Archives of Veterinary Research Vol. 1 (1): 16-26 (2024)

Open access

Contents lists available at Pensive Academic Publishing

Archives of Veterinary Research

Journal homepage: https://pensive-acadpub.com/

Original research

Human adenovirus type 5 expressing FMDV serotype O (strain R2/75) capsid proteins and its immunogenicity in guinea pigs

Ramesh Kumar ✉, B. P. Sreenivasa, Madhusudan Hosamani, Suresh H. Basagoudanavar and Mouttou Vivek Srinivas

FMD Vaccine Research Laboratory, ICAR- Indian Veterinary Research Institute, Hebbal, Bangalore, Karnataka - 560024, India

Article history:

Received 14 November 2024
Accepted 02 December 2024
Published 12 December 2024

Keywords:

Foot-and-mouth disease
Virus like Particles
FMD VLP vaccine
Adenoviral expression system
New generation vaccine
Guinea pigs

Correspondence:
Dr. Ramesh Kumar ✉
drrkvet13@gmail.com

DOI
https://doi.org/10.70964/avr.5

ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious illness that significantly impacts cloven-hoofed animals and is of transboundary importance. The inactivated FMD vaccine is commonly used in countries like India to control the disease. Regular vaccination has helped to eradicate FMD in some countries and there are several concerns associated with the production and use of inactivated vaccines for FMD, which includes high costs associated with bio-containment facilities, potential risk of the virus escaping into the environment, and the vaccine's sensitivity to temperature changes. Therefore, the present study was aimed to develop a new generation FMD VLP vaccine using a Human adenovirus type-5 based expression system. In our study, the recombinant adeno-FMD virus showed efficient expression of the FMDV capsid proteins, which self-assembled into VLPs was observed by electron microscopy. The adeno-FMD vaccines protected fewer animals than conventional inactivated virus vaccines after homologous challenge. Recombinant adenovirus containing mutant $3C^{pro}$ was found superior to its wild-type counterpart in terms of protection. As no vaccine in the present study has given 100% protection, additional dose-response studies may provide information regarding the protective dose in guinea pigs. The data generated in the study suggest that the adeno-FMD vaccines may be an ideal alternative to the conventional inactivated virus vaccine.

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of transboundary importance affecting cloven-hoofed animals (Grubman and Baxt, 2004). The disease is produced by a highly divergent small RNA virus of the genus Aphthovirus in the family Picornaviridae. It causes significant distress and suffering to animals, affecting the livelihood of farmers and producing huge economic losses. Inactivated FMDV vaccine has been widely used to control and prevent the disease in enzootic countries like India, and regular vaccination has contributed to eradicating FMD in some countries (Doel, 2003). However, due to certain concerns and limitations of the inactivated vaccine regarding its use and production, such as the high cost of bio-containment facilities to handle live FMDV potential risk of escape of the virus to the environment (Barteling and Vreeswijk, 1991), thermo labile (Rueckert, 1996), and not DIVA enabled; there is a need to develop an alternate vaccine that overcomes these limits and can produce an early and long-lasting protective immune response.

The development of a new generation of FMD vaccines has been extensively explored by various researchers all over the world. The basic concept followed in various approaches to design new generation vaccines has been the delivery of the FMD virus capsid protein genes responsible for forming viral empty capsid particles (VLPs) or part thereof (Srinivas et al., 2015). These VLPs mimic the virion regarding immunogenicity but lack infectivity, making it safe (Plummer and Manchester, 2011, Srinivas et al., 2016). The VLPs present the viral antigens in more authentic conformation than other subunit vaccines (Rweyemamu et al., 1979) and induce an efficient humoral and cellular immune response (Mayr et al., 1999; Kushnir et al., 2012). Therefore, the present study was aimed to

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develop a new generation FMD VLP vaccine using a Human adenovirus type-5 based expression system.

Materials and methods

Ethical approval

All genetic manipulations for the production of recombinant adenoviruses expressing FMDV capsid proteins were carried out after obtaining necessary approval from the Institute Biosafety Committee (IBSC) and Review Committee on Genetic Manipulation (RCGM), Department of Biotechnology, Government of India. Animal experiments were carried out after approval of the experiment in the prescribed format by the IAEC, IVRI Bangalore, India.

Cells and cell culture

The human embryonic kidney (HEK-293) cell line (Agilent Technologies, USA) was grown in Eagle's minimum essential medium (EMEM) (Lonza, Belgium) containing 10% fetal bovine serum (FBS), 25mM HEPES, non-essential amino acids (Lonza, Belgium), penicillin (100IU/ml) and streptomycin (100µg/ml). HeLa cells (ECACC, UK) and A549 cells (ECACC, UK) were grown in EMEM and Dulbecco's modified Eagle's medium (HiMedia, India) containing 10% FBS, respectively. All the cells were maintained in respective medium containing 1-2% FBS and incubated at 37°C in a humidified CO_2 (5%) incubator (Thermo Scientific, USA) during growth and maintenance.

Viruses and their propagation

The recombinant human adenovirus type 5 expressing FMDV serotype-O capsid proteins (hAd5/P1-2AB3BC$_{wt}$ and hAd5/P1-2AB3BC$_{m}$) were constructed in our laboratory using AdEasy adenoviral vector system (Kumar et al., 2015a) was used in this study. To check the presence of replication-competent adenoviruses (RCA) at a particular passage level, both the viruses were serially passaged in the HEK-293 cells for upto 12 passage level and purified by CsCl density gradient ultracentrifugation as described earlier (Kumar et al., 2015a).

Selection of the vaccine virus

Both viruses at passage levels 2, 4, 6, 8 and 12 were tested for the presence of RCA as described earlier (Kumar et al., 2015b) and the 2nd passage of both viruses free from RCA were selected for vaccination purposes.

