
	SD		0.00		0.68		2.21		0.48		2.97
Recombinant FMDV vaccine – neat/25	6175	–	0.00	–	6.66	–	5.40	–	5.53	–	5.71
	6059	–	0.00	–	6.80	+	6.59	–	5.20	–	5.45
	6113	–	0.00	–	6.30	–	5.16	–	5.28	–	5.62
	6064	–	0.00	–	6.29	–	5.79	–	5.12	–	5.57
	6156	–	0.00	–	5.98	–	5.65	–	5.51	–	5.61
	mean		0.00		5.34		5.72		5.33		5.59
	SD		0.00		2.63		0.55		0.18		0.10
Unvaccinated controls	3582	–	0.00	+	6.75	+	6.20	–	6.18	–	5.85
	3586	–	0.00	+	6.85	+	6.53	–	6.98	–	5.01
	mean		0.00		6.80		6.37		6.58		5.43

[a] Negative for virus isolation.
[b] Positive for virus isolation.
[c] Log10 copy numbers of FMDV RNA per swab.
* $p < 0.05$.
** $p < 0.01$.

the unvaccinated control animals (#3582 and #3586) were positive for NSP antibody response at either 5–18 dpc or 8–18 dpc.

4. Discussion

The baculovirus expression vector system (BEVS) using insect cells has been used for producing complex proteins including VLPs. Vaccines produced by BEVS are commercially available for veterinary and human use (Cox, 2012). Protein E2 based vaccine against classical swine fever virus (Porcilis Pesti®, Merck) and VLP based vaccine against porcine circovirus 2 (Porcillis® PCV, Merck; Circo-FLEX®, Boehringer Ingelheim) are produced in insect cell lines, and has been approved for veterinary use (Blanchard et al., 2003; Fachinger et al., 2008; van Aarle, 2003).

FMDV serotype O is the most prevalent serotype in India (PD FMD Annual Report, 2011) and many parts of the world. The FMDV O_1 Manisa strain is listed by world reference laboratory as the high priority strain to be included in FMDV antigen banks (World reference laboratory quarterly report, October–December 2011). Given the importance of the FMDV strain, we explored the possibility of preparing a recombinant VLP vaccine for use in animals. Additionally, the development of FMDV serotype O VLPs has not been reported so far.

In the present study, the P1-2A and 3C protein coding regions of O_1 Manisa strain was codon optimized for insect cell expression and synthesized as a single construct. This construct was used to express the capsid protein precursor P1-2A along with the protease 3C as a single polyprotein. The FMDV structural protein expression was confirmed by immuno-fluorescent test using VP1 specific mAbs and FMDV antigen ELISA (Hamblin et al., 1984). The expression of protease 3C and its ability to process the polyprotein was evident from the presence of individual structural proteins in the immune-blot, which was developed using VP1 or VP2 protein specific sera. Additional protein bands corresponding to the sizes of intermediary proteins of 3C cleavage were also observed in the immune-blot. As described by the previous reports, these intermediary bands were not uncommon (Cao et al., 2009; Oem et al., 2007) and a similar protein profile was also observed in FMDV whole virus preparations. The intact P1-2A protein band was not detectable in the immuno-blot, unlike the previous reports wherein the P1-2A and 3C proteins were expressed using two different promotors (polyhedrin and p10; Cao et al., 2009). In the present study, these proteins were expressed as a single polyprotein from polyhedrin promotor. The expression of equimolar concentration of P1-2A and 3C proteins *in-cis* could be the reason for near complete processing of P12A into its individual components or their intermediates like VP1 + VP3.

Western-blot developed with the VP2 specific polyclonal sera revealed a protein band corresponding to the size of VP2 in addition to the VP0 band, indicating a partial cleavage of VP0 even in the absence of genomic RNA packaging. Since the VP0 processing is often referred as maturation cleavage, that occurs at the time of capsid assembly and encapsidation, this cleavage event has to be studied further to know more about the possibilities of VP0 processing in the absence of RNA packaging (Curry et al., 1997). Despite the processing events, as described above, the assembled VLPs were not numerous when observed in transmission electron microscopy. Future studies have to be aimed at obtaining the FMDV VLPs which are efficiently assembled and stable.

Table 3
The mean FMDV RNA copy numbers and virus isolation results for the OP fluid collected on different dpc. The mean RNA copy numbers of vaccinated groups were compared with control animals and the significantly less mean values were indicated.

