**Recombinant human adenovirus-5 expressing capsid proteins of Indian vaccine strains
of foot-and-mouth disease virus elicits effective antibody response in cattle**

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ABSTRACT

Recombinant adenovirus-5 vectored foot-and-mouth disease virus (FMDV) constructs (Ad5-FMD) were made for three Indian vaccine virus serotypes O, A and Asia 1. Constructs co-expressing FMDV capsid and viral 3C protease sequences, were evaluated for their ability to induce a neutralizing antibody response in indigenous cattle (*Bos indicus*). Purified Ad5-FMD viruses were inoculated in cattle as monovalent (5×10^9 pfu/animal) or trivalent (5×10^9 pfu/animal per serotype) vaccines. Animals vaccinated with monovalent Ad5-FMD vaccines were boosted 63 days later with the same dose. After primary immunization, virus neutralization tests (VNT) showed seroconversion in 83, 67 and 33 per cent of animals vaccinated with Ad5-FMD O, A and Asia 1, respectively. Booster immunization elicited seroconversion in all of the animals (100%) in the monovalent groups. When used in a trivalent form, the Ad5-FMD vaccine induced neutralizing antibodies in only 33, 50 and 16 per cent of animals against serotypes O, A and Asia 1, respectively on primo-vaccination, and titers were significantly lower than when the same vectors were used in monovalent form. Neutralizing antibody titers differed by serotype for both Ad5-FMD monovalent and trivalent vaccines, with Asia 1 serotype inducing the lowest titers. Antibody response to Ad5 vector in immunized cattle was also assessed by VNT. It appeared that the vector immunity did not impact the recall responses to expressed FMDV antigens on booster immunization. In summary, the study suggested that the recombinant Ad5-FMD vaccine has a potential use in monovalent form, while its application in multivalent form is not currently encouraging.

Key words

Foot-and-mouth disease virus, adenovirus, vectored vaccine, Indian serotypes, capsid protein, monovalent, trivalent

1. Introduction

Foot-and-mouth disease (FMD), a highly contagious disease affecting cloven-hoofed animals such as cattle, pigs and small ruminants is considered a major global threat to animal health. It is caused by FMD virus (FMDV) an RNA virus belonging to the *Aphthovirus* genus of the *Picornaviridae* family. The disease is enzootic in India with outbreaks caused by three FMDV serotypes O, A and Asia 1. Mass immunization of animals with trivalent vaccine containing inactivated antigens is currently used to control the disease.

Though inactivated vaccines are widely applied for control of FMD, they have certain limitations, most importantly the safety concerns and the high cost of high containment vaccine manufacturing facility. To circumvent these limitations, recombinant vaccines have been considered as they are safe to produce, non-infectious, DIVA compliant and can be produced in a BSL2 facility (Grubman et al., 2010). Among the newer vaccine candidates, human replication-defective adenovirus 5 (Ad5) vectored FMD vaccines have been demonstrated as one of the successful molecular vaccines to date for protection against FMDV (Grubman et al., 2010 and Moraes et al., 2011).

Ad5-FMD vaccines have so far been developed against A24 Cruzeiro (Moraes et al., 2002), O1 Campos (Caron et al., 2005), O1 Manisa (Diaz-San Segundo et al., 2016; Fernandez-Sainz et al., 2017) and O/China/99 (Lu et al., 2008) with varying degrees of success. Ad5-A24 is by far the most successful recombinant vaccine conferring complete protection from challenge in swine and cattle as early as 7 days post vaccination (Moraes et al., 2002 and Pacheco et al., 2005) and the vaccine has been granted a conditional license in the U.S. for use in cattle in an emergency situation (Grubman et al., 2012).. The Ad5-FMD vaccine developed by U.S. Department of Agriculture (USDA) has not been tested for its efficacy in an FMD enzootic situation like India. Also, no immunization studies in cattle have been reported using the Asia 1 strain. Therefore, a proof-of-concept study was carried out to

evaluate the humoral immune response in Indian cattle to non-adjuvanted Ad5 vectored vaccines carrying the capsid sequences of Indian vaccine strains.