Processing of cells for expression studies

Before vaccination, both the recombinant adenoviruses were evaluated for their expression of FMDV capsid proteins in vitro. Both HEK-293 and A549 cells (~10^7 cells) were infected with the recombinant adenoviruses, and the infected cells were harvested at optimum CPE. The cells were lysed by re-suspending the cell pellet in 500µL 1% Triton X-100. The expression of the target proteins was examined using different assays.

Expression study of the target proteins

Sandwich ELISA

In sandwich ELISA, the ELISA plates were coated with polyclonal rabbit anti-146S FMDV type O antibody diluted in a coating buffer (carbonate-bicarbonate buffer, pH 9.6). After three washes with washing buffer (PBST, phosphate buffer saline containing 0.05% Tween-20), 50µL of the test antigen was added to each well of the ELISA plates in duplicate. The plates were incubated at 37°C for 1h. The plates were washed as above, and 50µL of polyclonal guinea pig anti-146S FMDV type O antibody diluted in blocking buffer (PBST with 5% adult bovine serum) was added to each well of the plates followed by incubation at 37°C for 1h. The plates were washed as above, and 50 µL of conjugate (polyclonal rabbit anti-guinea pig immunoglobulin HRP diluted as 1: 3000 in blocking buffer) was added to each well, followed by incubation at 37°C for 1h. The plates were washed as above, and 50µL of substrate solution (0.05% H2O2 and 0.05% OPD in citrate buffer) was added to each well, followed by incubation at 37°C for 10min. Then, the reaction was stopped by adding 50µL of 1M H_2SO_4, and the plates were read at 492 nm in the ELISA plate reader (Tecan, Austria).

Western blot

In western blot, clarified cell lysate was mixed with 3´ sample loading buffer and denatured at 95°C for 5 min. Then, 20 µL of each test sample was loaded per well of the SDS-poly-acryl-amide gel, and electrophoresis was carried out in a Mini-PROTEAN Tetra cell apparatus (Bio-Rad, USA). After completion of the SDS-PAGE, the gel proteins were transferred to the PVDF membrane using a semi-dry transfer unit (Amersham Biosciences, USA). Then, the membrane was removed and fixed with methanol for 30 sec, followed by blocking (PBST with 5% skim milk powder) at 37°C and 50 rpm for 1h. The membrane was washed thrice with PBST, and the antigens of interest were probed with polyclonal rabbit anti-146S FMDV type O serum at 37°C and 50 rpm for 1h. The membrane was washed with PBST and incubated with HRP-conjugated polyclonal goat anti-rabbit immunoglobulin (diluted 1:4000 in PBS) at 37°C and 50 rpm for 1h. Then, the membrane was washed and treated with DAB-urea substrate at room temperature for 1-2 min with continuous shaking. The reaction was stopped by discarding the solution and rinsing the membrane with distilled water. The membrane was air-dried, and the band size was recorded using the standard protein marker.

Immunofluorescence assay

An indirect immunofluorescence assay was also performed to detect the expression of the target protein.

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The HEK-293 and A549 cells were grown in 24 well tissue culture plates as monolayer and infected with the recombinant adenoviruses at multiplicity of infection (MOI) of 0.1 and 100, respectively. After 72h, when CPE appeared as viral plaques in HEK-293 cells and disturbed the morphology of A549 cells, the immunofluorescence assay was carried out. The spent media was removed, and the monolayer was washed twice with pre-warmed DPBS++ and air-dried. The cells were fixed with chilled acetone-methanol solution at RT for 10 min. The fixative was removed, and 500 µL of blocking solution (3% bovine serum albumin in PBS) was added to each well of the plate, followed by incubation at room temperature for 30 min. Polyclonal rabbit anti-146S FMDV type O serum was used to probe the expressed FMDV antigen at 37°C for 1h. The cells were washed thrice with PBST, and then 200 µL of Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, USA), diluted 1: 2000 in PBS, and was added to each well of the plate. The plate was incubated at 37° C for 45 min. The reaction mix was discarded, and the unbound antibodies were washed away. The cells emitting green fluorescence were observed under a fluorescence microscope (Nikon, Japan).

Transmission electron microscopy (TEM)

The HEK-293 and A549 cells (~5x10^8cells) grown as monolayer were infected with hAd5/P1-2AB3BCwt-P2 at an MOI of 10 and 100, respectively. The infected cells were harvested separately at the completion of CPE or latest, by 72h post-infection (hpi) and re-suspended in 30 mL of DPBS containing 1% Triton X-100 and protease inhibitors cocktail. The cell lysates were incubated on ice for 30 min and clarified by centrifugation. The supernatant was collected and overlaid on 5mL of 30% sucrose in DPBS in 36mL polyallomer ultracentrifuge tubes (Seton Scientific, USA). The FMDV capsid proteins were pelleted at 1,00,000g for 90 min at 4°C in an ultracentrifuge (Sorvall WX80, Thermo Scientific). The supernatant was discarded, and the pellet was re-suspended in 3.5mL of DPBS containing protease inhibitors and 1000U of benzonase® and incubated at RT for 30 min with intermittent shaking. The solutions were clarified, and the supernatant was overlaid on 2mL of 30% sucrose in DPBS in 6mL ultra-centrifuge tubes. The capsid proteins were pelleted as above by ultracentrifugation. The supernatant was discarded, and the pellet was re-suspended in 500 µl of PBS for TEM. The clarified supernatant after benzonase® treatment was also fractionated using a 30-60% sucrose (in PBS) gradient, and the fraction showing the highest expression in the western blot was concentrated and subjected to TEM.

