



Multiple efficacy studies of an adenovirus-vectored foot-and-mouth disease virus serotype A24 subunit vaccine in cattle using homologous challenge



Christopher Schutta^a, José Barrera^{b,1}, Melia Pisano^{b,c,1}, Laszlo Zsak^{a,2}, Marvin J. Grubman^d, Gregory A. Mayr^{d,3}, Mauro P. Moraes^{d,4}, Barbara J. Kamicker^e, David A. Brake^e, Damodar Etyreddy^f, Douglas E. Brough^f, Bryan T. Butman^f, John G. Neilan^{a,*}

^a Plum Island Animal Disease Center, U. S. Department of Homeland Security Science and Technology Directorate, P.O. Box 848, Greenport, NY 11944, USA

^b The McConnell Group, Inc., Plum Island Animal Disease Center, P.O. Box 848, Greenport, NY 11944, USA

^c Oak Ridge Institute for Science and Education, PIADC Research Participation Program, Oak Ridge, TN, USA

^d Plum Island Animal Disease Center, Foreign Animal Disease Research Unit, Agricultural Research Service, U. S. Department of Agriculture, P.O. Box 848, Greenport, NY 11944, USA

^e Leidos, Inc., Plum Island Animal Disease Center, P.O. Box 848, Greenport, NY 11944, USA

^f GenVec, Inc., 910 Clopper Road, Suite 220N, Gaithersburg, MD 20878, USA

ARTICLE INFO

Article history:

Received 1 September 2015

Received in revised form 4 December 2015

Accepted 8 December 2015

Available online 18 December 2015

Keywords:

Foot-and-mouth disease virus

FMDV serotype A24 Cruzeiro

Replication-deficient human adenovirus vectored vaccine

DIVA

Vaccine efficacy

ABSTRACT

The safety and efficacy of an experimental, replication-deficient, human adenovirus-vectored foot-and-mouth disease virus (FMDV) serotype A24 Cruzeiro capsid-based subunit vaccine (AdtA24) was examined in eight independent cattle studies. AdtA24 non-adjuvanted vaccine was administered intramuscularly to a total of 150 steers in doses ranging from approximately 1.0×10^8 to 2.1×10^{11} particle units per animal. No detectable local or systemic reactions were observed after vaccination. At 7 days post-vaccination (dpv), vaccinated and control animals were challenged with FMDV serotype A24 Cruzeiro via the intra-dermal lingual route. Vaccine efficacy was measured by FMDV A24 serum neutralizing titers and by protection from clinical disease and viremia after challenge. The results of eight studies demonstrated a strong correlation between AdtA24 vaccine dose and protection from clinical disease ($R^2 = 0.97$) and viremia ($R^2 = 0.98$). There was also a strong correlation between FMDV A24 neutralization titers on day of challenge and protection from clinical disease ($R^2 = 0.99$). Vaccination with AdtA24 enabled differentiation of infected from vaccinated animals (DIVA) as demonstrated by the absence of antibodies to the FMDV nonstructural proteins in vaccinates prior to challenge. Lack of AdtA24 vaccine shedding after vaccination was indicated by the absence of neutralizing antibody titers to both the adenovector and FMDV A24 Cruzeiro in control animals after co-mingling with vaccinated cattle for three to four weeks. In summary, a non-adjuvanted AdtA24 experimental vaccine was shown to be safe, immunogenic, consistently protected cattle at 7 dpv against direct, homologous FMDV challenge, and enabled differentiation of infected from vaccinated cattle prior to challenge.

Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Abbreviations: FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; AdtA24, replication-deficient human adenovirus vectored FMDV serotype A24 Cruzeiro vaccine; DIVA, differentiate infected from vaccinated animals; PU, particle units; dpv, days post-vaccination; dpc, days post challenge; USDA, United States Department of Agriculture; VNT, virus neutralization test; GMT, geometric mean VNT titer; NSP, nonstructural protein.

* Corresponding author. Tel.: +1 6313233025; fax: +1 6313233097.

E-mail address: john.neilan@st.dhs.gov (J.G. Neilan).

¹ Current address: Leidos, Inc., Plum Island Animal Disease Center, P.O. Box 848, Greenport, NY 11944.

² Current address: Southeast Poultry Research Laboratory, Southeast Area, Agricultural Research Service, U.S. Department of Agriculture, 934 College Station Road, Athens GA 30605.

³ Current address: Plum Island Animal Disease Center, Animal and Plant Health Inspection Service, U. S. Department of Agriculture, P.O. Box 848, Greenport, NY 11944.

⁴ Current address: Ceva Biomune, 8906 Rosehill Road, Lenexa, KS 66215.

<http://dx.doi.org/10.1016/j.vaccine.2015.12.018>

0264-410X/Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Foot-and-mouth disease (FMD) is the most highly contagious disease affecting livestock resulting in significant adverse economic impact worldwide [1]. FMD is caused by a *Picornaviridae* virus, foot-and-mouth disease virus (FMDV). FMDV affects domestic and wild-life cloven-hoofed ruminants as well as swine. Typical FMDV clinical signs, although age and species dependent, include fever and lesions on the mouth, hooves, and teats. Most susceptible animals survive infection, but often exhibit decreased production due to debilitation from the lesions. The FMDV infection pathways and host response in cattle are reviewed by Arzt et al. [2].