2. Materials and methods

2.1. Cells and viruses

Human 293 cells (ATTC CRL-1573) (Graham et al., 1977) at passage numbers 25-40 were used to generate, grow recombinant Ad5 viruses and for virus neutralization test (VNT). Baby hamster kidney cells (BHK-21) at passage numbers 20-40 were used to measure serum neutralization (SN) antibody titre against FMDV. The FMD viruses used in this study included current Indian vaccine strains *viz.*, O IND R2/1975, A IND 40/2000 and Asia 1 IND 63/1972.

2.2. Construction of adenovirus recombinants containing capsid coding sequences of Indian FMDV vaccine strains

The P1-2A-2B region (~2.7 kb) of the FMDV genome from the three Indian FMDV strains was obtained by PCR amplification of cDNA derived from RNA extracted from BHK-21 grown viruses. The primers used for PCR contained appropriate restriction enzyme sites to enable subcloning into transfer vector (The details on the primers are available upon request). The cloned products were confirmed by sequencing.

The P1-2A-2B fragment from pKS-II-A24-P12AB-A12-3BC transfer vector (Moraes et al., 2002) was replaced by P1-2A-2B of Indian vaccine strains, using Fast-link DNA ligation kit (Epicentre Biotechnologies, USA). Recombinant viruses were made using two

types of Ad5 vectors, pAd5-Blue (Moraes et al., 2001) and pAd5-CI (Moraes et al., 2007) as described earlier (Moraes et al., 2011). They were designated as Ad5-OR2/1975, Ad5-A40/2000, and Ad5-Asia1 63/1972 (hereafter collectively referred as Ad5-FMD viruses).

2.3. Confirmation of expression of FMDV capsid proteins in Ad5-FMD infected cells

FMDV protein expression was analyzed using the lysates prepared from cells infected with Ad5-FMD virus by SDS-PAGE followed by western blotting. A mixture rabbit polyclonal antibodies raised against the FMDV A12 capsid proteins VP1, VP2, and VP3 was used as primary antibody and the human serum adsorbed goat anti-rabbit IgG (H+L) F(ab')₂ fragment as a secondary antibody (KPL, USA).

To confirm the serotype specificity of FMDV antigen expressed in Ad5-FMD virus infected HEK-293 cells, a sandwich ELISA (OIE, 2012) was carried out using type specific anti-sera.

2.4. Recombinant vaccine and vaccination

Recombinant Ad5-FMD viruses and Ad5-Blue virus, grown in bulk cultures were purified using non-linear followed by linear CsCl gradient centrifugation (Graham and Prevec, 1991).

Indigenous bull calves (*Bos indicus*) aged 6 to 12 months and free from neutralizing antibodies ($\log_{10}$ SN titre <0.9) against the three serotypes of FMDV (O, A and Asia 1) were used in this study. Animals were also screened by 3ABC ELISA, a non-structural protein antibody detection test (Hosamani et al., 2015) to rule out previous exposure to FMDV infection. All the procedures for use of experimental animals were approved by the Institute Animal Ethics Committee.

Animals were divided into six groups containing six animals per group (Table 1). The

animals were inoculated subcutaneously at two sites on either side of the neck with Ad5-FMDV monovalent (T-02, T-03 & T-04) or Ad5-FMDV trivalent (T-05) vaccine in 2 ml (1 ml per site) of phosphate buffered saline (PBS). Ad5-Blue virus was used as vector control in one group (T-01), while another group (T-06) which acted as positive control received conventional inactivated oil-adjuvanted trivalent vaccine intramuscularly. Monovalent Ad5-FMD vaccine groups were inoculated with 5×10^9 pfu virus per animal, while the trivalent vaccine group was given 5×10^9 of each monovalent vaccine. Animals vaccinated with monovalent Ad5-FMD (T-02, T-03 & T-04) were boosted 63 days later with the same dose.

2.5 Titration of FMDV neutralizing antibodies

Post-vaccinate sera were collected from animals on days 7, 14, 21, 28 and 63. On day 63, animals in T-01 through T-04 and T-06 received the same dose of vaccine as in primary vaccination. Serum samples were collected on 14 and 28 days post booster (dpb) injections.