Ten µL of the test sample was applied on the copper grids coated with formvar-carbon film and allowed to adsorb for 10 min at room temperature. The residual volume of the sample was blotted out. The grid was washed twice with distilled water and stained with 1% uranyl acetate (w/v in distilled water) for 20 sec at room temperature. Excess stain was blotted out. The grid was washed four times with distilled water and dried for 2-3 hours. Then, the grid was placed on the grid holder and secured properly. The grid holder was fitted to the transmission electron microscope (Tecnai G212 BioTwin, FEI, USA). The microscope was operated at 120 kV, and the specimen was observed. The image was captured and analyzed with a CCD camera coupled with the microscope.

Experimental animals and immunization

The animal experiment was carried out with prior permission and approval from the Institutional Animal Ethical Committee (IAEC) as per the CPCSEA guidelines. Male guinea pigs weighing about 450 grams were selected and divided into four groups, each with six animals. The animals were immunized intramuscularly as per the schedule shown in Table 1, following a homologous prime-boost regimen. Mimicking the conventional FMD vaccine, an inactivated vaccine was formulated by blending inactivated FMDV antigen with Montanide® ISO-206 (Seppic, France). All the immunized animals were boosted at 28th day post vaccination (dpv) and challenged with 100GPID$_{50}$ of homologous virus at 14th day post booster (dpb). A schematic representation of immunization is given in Fig 1.

Sera from blood samples were collected at 0, 7, 14, 28 dpv, and on 7 and 14 dpb to analyze the humoral immune response.

Table 1. Immunization of guinea pigs

Group	Vaccine	Adjuvant	Dose /animal	Route
T-01	hAd5/P1-2AB-3BC^{wt}	-	5×10^8 pfu	i/m
T-02	hAd5/P1-2AB-3BC^{m}	-	5×10^8 pfu	i/m
T-03	Inactivated FMD vaccine	M-206	1.1 µg	i/m
T-04	dAd5	-	5×10^8 pfu	i/m

hAd5: human adenovirus type 5; wt: wild-type; m: mutant; dAd5: replication deficient hAd5; M-206: Montanide ISO-206, Seppic®; pfu: plaque forming units; i/m: intramuscular.

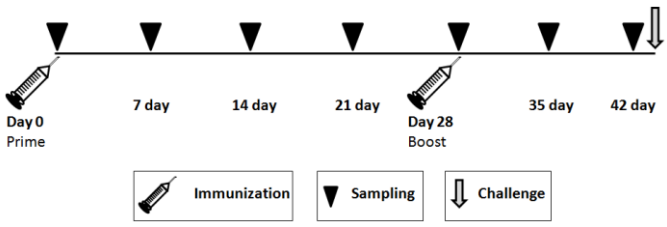

Fig 1. A schematic representation of immunization

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Virus neutralization assay to detect antibodies against FMDV

Starting from a 1/8 dilution, two-fold dilutions of the serum samples were prepared and dispensed in a 96-well tissue culture plate (50µL/well). Previously titrated FMDV containing 100$TCID_{50}$ (50% tissue culture infective dose) was added to each well. Different types of controls, such as a standard serum of known titre, a cell control, and a virus titration, are included in the test. The plates were incubated at 37°C for 1h in the CO_2 incubator. The BHK-21 cell suspension containing 10^6 cells/mL of the medium with 10% FBS was prepared. A volume of 50 µL of the BHK-21 cell suspension was added to each well, and the plates were incubated at 37°C for 2 days in the CO_2 incubator. The plates were read after 48h microscopically. A serum-neutralizing antibody titer was expressed as reciprocal to the highest dilution of serum, which protected 50% of the wells (Kärber, 1931).

Isotype ELISA to quantify IgG_1 and IgG_2 molecules

ELISA plates were coated with inactivated FMDV 'O' antigen (50ng/well). After washing and blocking steps, 100µl of test serum samples (diluted 1: 100 in PBST) were added in duplicate, and the plates were incubated at 37°C for 1h. FMDV type 'O' hyperimmune sera raised in guinea pigs were used as a positive control. Following washing, 1: 1000 dilutions of HRP-conjugated goat anti-guinea pig IgG_1 or IgG_2 were added to the respective plate wells. The plates were then incubated at 37°C for 1h, then washed and added 100µl of OPD substrate to each well. The reaction was stopped after 10 min of incubation at RT by adding 50 µL of 1M H_2SO_4, and absorbance of the wells was measured at 492nm by ELISA reader.

Challenge of the immunized animals

The immunized guinea pigs were challenged at 14 dpi with 100$GPID_{50}$ (50% guinea pig infectious dose) of FMDV serotype O by intradermal inoculation into one of the hind foot pads. All animals were monitored for major clinical signs of FMD, particularly characteristic vesicular lesions on the foot pads, for 7 consecutive days after the challenge. Animals showing primary lesions at the virus injection site without spreading to other feet throughout the observation period were considered protected. Animals showing secondary lesions on foot pads other than inoculated ones were considered unprotected.

Statistical analysis

ELISA absorbance readings from duplicate wells were used to calculate mean ± SD upon testing of expressed antigens collected at various time points. An absorbance of 0.1 or more in ELISA was considered positive. Serum-neutralizing antibody titre ($log_{10}SN_{50}$) of different groups of immunized guinea pigs at different time intervals are expressed as mean ± SE. A 't-test was used to compare the means of the two groups.

Results

Expression analysis of the target protein in the adenovirus expression system

The HEK-293 cells (~10^7 cells) infected with the adenoviruses at an MOI of 0.1, 1.0 and 10 were harvested at 96, 36, and 24 hpi, respectively, after completion of CPE, and the cell lysate was prepared as described in methods. The expression level of the target protein in HEK-293 cells was comparatively higher in the sample of 10 MOI compared to 0.1 and 1.0 MOI as detected by sandwich ELISA and presented in Table 2. Further, the expression of the target proteins was confirmed in western blot and immunofluorescence assay. Western blot result demonstrated that expression of the target protein in HEK-293 was very less (Fig. 2) and only detected in the HEK-293 cells infected with the virus at an MOI of 10. In the immunofluorescence assay, the infected cells emitted green fluorescence, indicating expression of the target proteins, while there was no fluorescence in non-infected control cells (Fig. 3).