FMD is enzootic in many developing countries in Asia and Africa, and periodic FMD vaccination campaigns are often practiced to prevent outbreaks or mitigate spread. Current FMD serotype-specific vaccines are made by growing live FMDV in cell culture, following by chemical inactivation and purification of 140S virions. Concerns about FMDV introduction into the United States have prompted government research and development into producing next generation vaccines that can be manufactured in the United States.

Mayr et al. initially described the construction of an E1/E3 replication deficient human adenovirus 5 (Ad5) recombinant virus that expressed the FMDV serotype A12 P1 capsid coding region, portions of the nonstructural protein coding regions, and the 3C protease, essential for P1 polypeptide processing [3]. Moraes et al. produced a similar virus containing the capsid coding region of FMDV serotype A24 Cruzeiro (Ad5-A24) that conferred protection in swine [4]. In a subsequent swine study, de Avila Botton et al. showed that higher Ad5-A24 doses resulted in a better clinical outcome, higher serum antiviral activity, no viremia, and lower amounts of FMDV in nasal secretions [5]. Cattle vaccinated with Ad5-A24 were also protected against FMD clinical disease following homologous challenge at one week post-vaccination [6].

Details of the construction of the adenovector E1, E3 deleted backbone (Adt) used in the studies reported herein have been previously described [7,8]. Additionally, a human cytomegalovirus promoter was added to control expression of the target FMDV gene cassette. The FMD vaccine used in our studies (AdtA24) is based on the FMDV strain A24 Cruzeiro P1-2A capsid and serotype A12 3C protease cloned into a replication deficient human adenovirus C, serotype 5 vector [9].

Based on the working hypothesis that a lead vaccine candidate and method of production could be identified for transition to a full development regulatory program, we conducted eight independent AdtA24 vaccine safety and efficacy studies using FMDV A24 Cruzeiro experimental challenge at one week post-vaccination. The primary goals were prevention of FMD clinical disease without adverse effects. The study series started with initial proof-of-concept studies using a research restricted vaccine production method and culminated with the identification of a vaccine purification method that could be scaled up and used in a manufacturing process and a vaccine dose that met the requirements for advancement to a regulatory-based product licensing program.

2. Materials and methods

2.1. Animals

Healthy Holstein cross-bred steers four to ten months of age and 160–260 kg were purchased from an Association for the Assessment and Accreditation of Laboratory Animal Care accredited livestock facility. Steers were acclimated and housed in the Plum Island Animal Disease Center (PIADC) BSL-3Ag animal facility. Prior to vaccination, steers were randomly allocated to treatment groups and allowed to freely co-mingle in assigned rooms throughout the

duration of the study. Animal care and study protocols were in accordance with the institutional guidelines of the PIADC Institutional Animal Care and Use Committee.

3. Experimental AdtA24 vaccines

The AdtA24 vaccine vector was constructed by GenVec, Inc. (Gaithersburg, MD) as summarized by Brake et al. [9] and grown in the M2A cell line in adherent flasks, shaker flasks, or bioreactors. Following lysis of AdtA24-infected host cells, sequential downstream purification steps were used to prepare the four experimental vaccines of varying purity used in these studies as follows: (1) three cycles of centrifugation on cesium chloride (CsCl) gradients (research restricted method); (2) benzonase treatment followed by clarification by centrifugation to produce downstream fraction 3 (DS3); (3) DS3 purification using ultrafiltration/diafiltration to produce downstream fraction 5 (DS5); (4) DS5 purification through anion exchange chromatography (Ciphaer Q Hyper-D) to produce downstream fraction 7 (DS7). For each vaccine lot, FMDV capsid expression and processing was confirmed by Western blot of transfected cell lysates using a VP2-specific monoclonal antibody [10] (kindly provided by CFIA, Winnipeg, Canada), and particle units (PU) were quantified [8]. AdtA24 preparations were stored at -80°C . On the day of vaccination, thawed vaccines were diluted with final formulation buffer (FFB; Lonza, Walkersville, MD) to the final target dose. For each study, baseline serum samples from each animal were collected immediately prior to vaccination (Day 0). Steers were inoculated intramuscularly (IM) in the cleidocapitalis muscle with a single 2 ml injection in the right side of the neck with either placebo (FFB alone or an Adt.Null vector) or AdtA24 (Table 1). Individual blood samples were collected weekly and used for serum virus neutralization tests or plasma virus isolation. Steers were assessed for potential adverse reactions, including core body temperature and observations of overall gross physical and injection site reactions.

4. Challenge virus preparation and administration

FMDV serotype A24 Cruzeiro (SGD variant) (isolate originated in Brazil, approximately 1950) challenge virus stock (1×10^6 bovine infectious dose 50% [BID₅₀]/ml) was prepared following one time passage in BHK-21 cell culture [11] and two amplifications in bovine tongue. For challenge, virus stock was diluted 1:40 in Dulbecco's Modified Eagle Medium (DMEM) with 1% antibiotics/antimycotics to obtain the target titer, 5.6–6.0 log₁₀ tissue culture infective dose 50% (TCID₅₀/ml), based on titration on a porcine cell line highly permissive for FMDV, LF-BK [12], or LF-BK $\alpha\text{v}\beta_6$, [13,14].

One week post-vaccination, steers were sedated with 0.22 mg/kg of xylazine IM in the hindquarter and challenged via the intradermal lingual (IDL) route using a minor modification of the World Organisation for Animal Health (OIE) guidelines [15]. FMDV challenge (1×10^4 BID₅₀/0.4 ml) was delivered by inoculation of 0.1 ml into each of four sites on the upper surface of the tongue. Sedation was reversed by administering 2–4 mg/kg of tolazoline intravenously.