Sera were screened for humoral response against homologous FMD viruses by VNT (OIE, 2012) to determine the SN antibody titres, expressed as $\log_{10}$ SN₅₀ values.

2.6 Titration of adenovirus antibodies

The VNT to quantify neutralization antibodies against adenovirus was essentially similar to FMD VNT, except that the 293 cell suspension was diluted to have 10,000 cells/well in 50 μ l volume and 100 TCID₅₀ non-recombinant Ad5-blue was added to each dilution of serum samples and final reading was taken after 6 to 7 days of incubation.

2.5. Data analysis

Mean antibody titers from Day 7 to Day 91 estimated by VNT were analyzed for

statistical significance using t-test. P values from the t-test were adjusted for multiple comparisons using Stepdown Bonferroni method. An adjusted *p* value of 0.05 or less was considered as statistically significant.

3. Results

3.1 Construction and characterization of Ad5-FMD recombinants

Ad5-FMD recombinants were recovered by picking individual plaques at 5–12 days post- plasmid transfection and amplified in HEK 293 cells. In western blotting, two bands of approximately 36 and 27 kDa were detected in the lysates of Ad5-FMD but not in the control Ad5-Blue infected cells and mock-infected HEK 293 cells (Fig. 1A), demonstrating that recombinant P1–2A polyproteins were synthesized and processed by the 3C protease into VP0 (36 kDa) and VP3/VP1 (27 kDa). The serotype specific sandwich-ELISA confirmed that each of the recombinant Ad5-FMD viruses expressed the capsid sequences with serotype specificity (Fig. 1B).

3.2. Ad5-FMD vaccine in monovalent form elicits better FMDV neutralizing antibody response compared to trivalent form

In the monovalent Ad5-O group, when sera were assayed by VNT, five of six animals (83%) showed seroconversion after the first vaccination. Out of five responders, the peak mean antibody titre ($\log_{10}$ SN₅₀) was 2.1 in two animals and in the range of 1.36 to 1.65 in the remaining three animals. One animal maintained a peak titre of 2.1 up to 63 dpv, whereas the antibody titres declined in all the other responders and by 63 dpv, the titres were below 1.65 (Fig. 2A). The levels of antibody titres in the monovalent Ad5-O group was found to be significantly higher ($p<0.05$) compared to titres in the Ad5-vector control group on all time points post vaccination. In comparison to inactivated FMD vaccine, monovalent Ad5-O titres

showed no significant difference in mean titres before booster. However, after booster in the inactivated vaccine group, a significant increase ($p<0.01$) in the titres was observed at 14 dpb.

In the monovalent Ad5-A group after the first vaccination, four of six animals (67%) developed a specific SN antibody response as assayed by VNT. These four responders showed peak antibody titres of 1.5, 1.65, 1.8 and 2.4. While one of these animals maintained an elevated titre throughout the first vaccination (peak titre = 2.4), the antibody titres declined in the other responders and the titres were below 1.65 by 63 dpv (Fig. 2A). The increase in the mean titre in monovalent Ad5-A group was significant ($P<0.05$) on 14 and 21 dpv as compared to Ad5-vector control group. In comparison to inactivated FMD vaccine, monovalent Ad5-A titres showed a significant lower mean titres after primary vaccination ($p<0.05$) and maintained the trend even after booster vaccination.

In the monovalent Ad5-Asia 1 group, two of six animals (33%) developed SN antibody response with peak titres of 1.36. One animal maintained a peak titre of 1.20, while the titres declined in the other responder and was below the basal level by 63 dpv (Fig. 2A). Statistically, significant increase in mean antibody titres could be observed in the monovalent Ad5-Asia 1 vaccine group on 21 dpv compared to Ad5-vector control group ($P<0.05$). However, in comparison with inactivated vaccine group, the anti-FMDV Asia 1 titres remained significantly lower before booster, though booster vaccination elevated the antibody titres.