Expression of the target protein was better at 3 dpi in A549 cells infected with the hAd5/P12AB3BC_{wt}-P2 virus at an MOI of 100 compared to infection at an MOI of 10. Sandwich ELISA and western blot results have demonstrated that the expression of the target protein was good at 3 dpi in A549 cells infected with the adenovirus at an MOI of 100, as presented in Table 2 and Fig. 2. Immunostaining of A549 cells infected with the adenoviruses has been shown in Fig. 4. The A549 cells emitting the green fluorescence represent the target protein expression in the adenovirus expression system.

Table 2. Expression analysis of the target protein in HEK-293 and A549 cells by sandwich ELISA

PC	NC	HEK-293			A549 cells (MOI 10)					A549 cells (MOI 10)		
FMDV 'O'	DPBS	MOI 0.1	MOI 1.0	MOI 10	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	1 dpi	2 dpi	3 dpi
0.501	0.062	0.318	0.403	0.520	0.107	0.161	0.399	0.708	0.813	0.237	0.905	1.348

PC: positive control; NC: negative control

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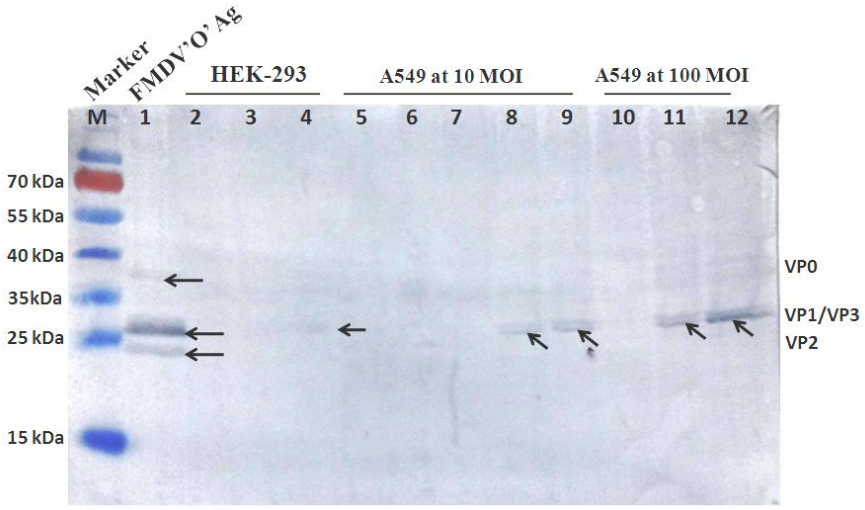

Fig 2. Western blot result demonstrated that expression of the target protein in HEK-293

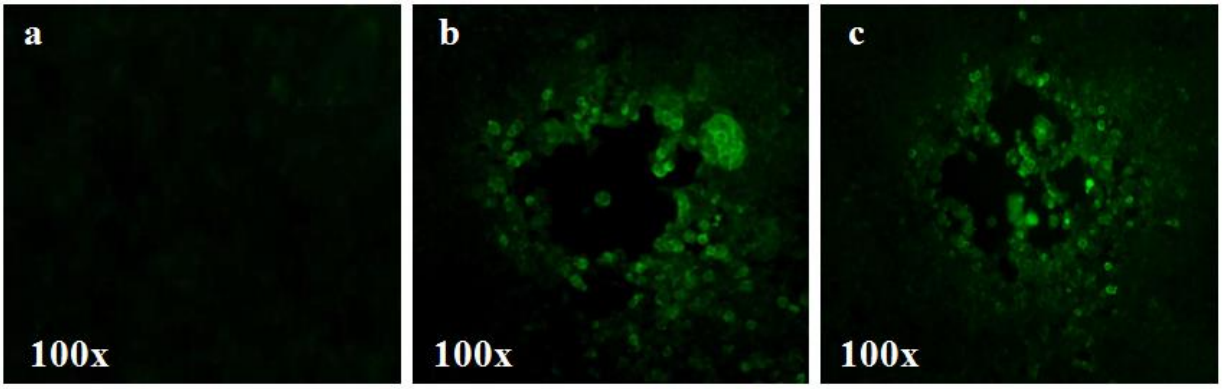

Fig 3. In the immunofluorescence assay, the infected cells (b & c) emitted green fluorescence, indicating expression of the target proteins, while there was no fluorescence in non-infected control cells (a)