5. Clinical observations

Individuals, through masked treatment allocation, performed immunizations, clinical observations (lesions), and laboratory assays (plasma virus isolation, plasma rRT-PCR, and virus neutralization tests). We assessed the presence or absence of FMD clinical disease in sedated steers at 3, 7, 10, and 14 dpc. We noted FMD clinical signs and lesions using the following criteria: negative, no

Table 1

Summary of eight independent AdtA24 vaccine efficacy studies in which steers were challenged at 7 days post-vaccination with FMDV serotype A24 Cruzeiro.

Study no.	AdtA24 dose (PU/steer) and purification method	N	% Protection from clinical disease	% Protection against viremia	FMDV mean VNT (day of challenge)		Ad5 mean VNT titer ($\log_{10}$) $\pm$ S.D. (day of challenge)
					% Positive steers	($\log_{10}$) $\pm$ S.D.	
1	FFB	4	0	ND	0	0.6 $\pm$ 0.0	ND
	2.1 $\times 10^{11}$ CsCl	7	100**	ND	100**	1.7 $\pm$ 0.3**	ND
2	FFB	7	14	0	0	0.6 $\pm$ 0.0	0.6 $\pm$ 0.0
	1.0 $\times 10^{11}$ CsCl	7	100**	100**	100**	1.6 $\pm$ 0.3**	0.9 $\pm$ 0.3*
	2.1 $\times 10^9$ CsCl	7	57	71*	43	1.1 $\pm$ 0.6	0.7 $\pm$ 0.2
	1.0 $\times 10^8$ CsCl	7	14	29	0	0.6 $\pm$ 0.0	0.6 $\pm$ 0.1
3	FFB	6	0	ND	0	0.6 $\pm$ 0.0	ND
	4.2 $\times 10^{10}$ CsCl	5	60	ND	80*	1.1 $\pm$ 0.4*	ND
4	FFB	4	0	0	0	0.6 $\pm$ 0.0	0.6 $\pm$ 0.0
	5.0 $\times 10^{10}$ DS5	5	80*	80*	80*	1.4 $\pm$ 0.6*	1.3 $\pm$ 0.4**
	1.3 $\times 10^{10}$ DS5	6	100**	100**	100**	1.6 $\pm$ 0.5**	1.2 $\pm$ 0.5*
	3.1 $\times 10^9$ DS5	5	20	80*	40	0.8 $\pm$ 0.3	0.9 $\pm$ 0.3*
	5.0 $\times 10^{10}$ DS7	5	100**	80*	100*	1.5 $\pm$ 0.6*	1.1 $\pm$ 0.4*
	1.3 $\times 10^{10}$ DS7	6	83*	100**	83**	1.1 $\pm$ 0.5*	1.0 $\pm$ 0.4*
	3.1 $\times 10^9$ DS7	5	60	80*	60	1.1 $\pm$ 0.5*	0.8 $\pm$ 0.2*
5	2.0 $\times 10^{10}$ Adt.Null	10	0	0	0	0.6 $\pm$ 0.0	1.2 $\pm$ 0.5
	2.0 $\times 10^{10}$ DS5	10	90**	100**	50*	0.9 $\pm$ 0.4*	1.4 $\pm$ 0.5
	1.0 $\times 10^{10}$ DS5	10	70**	100**	40	0.8 $\pm$ 0.2*	0.9 $\pm$ 0.4
	5.0 $\times 10^9$ DS5	10	70**	100**	10	0.7 $\pm$ 0.2	1.0 $\pm$ 0.3
6	FFB	2	0	0	0	0.6 $\pm$ 0.0	0.6 $\pm$ 0.0
	6.0 $\times 10^9$ DS5	11	82	100*	100*	1.5 $\pm$ 0.3**	0.7 $\pm$ 0.2*
7	FFB	10	0	0	0	0.6 $\pm$ 0.0	0.6 $\pm$ 0.0
	5.7 $\times 10^9$ DS5	20	55*	90**	65**	1.0 $\pm$ 0.4**	1.3 $\pm$ 0.4**
8	FFB	2	0	0	0	0.6 $\pm$ 0.0	0.6 $\pm$ 0.0
	1.8 $\times 10^{10}$ DS3	6	67	83	67	1.3 $\pm$ 0.6*	0.7 $\pm$ 0.2
	4.5 $\times 10^9$ DS3	6	83	83	50	0.9 $\pm$ 0.5	0.7 $\pm$ 0.2
	1.1 $\times 10^9$ DS3	6	67	67	33	0.9 $\pm$ 0.5	0.6 $\pm$ 0.0
	2.8 $\times 10^8$ DS3	6	17	17	17	0.6 $\pm$ 0.1	0.8 $\pm$ 0.3
Total vaccinates	150						
Total controls	45						

Different from the control group within a study:

* *P*-value, 0.01 to 0.05.** *P*-value < 0.01.

pedal or secondary (lip, mouth, or nose) vesicular lesions; positive, one or more pedal vesicular lesions on one or more feet or any secondary vesicular lesions. For dose response studies using economically feasible purification methods, we calculated the bovine protective dose for 50% or 80% of steers (BPD₅₀ or BPD₈₀) using the Spearman/Kärber method with endpoint generalized lesion data obtained on 14 dpc [16].