Booster vaccination in the monovalent Ad5-O, A and Asia 1 groups resulted in seroconversion in all (100%) animals by 14 dpb. The VNT titres ranged from 1.65 to 2.25 for O and A, and 1.5 to 1.65 for Asia 1. The increase in mean titres for all the monovalent groups was highly significant ($p<0.01$) compared to their pre-booster titres.

In the trivalent Ad5-FMD group, there was a marginal increase ($\log_{10}$ 1.20-1.65) in the levels of SN antibodies in two animals (33%) for type O, three animals (50%) for type A and one animal (16%) for type Asia 1 antigen after primary vaccination (Fig. 2B). The titres were however significantly less ($p<0.05$) compared to monovalent immunization.

3.3. Antibody response to vector (hAD5 virus)

Ad5 virus specific neutralizing antibodies were detectable by 7 dpv, which reached peak levels by 14 dpv. The antibody levels declined thereafter and reached basal levels by 63 dpv. Booster immunization on 63 dpv resulted in an increase in antibody levels ($\log_{10}$ SN₅₀ 2.11). By 84 dpb, antibody levels further declined, yet the levels were found to be higher than those detected at 63 dpv earlier (Fig. 2C).

4. Discussion

Adenovirus-vectored vaccines support a ‘vaccinate-to-live’ strategy in the event of an FMD outbreak. The vaccine induces rapid protection against FMD and enables unequivocal differentiation of infected from vaccinated animals. No data of its efficacy in an FMD enzootic situation has been previously reported. The present study thus served as a proof-of-concept to explore the application of recombinant Ad5 vaccines against FMDV strains currently used in inactivated vaccines for Indian cattle.

Using the Ad5 system developed at the Plum Island Animal Disease Center (Mayr et al., 1999), we constructed recombinant vectors containing the coding regions of the FMDV capsids of vaccine strains from Indian serotypes O, A and Asia 1. We then examined the ability of the Ad5-FMD vaccines in monovalent and trivalent forms to induce type specific immune responses in Indian cattle. Analysis of FMD-specific antibody titres after primary and booster vaccination of cattle demonstrated that type O and A strains induced higher

antibody responses against FMDV capsid proteins than the Asia 1 construct, even though all three groups received the same vaccine dose. This was interesting since the humoral responses against the Ad5 viruses were comparable in each group when assessed at 7 and 14 dpv in all the monovalent Ad5-FMD groups (Fig. 2A). Thus it can be speculated that the differential FMDV-specific immune response among three serotype specific recombinant constructs are not attributable to the Ad5 delivery of the transgene in vivo and may be a function of the variable efficiency of polyprotein (P1-2A) processing or antigen processing and presentation of viral peptides in the host.

In a study involving Ad5-FMDV A24, Grubman and Mason (2002) reported a 10-20 fold increase in neutralizing antibody response after booster immunization. In our study, we observed a 3-5 fold increase in the titres after booster immunization of groups with respective monovalent vaccine. In India, a trivalent inactivated and adjuvanted vaccine (type O, A and Asia 1) is currently being used in vaccination programs. Therefore, we evaluated the serum antibody response in cattle of a trivalent Ad5-FMD vaccine. Immunized animals showed a marginal increase in antibody titres on primary vaccination in comparison to Ad5 vector inoculated control animals. The basis for poor antibody response in the trivalent Ad5-FMD group remains unclear and may be attributed to a higher Ad5 viral dose (3-fold higher as compared to the recommended dose of 5×10^9 pfu per animal) which may lead to a suppressed immune response to FMDV antigens. Interestingly, the higher dose of Ad5-virus in the trivalent vaccine did not lead to a commensurate increase in Ad5 virus-specific antibody response, compared to that of monovalent Ad5-FMD vaccines. The reason(s) for a reduced immune response to the trivalent vaccine requires further analysis.

Vector immunity plays an important role in transgene expression (Bangari and Mittal, 2006). An increase in Ad5 specific antibody levels did not apparently influence the immunogenicity to FMDV antigens. When the booster immunization was administered on

day 63, vector antibody levels had declined in all the animals. We demonstrated by VNT that at 9 weeks post-primary vaccination, Ad5-antibody levels almost returned to base levels. The vector immunity did not apparently impact the recall responses to expressed FMDV antigens, though adenoviral vector-specific antibody generated after priming is thought to hinder the booster effect. The data indicate that vector antibodies last for about 2-3 months after the first booster suggesting that the Ad5 vector can be used in vaccination campaigns. Further studies are required to understand the duration of vector immunity during repeated immunization and to plan immunization schedules which would allow an optimal immune response against FMDV.