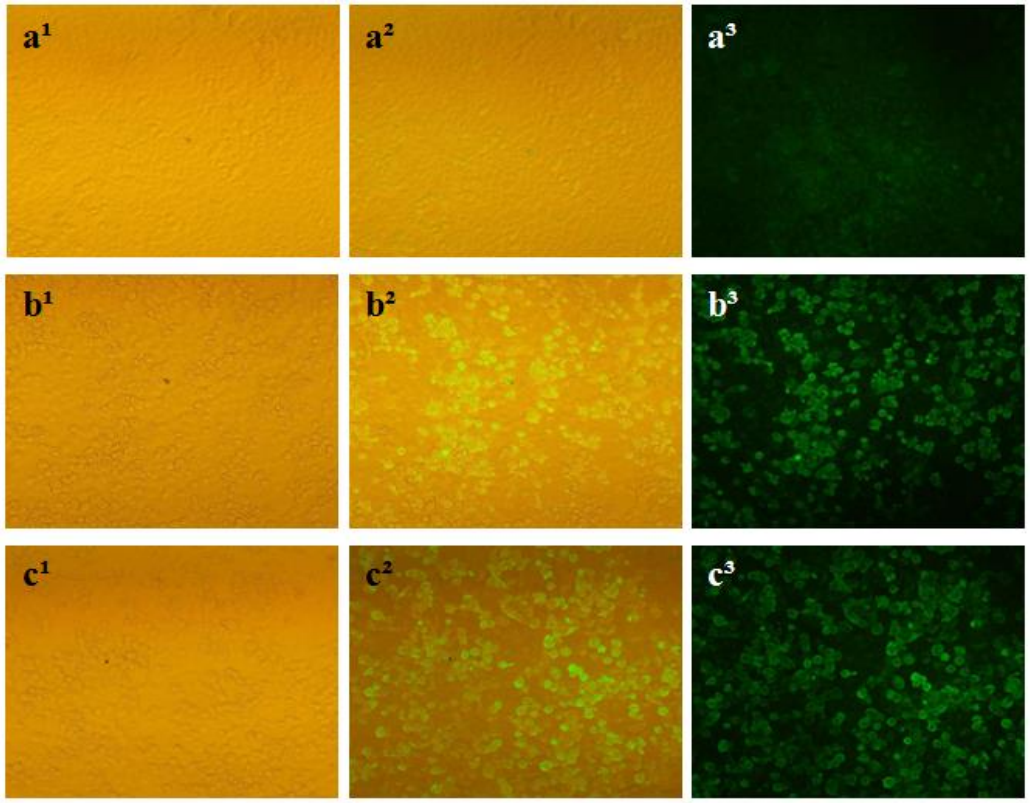

Fig 4. Immunostaining of A549 cells infected with the adenoviruses. The infected cells (b & c) emitted green fluorescence, indicating expression of the target proteins, while there was no fluorescence in non-infected control cells (a)

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Transmission electron microscopy reveals the self-assembly of FMDV capsid proteins into VLPs

The recombinant adenovirus hAd5/P1-2AB3BCwt-P2 was used to infect HEK-293 and A549 cells, and expressed target proteins and formation of virus-like-particles (VLPs) were analyzed by western blot and TEM, respectively. Fraction 5 of the sucrose gradient, which showed the highest expression of the target protein in the western blot, was processed for electron microscopy (Fig. 5). The TEM revealed the formation of FMDV empty capsid particles and pentameric structures of the capsid, as shown in Fig. 6a.

The purified and concentrated recombinant adenovirus expressing the FMDV capsid proteins was also processed for TEM. An icosahedral structure measuring approximately 80-90 nm in diameter was observed, as shown in Fig. 6b.

Evaluation of immune response in the vaccinated guinea pigs

There were no untoward reactions in the guinea pigs after immunization. The humoral immune response of the vaccinated guinea pigs was evaluated by virus neutralization test (VNT), and FMDV type 'O' specific IgG_1 and IgG_2 level was measured and expressed as absorbance (OD492nm) by indirect ELISA.

Virus neutralization test was carried out using serum samples collected on 0, 7, 14 & 28 dpv and 7 & 14 dpb as described in the methods. Neutralizing antibody titres against FMDV type 'O' antigen in all the immunized guinea pig serum samples were measured, and endpoint titres were calculated as the reciprocal of the highest dilution of serum that neutralizes $100TCID_{50}$ of FMDV 'O' in 50% of the wells. The virus-neutralizing antibody titre (mean±SE) was expressed as $\log_{10}SN_{50}$ per mL, as presented in Table 3 and Fig. 7. No detectable neutralizing antibody response was observed on 0 & 7 dpv in all the groups, including controls. A $\log_{10}$ SN titre of neutralizing antibodies on 14 dpv is 1.22, 1.40 and 1.42 by the groups T-01, T-02 and T-03, respectively. On the 7th dpb the neutralizing antibody titer was increased to 1.47, 1.62 and 1.74 in the groups T-01, T-02 and T-03, respectively. The difference in the antibody titre after the prime-booster dose was statistically significant in all the groups (P = 0.0036, 0.012 and 0.0008 for the group T-01, T-02 and T-03, respectively) by the 't' test**.** The negative control group (T-04) did not show neutralizing antibodies during the experiment.

Table 3. Serum neutralizing antibody titer ($\log_{10}$ SN_{50}) of different groups of immunized guinea pigs at different time intervals

Group	Log_{10} SN_{50} titer (Mean ± SE)					
	0 dpv	7 dpv	14 dpv	28 dpv	7 dpb	14 dpb
T-01	< 0.90 ± 0.00	< 0.90 ± 0.00	1.22 ± 0.10	1.20 ± 0.09	1.47 ± 0.10	1.43 ± 0.09
T-02	< 0.90 ± 0.00	< 0.90 ± 0.00	1.40 ± 0.10	1.34 ± 0.14	1.62 ± 0.11	1.60 ± 0.11
T-03	< 0.90 ± 0.00	< 0.90 ± 0.00	1.42 ± 0.07	1.36 ± 0.06	1.74 ±0.07	1.66 ± 0.07
T-04	< 0.90 ± 0.00	< 0.90 ± 0.00	< 0.90 ± 0.00	< 0.90 ± 0.00	< 0.90 ± 0.00	< 0.90 ± 0.00

T-01: hAd5/P1-2AB3BC^{wt} (5×10^8 pfu in PBS); **T-02:** hAd5/P1-2AB3BC^{m} (5×10^8 pfu in PBS); **T-03:** FMDV Serotype 'O' Ag (1.2 µg + MONTANIDE™ ISA 206 VG); **T-04:** dAd5 (5×10^8 pfu in PBS).