5.1. Virus neutralization test (VNT)

FMDV serotype A24 strain Cruzeiro and adenovirus serotype 5 (Ad5) antibody titers were determined using heat-inactivated (56 °C, 30 min) serum samples that were serially diluted four-fold (modified from 15). FMDV VNT titers were determined in duplicate wells after 3 days in BHK-21 cell cultures grown on DMEM supplemented with 10% fetal bovine serum, 1% Modified Eagle Medium non-essential amino acids (MEM NEAA), 1% MEM with sodium pyruvate, 1% antibiotic/antimycotic, and 0.15% sodium bicarbonate. Ad5 VNT titers were determined in duplicate wells after 10 days by a constant-virus decreasing-serum neutralization assay in HEK293 cells grown in MEM supplemented with 3% fetal bovine serum, 1% MEM NEAA, 1% antibiotic/antimycotic.

FMDV and Ad5 VNT titers were calculated using the Spearman–Kärber method based on cytopathic effect (CPE) using 50% as the neutralization endpoint, and the lower limit of assay detection was 0.6 $\log_{10}$ [17,18]. The geometric mean virus neutralization test titers (GMT) were calculated using a value of 0.6 $\log_{10}$ for samples that were below the limit of detection. A test sample was scored positive if the VNT titer was $\geq 0.9 \log_{10}$, since more concentrated samples were not reliable in this assay.

5.2. Detection of antibodies to FMDV non-structural protein (NSP)

The PrioCHECK® FMDV NS Antibody ELISA (ThermoFisher Scientific) was used to detect antibodies to FMDV NSP according to manufacturer's instructions. A sample was considered positive if the percent inhibition was $\geq 50\%$.

5.3. Detection of FMDV or FMDV nucleic acid

Plasma samples collected on 0 to 5 dpc were tested for the presence of viable FMDV [15]. Undiluted samples (20 μ l) were added to single wells of 24-well plates containing BHK-21 or LF-BK cell monolayers. A minimum of two blind passages on CPE-negative wells was conducted to confirm the absence of infectious virus. Plasma samples collected on 0 to 5 dpc were analyzed by Real-Time Reverse Transcriptase–Polymerase Chain Reaction (rRT–PCR) for the presence or absence of FMDV nucleic acid according to Callahan et al. [15,19]. A sample *C_t* value ≤ 40 was scored as positive. Any sample that was virus isolation or rRT–PCR positive during any of the first five dpc was scored as positive. In some instances, only one assay method was used on each sample collected.

5.4. Data analysis

VNT geometric means and standard deviations, and regression analyses of both AdtA24 dose and protection from clinical disease and viremia, as well as AdtA24 dose and VNT titer were conducted (Microsoft Excel). Comparisons between the control group and treatments groups within an experiment were done using the two-tailed Fisher's exact test (GraphPad). *P*-values ≤ 0.05 were considered significant.

6. Results

6.1. Safety

No adverse local or systemic reactions were observed during the first three days following AdtA24 IM administration to 150 steers.

7. Efficacy

7.1. Response of control steers to FMDV serotype A24 Cruzeiro infection

Following challenge with FMDV serotype A24 Cruzeiro, 98% (44/45) of FFB- or Adt.Null-inoculated, control steers developed pedal lesions by 3 dpc. Viremia was detected as early as 1 dpc, with most animals positive on 2 and 3 dpc, and rarely on 4 dpc. Serum VNT (typically 2.4 to 3.0 log₁₀) and NSP antibody responses in control steers were detected on 7 and 14 dpc (data not shown).

7.2. Vaccine efficacy studies at 7 dpv

A summary of results and statistical comparisons from testing AdtA24 preparations purified by one of four methods and spanning a 2100-fold dose range in eight studies are summarized in Table 1. Details are summarized below and grouped by AdtA24 purification method.

7.3. CsCl preparation: efficacy Studies 1, 2, and 3

In Efficacy Study 1, the highest immunizing dose tested in all eight studies, 2.1×10^{11} PU/steer, prevented clinical disease in all seven steers and resulted in the highest VNT titer at the time of challenge (Table 1). In Efficacy Study 2, we titrated three doses that spanned a 1000-fold range, and a clear dose-titration effect occurred in protection from clinical disease and viremia, and in the VNT titers and percent seropositive (Table 1). In Efficacy Study 3, a pilot study using 4.2×10^{10} PU/steer, was designed to evaluate efficacy at an earlier time point, 4 dpv, compared to 7 dpv. Despite the absence of detectable VNT to FMDV A24 Cruzeiro, protection against FMD generalized disease was 60% in both the 4 and 7 dpv groups (Table 1; data not shown for 4 dpv group).

7.4. DS5 and DS7 preparations: efficacy Studies 4, 5, 6, and 7

Efficacy Study 4 was designed to determine if there were differences in the efficacy of two production scale-able vaccine preparations, DS5 and DS7, tested with the identical three doses in the same study (Table 1). The two highest doses were nearly indistinguishable for prevention of clinical disease and viremia, as well as post-vaccination/pre-challenge geometric VNT titer. Although the DS7 low dose preparation induced a stronger response than an equivalent DS5 dose in terms of VNT titer and protection, the calculated DS5 BPD₅₀ (6.3×10^9 PU) and DS7 BPD₅₀ (3.5×10^9 PU) were similar enough that we chose to conduct further studies on the less purified preparation, DS5, which would be more economical for commercial production. In Efficacy Study 5, we examined the effects of a high dose of the placebo vector, Adt.Null, and another dose titration of the DS5 preparation for comparison to results in Efficacy Study 4. All Adt.Null-inoculated steers developed clinical disease and viremia following challenge (Table 1). For the three AdtA24 treatment groups, there was a relatively flat dose response with respect to seroconversion and to efficacy. All 30 vaccinates were protected from viremia. The DS5 calculated BPD₈₀ was 7.1×10^9 PU, similar to the calculated BPD₅₀ in Efficacy Study 4. In Efficacy Study 6, we tested an AdtA24 DS5 vaccine produced under an experimental outline of production at an immunizing dose that