Groups	Animal no.	0 dpc		8 dpc		18 dpc	
		VI	qRT-PCR[c]	VI	qRT-PCR[c]	VI	qRT-PCR[c]
Recombinant FMDV vaccine – neat	5798	–[a]	0.00	–	6.13	–	6.34
	5871	–	0.00	+[b]	6.72	–	0.00
	5859	–	0.00	–	6.40	–	0.00
	5944	–	0.00	–	0.00	–	0.00
	6019	–	0.00	–	0.00	–	0.00
	6073	–	0.00	+	7.01	–	0.00
	mean		0.00		4.38		1.06**
	SD		0.00		3.4		2.59
Recombinant FMDV vaccine – neat/5	6047	–	0.00	–	0.00	–	6.03
	6166	–	0.00	+	7.57	–	0.00
	6119	–	0.00	+	7.44	–	0.00
	6057	–	0.00	+	6.29	–	0.00
	6115	–	0.00	+	6.30	–	0.00
	6169	–	0.00	+	0.00	–	7.08
	mean		0.00		4.60		2.18*
	SD		0.00		3.6		3.4
Recombinant FMDV vaccine – neat/25	6175	–	0.00	–	6.36	–	5.12
	6059	–	0.00	+	0.00	–	0.00
	6113	–	0.00	+	7.93	–	0.00
	6064	–	0.00	–	6.22	–	0.00
	6156	–	0.00	–	7.01	–	0.00
	mean		0.00		5.50		1.02**
	SD		0.00		3.15		2.29
Unvaccinated controls	3582	–	0.00	+	7.22	+	6.32
	3586	–	0.00	+	7.22	+	7.12
	mean		0.00		7.72		6.72

[a] Negative for virus isolation.
[b] Positive for virus isolation.
[c] Log_{10} copy numbers of FMDV RNA per ml of probang samples.
* $p < 0.05$.
** $p < 0.01$.

Table 4
The serum NSP antibody response in the cattle used for challenge experiment. Antibodies against FMDV NSP were detected using PrioCHECK® FMDV NS ELISA kit.

Groups	Animal no.	0 dpv	0 dpc	5 dpc	8 dpc	18 dpc
Recombinant FMDV vaccine – neat	5798	N	N	N	N	N
	5871	N	N	N	N	N
	5859	N	N	N	N	N
	5944	N	N	N	N	N
	6019	N	N	N	N	P
	6073	N	N	N	P	P
Recombinant FMDV vaccine – neat/5	6047	N	N	N	P	N
	6166	N	N	N	P	N
	6119	N	N	N	N	P
	6057	N	N	P	P	P
	6115	N	N	N	N	P
	6169	N	N	N	N	N
Recombinant FMDV vaccine – neat/25	6175	N	N	N	N	N
	6059	N	N	N	N	N
	6113	N	N	N	N	N
	6064	N	N	N	P	P
	6156	N	N	N	N	N
Unvaccinated controls	3582	N	N	N	P	P
	3586	N	N	P	P	P

N – negative for NSP antibody; P – positive for NSP antibody.

The insect cell expressed P1-2A-3C was formulated as vaccine and potency of the vaccine was evaluated in cattle. The partially purified VLP preparation having total protein content of 75 μg was used in preparing the vaccine. The VLP content of the preparation was 2.5 μg per dose as estimated by IC-ELISA. The mean serum neutralization titers were highest in the full dose vaccine group after one booster vaccination. Virus could be isolated from the OP fluid/nasal swab of the cattle, which were challenged with live vius. Similarly, the viral RNA also could be detected from the OP fluid and nasal swabs using qRT-PCR. However, the virus isolation and qRT-PCR results did not correspond and similar observations were made by others also using inactivated vaccine (Cox et al., 2005). Moreover, virus isolation and qRT-PCR results were positive in many animals irrespective of the systemic infection and these

results indicate that the virus could replicate locally even in the absence of generalization of the infection. Similar results were obtained with inactivated vaccines also, despite employing a high potency vaccine (18 PD_{50}; Cox et al., 2007, 2005). Further, the percentage protection values had not decreased in a dose dependent manner, in the cattle challenge experiment. This observation is also consistent with the earlier reports in the FMDV potency tests using inactivated vaccines (Goris et al., 2007; Vianna Filho et al., 1993). However, the mean serum antibody titers and the number of cattle with substantial serum neutralization titers on 0 dpc (28 dpv) were higher in full dose vaccinated group of cattle compared to the cattle in 1/5 and 1/25 dose vaccinated groups. It was intriguing that few of the cattle with less serum neutralizing titers were protected against the virus challenge. One cattle (#6057) in 1/5 dose group, in particular, did not develop clinical disease, though there was no neutralizing antibody titer on the day of challenge. The PD_{50} per dose of the recombinant vaccine was 5.01 which is similar to the PD_{50} value obtained using a vaccine preparation containing silk worm expressed FMDV serotype Asia1 VLPs. When the hemolymph of the silk worms containing the serotype Asia 1 VLPs were tested as vaccine, PD_{50} values ranging from 5.2 to 6.5 was reported, depending on the challenge virus used (Li et al., 2011, 2008).

5. Conclusion

As per the OIE recommendation and pharmacopoeia (British Pharmacopoeia vet, 2010; European Pharmacopoeia 2010; Indian Pharmacopoeia, 2010), the minimum potency required for the inactivated FMDV vaccine is $\geqslant 3$ PD_{50}. The recombinant P1-2A-3C vaccine containing $\sim$2.5 μg of VLPs produced 5.01 PD_{50} value in a cattle challenge experiment. Therefore, producing VLP based vaccine against FMDV using the insect cell expression platform holds the promise of a safe and potent vaccine. However, the commercial application of such a vaccine will depend on the cost of the vaccine, its utility as a marker vaccine and also on finding a method to increase the percentage protection or potency of the vaccine.

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