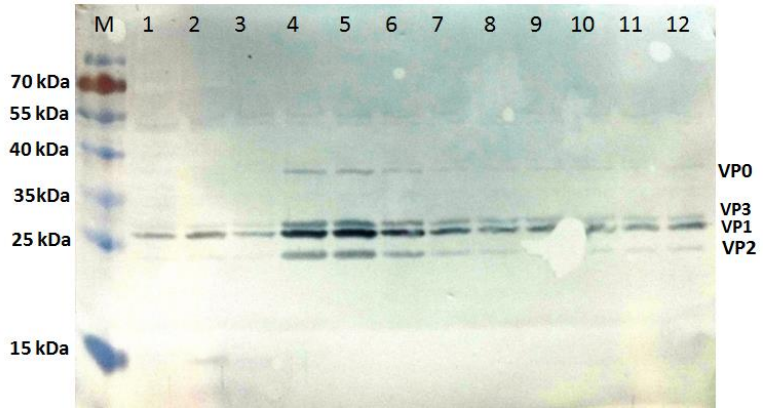

Fig. 5. Western blot analysis of FMDV capsid protein expressed in adenoviral expression system and purified by sucrose gradient. Lane M: Pre-stained protein ladder (Thermo Scientific), and Lane 1-12: Sucrose fractions 1-12 from top to bottom (20 µL of each fraction was analysed).

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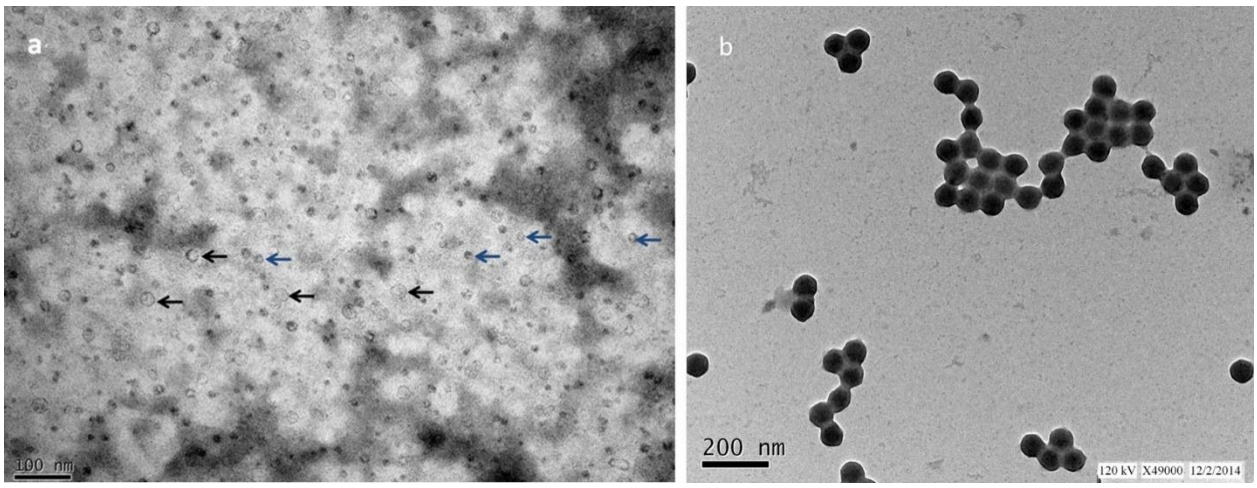

Fig. 6.a. Transmission electron micrograph of empty capsid particles (VLPs) of FMDV expressed in HEK-293 and A549 cells by adenovirus expression system. The black arrow indicates VLPs (~ 20-25 nm in diameter) while blue arrow indicates pentamer (~ 8-10 nm in diameter) of the FMDV. Magnification 98000×; **6.b.** Transmission electron micrograph of human adenovirus type 5 carrying FMDV serotype 'O' capsid protein coding gene cassette. The CsCl density gradient ultra-purified virus suspended in PBS was used for the microscopy. Magnification 49000×

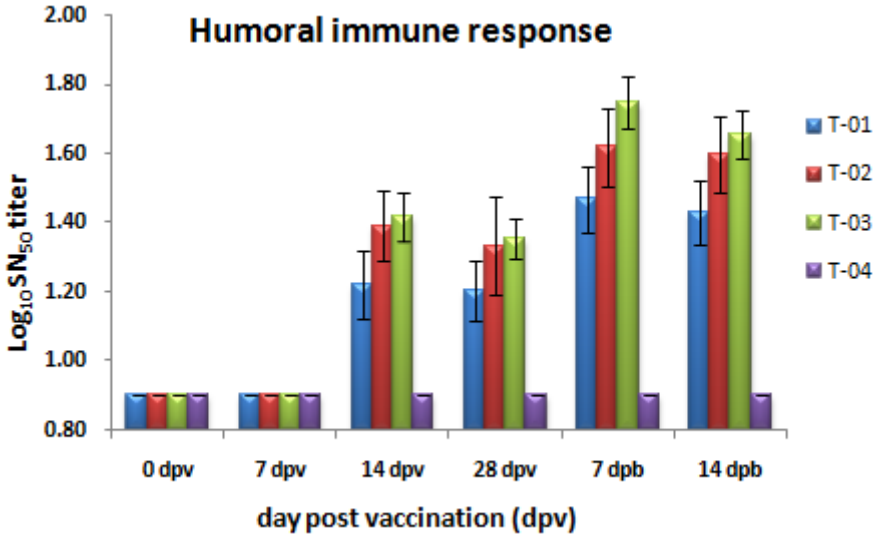

Fig. 7. Serum neutralizing antibody titer (Log_{10} SN_{50}) of different groups of immunized guinea pigs at different time intervals. **T-01:** hAd5/P1-2AB3BC^{wt} (5×10^8 pfu in PBS); **T-02:** hAd5/P1-2AB3BC^{m} (5×10^8 pfu in PBS); **T-03:** FMDV Serotype 'O' Ag (1.2 µg + MONTANIDE™ ISA 206 VG); **T-04:** dAd5 (5×10^8 pfu in PBS)

IgG_1 and IgG_2 level depicts a cellular immune response to FMDV

FMDV type 'O' specific total IgG_1 response started increasing at 7th dpv and maintained till 28th dpv in all the immunized groups except group T-04, which received non-recombinant hAd5. The IgG_1 level slightly increased after the booster dose in all groups except group T-04. The highest IgG_1 immune response was recorded in the group T-03, followed by T-02 and T-01. There was no statistically significant difference in IgG_1 level after booster dose in all the groups (P = 0.0598, 0.3332, and 0.4871 for the groups T-01, T-02 and T-03, respectively).