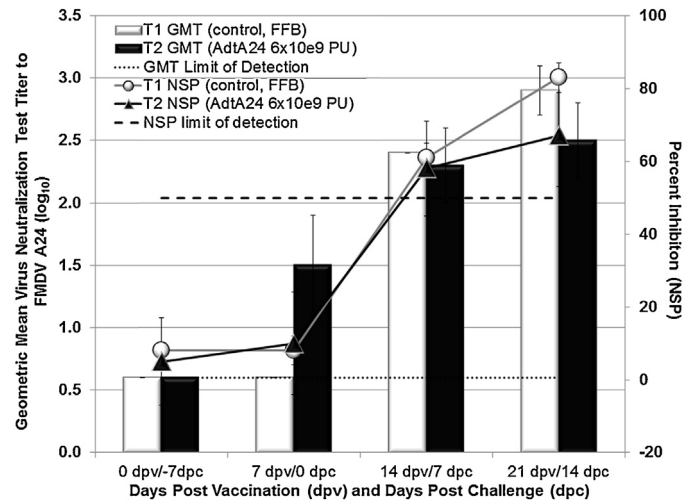


Fig. 1. FMDV virus neutralization test (VNT) and nonstructural protein (NSP) serological responses of steers immunized with AdtA24 (Efficacy Study 6). Steers were administered 6.0×10^9 particle units of AdtA24 (treatment group 2 [T2], $n = 11$) or final formulation buffer (FFB) (control treatment group [T1], $n = 2$). All steers were challenged 7 days later with virulent FMDV serotype A24. The virus neutralization test (VNT) titers for FMDV A24 were measured prior to vaccination and every 7 days for 21 days and shown by the bars with geometric mean VNT titer (GMT) values on the left vertical axis ($\pm$ standard deviation; limit of detection range: >0.6 – 3.6 log₁₀). Antibody production to the FMDV NSP was measured weekly, and data are recorded as lines with values on the right vertical axis. A positive response for antibodies to the FMDV NSP is indicated by $\geq 50\%$.

was slightly below the DS5 BPD₈₀ value (7.1×10^9 PU) determined in Efficacy Study 5 (Table 1). All vaccinates were FMDV seropositive at 7 dpv, and 64% of these had VNT titers ≥ 1.5 log₁₀. Following challenge, 82% and 100% were protected against clinical disease and viremia, respectively. In Efficacy Study 7, the same AdtA24 DS5 experimental lot used in Efficacy Study 6 was further evaluated at an immunizing dose of 5.7×10^9 PU in two treatment groups that were challenged at 7 dpv (Table 1) or 14 dpv. The GMT ($\pm$ standard deviation) and percent VNT positive were higher at 14 dpv (1.3 ± 0.5 log₁₀; 80%) compared to 7 dpv (1.0 ± 0.4 log₁₀; 65%), and clinical protection was higher in vaccinates challenged at 14 dpv (75%) compared to 7 dpv (55%).

7.5. DS3 preparation: efficacy study 8

The goal of Efficacy Study 8 was to determine if the least purified DS3 vaccine preparation had a similar BPD₅₀ to the DS5 and DS7 vaccines used in Efficacy Study 3 (Table 1). Although the DS3 preparation had a lower BPD₅₀ (1.4×10^9 PU) compared to DS5 and DS7 (Study 4), concerns related to assay consistency and long term product stability associated with the vaccine potency release led to a decision to not advance the DS3 production method.

7.6. Serological responses to FMDV and nonstructural proteins

In eight efficacy studies, 66% (99/150) of AdtA24 vaccinates had detectable FMDV neutralizing antibodies, but none of the vaccinates had antibodies to NSPs at the time of challenge. To illustrate this point, in Efficacy Study 6 at 7 dpv (day of challenge), all eleven steers immunized with 6.0×10^9 PU of AdtA24 were VNT positive and NSP negative (Fig. 1). At 7 and 14 dpc, all vaccinated and naive steers were VNT and NSP seropositive.

8. Discussion

This series of eight AdtA24 independent vaccine studies greatly expands upon our previous proof-of-concept cattle experiment [6].