Previous studies have shown that the humoral immune response has a major role in providing protection against FMD (McCullough et al., 1992). Measuring antibodies against structural antigens is a serological biomarker for FMD vaccine efficacy. Therefore, this study was focussed on the antibody response to both the monovalent and trivalent vaccines. The results clearly suggested that the monovalent Ad5-FMD vaccine induced a better humoral immune response compared to the trivalent vaccine. The antibody response to monovalent Ad5-FMD vaccines was lower than the conventional inactivated virus antigen based vaccine. This can be partially attributed to the absence of an adjuvant with the Ad5 vaccines. Recent studies in swine and cattle have shown that addition of adjuvants to Ad5-FMD vaccines allowed dose sparing of vaccine or early protection (Diaz-San Segundo et al., 2014; Diaz-San Segundo et al., 2016). Therefore, further potency and challenge studies using a combination of adjuvants with the Ad5-FMD vaccine will be necessary to evaluate the suitability of this new generation vaccine in cattle.

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Conflict of interest statement

The authors declare no conflicting interests.

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Legend to Figures

Fig.1. Analysis of expression of FMDV capsid proteins by recombinant Ad5-FMD viruses.

(A) Western blot showing the expression of the capsid proteins: Lane 1, HEK 293 cell lysate; lane 2, FMDV type O infected BHK-21 cell lysate; lane M, protein molecular weight marker; lane 3, Ad5-FMD serotype O IND R2/1975; lane 4, Ad5-FMD serotype A IND 40/2000; lane 5, Ad5-FMD serotype Asia1 IND 63/1972. (B) Sandwich-ELISA confirming the indicated serotype specific reactivity of lysates from cells infected with recombinant Ad5-FMD viruses.

Fig.2. Serum neutralizing (SN) antibody response in the vaccinated animals. (A) Geometric mean FMDV specific SN antibodies in post-vaccinate sera of indicated monovalent Ad5-FMD vaccine groups at different time intervals, compared to trivalent inactivated vaccine. Error bars shown refer to the standard error (S.E.) for each mean data point. (B) Geometric mean FMDV specific SN antibodies in post-vaccinate sera of indicated trivalent Ad5-FMD vaccine groups at different time intervals, compared to trivalent inactivated vaccine. Error bars shown refer to the standard error (S.E.) for each mean data point. (C) Adenovirus specific SN antibody response in Ad5-FMD vaccinated animals following primary and booster immunization on day 63 of primo-vaccination with monovalent and trivalent constructs. Error bars shown refer to the standard error (S.E.) for each mean data point representing triplicate analyses.

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366 **Table 1:** Experimental design used in Ad5-FMD vaccination trial in cattle

Group	No. of Animals	Vaccine	Vaccination		
			Study day	Dose	Route
T-01	6	Ad5-Blue	0,7,14,21, 28 & 63 dpv; 14 & 28 dpb	5×10^9	Sub-cutaneous
T-02	6	Ad5-O R2/75	do.	do.	do.
T-03	6	Ad5-A 40/00	do.	do.	do.
T-04	6	Ad5-Asia1 63/72	do.	do.	do.
T-05	6	Ad5-FMD Trivalent	0,7,14,21, 28 & 63 dpv	$3 \times (5 \times 10^9)$	do.
T-06	6	Inactivated oil adjuvanted vaccine	0,7,14,21, 28 & 63 dpv; 14 & 28 dpb	As recommended by the manufacturer	Intra-muscular

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368

369 do.=ditto

370 Dose of inoculum per animal expressed as number of PFU in 2 ml of PBS

371 Day 77 and 91 correspond to Day 14 and 28 post-booster

372 dpv= days post vaccination

373 dpb= days post booster

374

A **B**

A **B** **C**