FMDV type 'O' specific total IgG_2 response also started increasing at the 7th dpv and further increased till the 28th dpv in the groups T-01, T-02 and T-03 except group T-04. The highest IgG_2 immune response was recorded in the group T-03, followed by T-02 and T-01 at 28th dpv. After the booster dose, there was a slight increase in the IgG_2 level in all groups except group T-04. There was no statistically significant difference in IgG_2 level after booster dose in all the groups (P = 0.212, 0.1379 and 0.8546 for the groups T-01, T-02 and T-03, respectively).

A predominance of IgG_2 was observed in all the vaccinated groups regardless of the booster immunization, with a much lower level of IgG_1

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(IgG_1/IgG_2 = 0.2-0.4), indicating a Th_1 type of immune response.

Viral challenge and protection score

The immunized guinea pigs were apparently healthy at the time of challenge. The guinea pigs, including the control groups, were challenged with $100GPID_{50}$ at 14 dpb. FMD lesions on the foot pads were observed daily for 7 days and scored accordingly, as shown in Table 4. The animals found to be positive for secondary lesions were considered unprotected. The secondary lesions appeared 2-3 days post-challenge, and no lesions were observed for up to 7-day post-challenge. About 50, 67, and 83% of animals were protected in the groups T-01, T-02 and T-03, respectively, while all animals of the negative control group (T-04) were unprotected (Table 4).

Table 4. Protection of immunized Guinea pigs after homologous challenge virus

Group	No. of unprotected animals	No. of protected animals	Total	% of protected animals
T-01	3	3	6	50.00
T-02	2	4	6	66.67
T-03	1	5	6	83.33
T-04	6	0	6	00.00

T-01: hAd5/P1-2AB3BC^{wt} (5×10^8 pfu in PBS); **T-02:** hAd5/P1-2AB3BC^{m} (5×10^8 pfu in PBS); **T-03:** FMDV Serotype 'O' Ag (1.2 µg + MONTANIDE™ ISA 206 VG); **T-04:** dAd5 (5×10^8 pfu in PBS)

Discussion

There is no effective treatment for FMD, so it is necessary to control and prevent the disease by vaccination and/or stamping-out policy. In endemic countries like India, vaccination is the only possible way to control or eradicate the disease. Due to certain limitations of the conventional inactivated virus vaccine, various approaches have been followed to develop a better and safer vaccine for FMD control and prevention (Sanz-Parra et al., 1999). The development of different kinds of recombinant vaccines has been explored by various researchers worldwide. Although they are not as effective as conventional vaccines, they may have other valuable properties such as safety, type of immune response, duration of immunity, stability, etc. (Romanutti et al., 2013). It has been proved that a booster dose of the same or different vaccine sharing a common antigen is required to mount a better immune response (Lu et al., 2008; Magalhaes et al., 2008; Zhou et al., 2013; Romanutti et al., 2013).

The basic concept followed in various approaches to design new generation vaccines has been the delivery of the FMD virus capsid protein genes responsible for forming viral empty capsid particles (VLPs) (Srinivas et al., 2015). The VLPs present the viral antigens in more authentic conformation than other subunit vaccines (Rweyemamu et al., 1979, Srinivas et al., 2016) and induce an efficient humoral and cellular immune response (Mayr et al., 1999; Kushnir et al., 2012). The adenovirus vector expressing the FMDV capsid coding genes along with the $3C^{pro}$ gene has been widely used to deliver the target antigen. It has been proven to induce a good humoral and a strong cellular immune response. The present study aimed to develop an adenoviral vaccine using an Indian FMD vaccine virus and to test its properties in terms of expression of the capsid proteins, stability and protective immune response.

The expression of the target proteins is the ultimate aim of gene delivery, which is to produce an immune or therapeutic response. The present study demonstrated the expression of the FMD virus capsid proteins in the adenovirus expression system. Initially, the expression of the target protein in recombinant adenovirus-infected HEK-293 cells was so low that it was not detected by western blot. However, it was detected in sandwich ELISA and confirmed by immunostaining of the infected HEK-293 cells. However, later on, the expression of the target protein was detected in the HEK-293 cells, and it was concluded that the HEK-293 cells infected with a MOI of 10 of the recombinant adenovirus better express target protein as compared to low MOI. A549 cells (non-complementing) were also used to check the expression of the target protein, and it was observed that the expression of the capsid proteins was better in A549 cells as compared to HEK-293 cells as demonstrated by sELISA and western blot. Our expression results do not correlate with the results of Turner et al. (2007), who demonstrated two-fold viral expression in HEK-293 cells compared to A549 cells. At MOI ≥ 1.0, early CPE was observed in the HEK-293 cells infected with the recombinant adenoviruses (hAd5/P1-2AB3BC^{wt} and hAd5/P1-2AB3BC^{m}) due to supplement of E1 gene by the HEK-293 cells for replication of the replication-deficient adenoviruses. The expression of FMDV capsid proteins was found maximum in HEK-293 cells infected with an MOI of 10 at 24 hpi. On the other hand, A549 cells do not

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supplement the adenovirus with the E1 gene necessary for the virus replication and can withstand for a longer time even at higher MOI. An MOI of 10 and 100 of the adenoviruses were tested in the A549 cells, and the expression of FMDV capsid proteins was found to be maximum with the MOI of 100 at 3 days post-infection. There were CPE-like changes in the A549 cells, possibly due to the adenovirus toxicity and accumulation of foreign proteins in the host cells. Transmission electron microscopy of the expressed proteins revealed the formation of FMDV empty capsid particles in the adenovirus expression system.