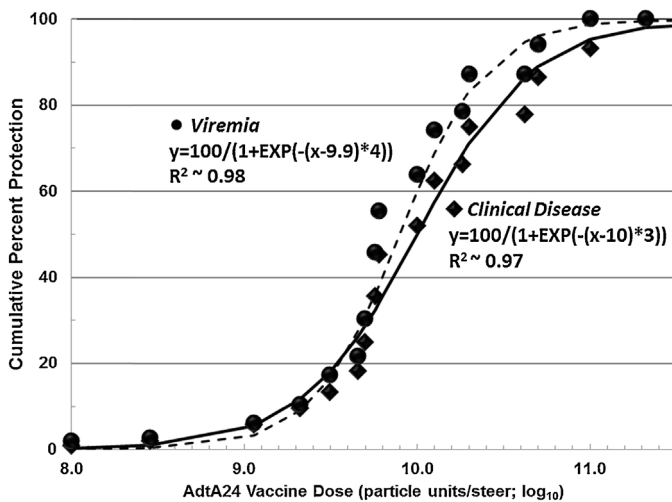


Fig. 2. Correlation between AdtA24 vaccine dose and cumulative protection from clinical disease and viremia. Protection from clinical disease was evaluated in steers vaccinated with one of 17 doses of AdtA24 and challenged at 7 dpv in eight studies. Protection from viremia was evaluated in steers vaccinated with one of 15 doses in six studies. 69% (103/150) of vaccinates were protected from clinical disease, and 84% (116/138) were protected from viremia (Steers in two studies were not tested for viremia.). The calculated dose for 50% and 90% prevention of clinical disease were 1.0×10^{10} and 5.6×10^{10} PU/steer, respectively. The calculated dose for 50% and 90% prevention of viremia were 8.0×10^9 and 3.0×10^{10} PU/steer, respectively. The coefficient of determination (R^2) between cumulative percent protection from clinical disease or viremia and the AdtA24 dose was calculated as $R^2 = 0.97$ or 0.98 , respectively, and the equations describing the relationships are on the graph.

The AdtA24 non-adjuvanted, IM-delivered vaccine was safe over the 2100-fold dose range evaluated as evidenced by an absence of local or systemic untoward reactions. Additional safety of AdtA24 vaccine with respect to the absence of vaccine shed and spread is inferred from the lack of FMD VNT seroconversion in the comingled, placebo controls.

Over the 1.0×10^8 to 2.1×10^{11} PU/steer dose range tested, 69% (103/150) of all AdtA24 vaccinates were protected from clinical disease. The best fit curve for data correlating AdtA24 dose with cumulative percent protection from clinical disease had an $R^2 = 0.97$ (Fig. 2). The estimated non-adjuvanted AdtA24 dose to prevent clinical disease in 50% or 90% of the protected steers was 1.0×10^{10} PU/steer and 5.6×10^{10} PU/steer. Similarly, for the 116 vaccinated steers protected from viremia (84% of total), the best fit curve for data correlating vaccine dose with cumulative percent protection had an $R^2 = 0.98$ (Fig. 2). The estimated AdtA24 dose to prevent viremia in 50% or 90% of steers was 8.0×10^9 PU/steer and 3.0×10^{10} PU/steer.

The FMDV A24 Cruzeiro GMT at time of challenge in protected steers were not strongly correlated with vaccine dose ($R^2 = 0.51$) (Fig. 3). This finding may be due to the relatively short, one week time period between vaccination and challenge used in these studies compared to the OIE standard three week time interval between vaccination and challenge reported in most cattle potency studies using conventional, inactivated FMD vaccines [15]. Other possible explanations for the lack of strong correlation may be due to the potency differences in the different vaccine preparations, health status of the steers, cellular immune response differences, or differences among individual steers in their antibody response levels to FMDV A24 capsid proteins expressed by AdtA24-infected host cells. Importantly, on the day of challenge, 96% (63/66) of vaccinates with VNT titers $\geq 1.2 \log_{10}$, and 87% (81/93) of vaccinates with VNTs $\geq 0.9 \log_{10}$ were protected from clinical disease (Fig. 4). Among vaccinated, protected animals, the correlation between individual VNT titers and cumulative protection from clinical

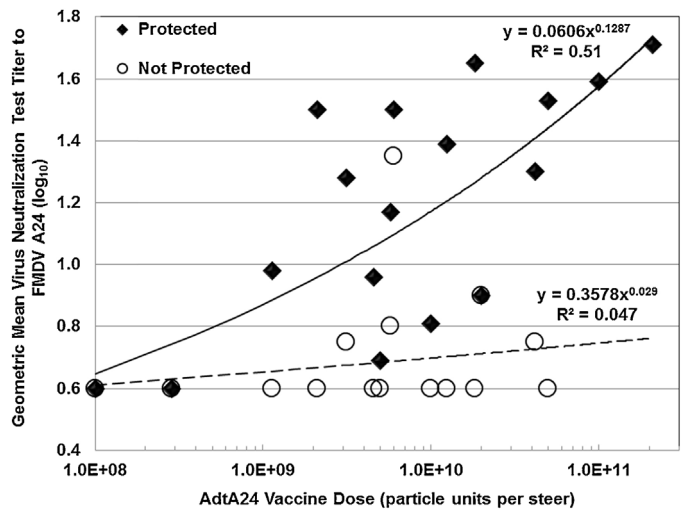


Fig. 3. Correlation between AdtA24 vaccine dose and the geometric mean virus neutralization test titer (GMT). Steers were vaccinated with one of 17 doses of AdtA24 and challenged at 7 days post vaccination (dpv) in eight studies. Separate GMTs are plotted for protected and not protected steers for each AdtA24 dose. The equations describing the relationships between GMT and AdtA24 dose for the protected and not protected steers are on the graph. From the best-fit line for protected steers, the GMT was calculated as $1.6 \log_{10}$ for 90% of the steers, and $1.2 \log_{10}$ for 50% of the steers.

disease was $R^2 = 0.99$ (Fig. 5). The predicted response for 50% and 90% cumulative protection from clinical disease at 7 dpv required VNT titers of $1.0 \log_{10}$ and $1.9 \log_{10}$, respectively.

In a four study subset analysis in which at least 90% protection against clinical disease was observed, there was a poor correlation ($R^2 = 0.22$) between individual VNT titers to FMDV A24 Cruzeiro and to Ad5 (data not shown). Although Ad5 seroconversion is indicative of AdtA24 vaccine exposure, these results suggest the low feasibility of the future development of an Ad5-based antibody assay as a surrogate correlation of protection at one week post-vaccination.

Since CsCl purification methods for AdtA24 are impractical for large-scale manufacturing, several feasibility efficacy studies were conducted using material collected at different vaccine production downstream steps. The DS3 preparation, at a 1.1×10^9 PU

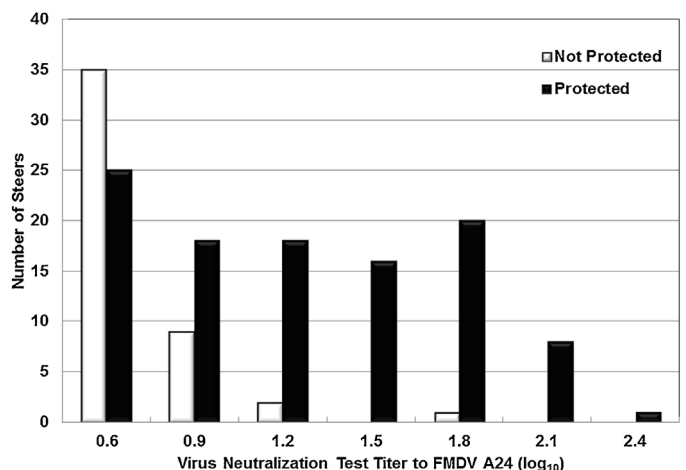


Fig. 4. Protection from clinical disease of all 150 AdtA24 vaccinated steers challenged at 7 days post vaccination (dpv) in eight studies. The number of steers protected and unprotected from clinical disease are displayed based on FMDV A24 VNT titer. For the 103 protected steers, the GMT for 50% and 90% of the steers were 1.2 and $1.8 \log_{10}$, respectively. For the 47 unprotected steers, the GMT for 50% and 90% were 0.6 and $0.9 \log_{10}$, respectively.

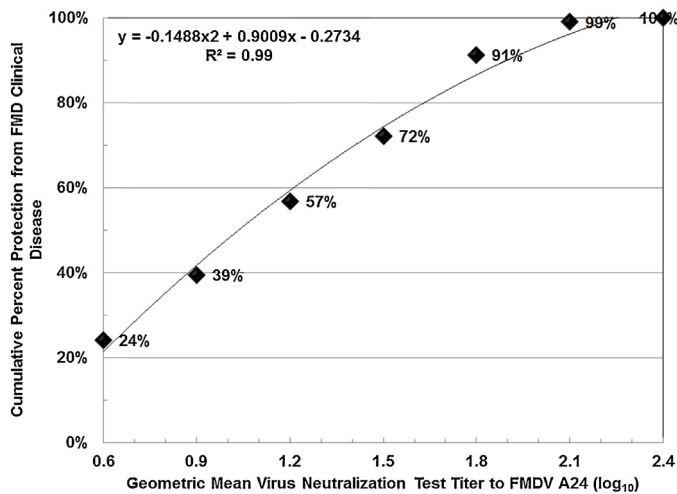


Fig. 5. For 103 steers protected from clinical disease, correlation between cumulative protection from clinical disease and FMDV A24 geometric mean virus neutralization test titer (GMT). Steers ($n = 150$) were vaccinated with one of 17 doses of AdtA24 and challenged at 7 days post vaccination (dpv) in eight studies. The relationship between the GMT and cumulative protection from clinical disease for the 103 protected steers are presented along with the equation describing this relationship. The calculated FMDV A24 GMT titer to prevent clinical disease in 90% of the 103 steers that were protected from clinical disease was 1.9 log₁₀ and for 50% of the steers, was 1.0 log₁₀.

dose, protected at a similar level (67%) to that obtained with a three-fold higher DS5 dose (60%), and at a much higher level compared to a three-fold higher DS7 dose (20%). However, these DS3 results were obtained in a very limited number of animals (5–6/group), and in consideration of other important production criteria (e.g., potency release testing and stability), the AdtA24 DS5 preparation was selected as the basis for future studies.

None of the AdtA24 vaccinates were NSP positive prior to challenge. This result is likely because the AdtA24 FMDV gene cassette was designed to remove the FMDV 3A gene and the coding region for the first six amino acids of FMDV 3B which may prevent antibody inhibition in FMDV NS competitive ELISA. Although AdtA24 encodes for the 3C protein, it is likely that the 3C protein is either non-immunogenic in cattle following single dose administration and/or antibodies to the 3C protein are not inhibitory in the FMDV NS competitive ELISA. Thus, in addition to the manufacturing safety aspect of the Adt-based FMD platform, another important feature is its potential for use in a FMD outbreak response in which a DIVA-based vaccination strategy may be required. Additional studies using higher potency AdtA24 vaccines, prime-boost immunization regimens, and serological testing at monthly time points will be necessary to validate and expand the encouraging DIVA results obtained in these pilot studies.