In the present study, we followed a homologous prime-boost regimen in guinea pig immunization by the adeno-FMD vaccines along with control vaccines-conventional and non-recombinant adenovirus vaccine. The highest neutralizing antibody titer was found in the guinea pigs immunized with inactivated FMDV vaccine both after priming and booster dose. The adeno-FMD vaccine containing mutant $3C^{pro}$ has been found to induce more neutralizing antibodies compared to its wt counterpart. The antibody titre further increased after homologous booster dose in all vaccinated groups and was found to be statistically significant as compared to post-primary vaccination. In this context, it is apt to refer to the findings of Romanutti et al. (2013), Zhou et al. (2013a) and Mayr et al. (1999), who also reported a strong immune response following homologous prime-boost strategy in mice model. Du et al. (2007, 2008) have demonstrated that despite low neutralizing antibody titre, the adeno-FMD vaccine could protect as many guinea pigs as killed vaccine. In a study, immunization with the adenovirus vector vaccine resulted in significant humoral antibody responses and was found to be dose-dependent, reaching 3–4 $\log^{10}$ titres in animals receiving the two higher vector doses (Wang et al., 2006). However, in most immunization strategies, the adenovirus vector is used either to prime or boost to avoid the possibility of neutralizing the effects of adenoviral antibodies developed after primary immunization.

In this study, we also analyzed the IgG isotype profile against FMDV antigen, which revealed that both IgG_1 and IgG_2 were induced in all the vaccinated animals except the negative control group, consistent with a T cell-dependent immune responses (Collen et al., 1989; Romanutti et al., 2013). Although both IgG isotypes levels in serum were found to be low, a predominance of the Th_1 type of immune response (IgG_1/IgG_2 = 0.2-0.44) was observed in all the vaccinated groups regardless of the booster immunization, indicating a strong cellular immune response. As was not expected, the IgG_1 response was less than IgG_2, even in the case of an inactivated FMDV vaccine strategy. In other studies, including heterologous immunization strategy, adenovirus priming may play a role in shaping the qualitative immune response after boosting with different kinds of vaccine expressing the same transgene (Rodriguez et al., 2008; Lin et al., 2011; Romanutti et al., 2013).

The animals receiving the inactivated FMDV vaccine had the highest neutralizing antibody titer and IgG_2 levels. They were able to protect 83% of animals as compared to 50% and 67% protection by hAd5/P1-2AB3BC^{wt} and hAd5/P1-2AB3BC^{m} vaccine, respectively, against the FMDV homologous challenge ($100GPID_{50}$). The results suggested that adenovirus containing mutant $3C^{pro}$ offers marginally increased protection levels compared to its wild-type counterpart. In a study conducted by Du et al. (2007), the adeno-FMD vaccine (containing VP1 of FMDV and GMCSF) had been proven to protect 67% of guinea pigs against 100% protection by killed FMD vaccine after homologous challenge at 35 dpv. However, the guinea pigs had received a comparatively large vector dose of $2x10^9$ $TCID_{50}$ and 2.4µg of inactivated FMDV antigen. In contrast, Lu et al. (2008) demonstrated that a vector dose of 3×10^8 is sufficient to protect all the immunized guinea pigs at 25 dpv with homologous challenge. In the case of other viral disease trials, the adenovirus vectored vaccines are 100% efficacious in guinea pigs (Wang et al., 2006; Richardson et al., 2011; Wong et al., 2015). However, in the present study, the low protection rate of the adeno-FMD vaccine compared to the inactivated FMDV vaccine may be due to insufficient *in vivo* expression and processing of the FMDV capsid protein or the insufficient dose of recombinant vaccine. So, additional dose-response studies may provide information regarding the protective dose in guinea pigs. Also, further modification of the adenovirus vector and expression cassette may be required to obtain optimal expression of target proteins and to mount a better immune response. The use of adenovirus vectors for *in vivo* gene expression or vaccine delivery has been carried out in the veterinary field since 1999. However, with certain modifications, the present study was started, and generated data suggest that adeno-FMD vaccines may be an ideal alternative to the conventional inactivated virus vaccine. Although the booster with the homologous vaccine induced an increased immune response, other studies suggest that boosting with different vector/protein/DNA vaccines elicited a better immune response.

Conclusion

The recombinant adeno-FMD virus showed efficient expression of the FMDV capsid proteins, which self-assembled into VLPs as demonstrated by electron microscopy. The adeno-FMD vaccines protected fewer animals than conventional inactivated virus vaccines after homologous challenge. Recombinant adenovirus containing mutant $3C^{pro}$ was found superior to its wild-type counterpart in terms of protection. As no vaccine in the present study has given 100% protection, additional dose-response studies may provide

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information regarding the protective dose in guinea pigs. The data generated in the study suggest that the adeno-FMD vaccines may be an ideal alternative to the conventional inactivated virus vaccine.

Disclosure statement

The author reported no potential conflict of interest.

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Article citation

Kumar R, Sreenivasa BP, Hosamani M, Basagoudanavar SH and Srinivas MV (2024). Human adenovirus type 5 expressing FMDV serotype O (strain R2/75) capsid proteins and its immunogenicity in guinea pigs. *Archives of Veterinary Research*, 1(1): 16-26.

Article DOI: https://doi.org/10.70964/avr.5

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