AdtA24 immunization 7 days prior to challenge with virulent FMDV serotype A24 Cruzeiro prevented clinical disease in 100% of steers at doses $\geq 5.0 \times 10^{10}$ PU. More recently, cattle efficacy studies using adjuvant systems compatible with the Adt-based FMD platform are very encouraging with respect to lowering the AdtA24 BPD₅₀ and BPD₈₀ vaccine dose (Schutta et al., manuscript in preparation). Lastly, the relatively rapid onset of protection following single dose immunization is another important aspect of the AdtA24 product profile. The mechanism(s) of host protection following single dose AdtA24 vaccine administration requires further study and may involve antibody-independent pathways or non-neutralizing antibodies, since 24% (25/103) of the protected steers had undetectable VNT titers at time of challenge.

Conflict of interest statement

All authors have read and approved the manuscript. D. Etyreddy, D. Brough, and B. Butman were employed by GenVec Inc. while the studies were performed.

Acknowledgments

We thank K. Addorisio, A. Zsak, W. Hurtle, T. Hester, and K. Floyd for their technical assistance. We thank the PIADC Animal Research Branch for their professional support and assistance and M. Rasmussen for critical review of this manuscript. GenVec, Inc., Leidos, Inc., The McConnell Group, Inc., and M. Moraes were funded through separate agreements with the Science and Technology Directorate of the U.S. Department of Homeland Security (DHS S&T) under Award Numbers HSHQDC-07-9-00004, GS-23F-8006H, HSHQDC-8-C-00011, and HSHQPD-07-X-00003. This research was supported in part by an appointment of M. Pisano to the PIADC Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the U.S. DHS. ORISE is managed by ORAU under DOE contract number DE-AC05-06OR23100. DHS S&T Agriculture Defense Branch, as the funding source, had no involvement in the study design; collection, analysis and interpretation of data; writing of the report; and in the decision to submit the article for publication. All opinions expressed in this paper are the authors' and do not necessarily reflect the policies and views of DHS, ARS, DOE, or ORAU/ORISE.

References

- [1] OIE. World Organisation for Animal Health. Foot and Mouth Disease 2007. Available from: (http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/foot_and_mouth_disease.pdf); [accessed 23.11.15].
- [2] Arzt J, Juleff N, Zhang Z, Rodriguez LL. The pathogenesis of foot-and-mouth disease I: viral pathways in cattle. *Transbound Emerg Dis* 2011;58(4 (Aug));291–304 [PubMed PMID: 21366894. Epub 2011/03/04. eng.].
- [3] Mayr GA, Chinsangaram J, Grubman MJ. Development of replication-defective adenovirus serotype 5 containing the capsid and 3C protease coding regions of foot-and-mouth disease virus as a vaccine candidate. *Virology* 1999;263(2):496–506 [Oct 25 PubMed PMID: 10544121. Epub 1999/11/02. eng.].
- [4] Moraes MP, Mayr GA, Mason PW, Grubman MJ. Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. *Vaccine* 2002;20(11–12):1631–9 [Feb 22 PubMed PMID: 11858872. Epub 2002/02/23. eng.].
- [5] de Avila Botton S, Brum MC, Bautista E, Koster M, Weiblen R, Golde WT, et al. Immunopotential of a foot-and-mouth disease virus subunit vaccine by interferon alpha. *Vaccine* 2006;24(17):3446–56 [PubMed PMID: 16522345. Epub 2006/03/09. eng. Apr 24].
- [6] Pacheco JM, Brum MC, Moraes MP, Golde WT, Grubman MJ. Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. *Virology* 2005;337(2):205–9 [PubMed PMID: 15893355. Epub 2005/05/17. eng. Jul 5].
- [7] Gall JG, Lizonova A, Etyreddy D, McVey D, Zuber M, Kovessdi I, et al. Rescue and production of vaccine and therapeutic adenovirus vectors expressing inhibitory transgenes. *Mol Biotechnol* 2007;35(3 (Mar)):263–73 [PubMed PMID: 17652790. Epub 2007/07/27. eng.].
- [8] Brough DE, Lizonova A, Hsu C, Kulesa VA, Kovessdi I. A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. *J Virol* 1996;70(9 (Sep)):6497–501 [PubMed PMID: 8709289. Pubmed Central PMCID: PMC190687. Epub 1996/09/01. eng.].
- [9] Brake DA, McIlhenny M, Miller T, Christianson K, Keene A, Lohns G, et al. Human adenovirus-vectored foot-and-mouth disease vaccines: establishment of a vaccine product profile through in vitro testing. *Dev Biologicals* 2012;134:123–33 [PubMed PMID: 22888605. Epub 2012/08/15. eng.].
- [10] Yang M, Clavijo A, Suarez-Banmann R, Avalo R. Production and characterization of two serotype independent monoclonal antibodies against foot-and-mouth disease virus. *Vet Immunol Immunopathol* 2007;115(1–2):126–34 [PubMed PMID: 17109972. Epub 2006/11/18. eng. Jan 15].
- [11] Mowat GN, Brooksby JB, Pay TW. Use of BHK 21 cells in the preparation of mouse attenuated live foot-and-mouth disease vaccines for the immunization of cattle. *Nature* 1962;196:655–6 [PubMed PMID: 13936398. Epub 1962/11/17. eng. Nov 17].

- [12] Swaney LM. A continuous bovine kidney cell line for routine assays of foot-and-mouth disease virus. *Vet Microbiol* 1988;18(1 (Sep)):1–14 [PubMed PMID: 2847400. Epub 1988/09/01. eng.].
- [13] LaRocco M, Krug PW, Kramer E, Ahmed Z, Pacheco JM, Duque H, et al. A continuous bovine kidney cell line constitutively expressing bovine alphavbeta6 integrin has increased susceptibility to foot-and-mouth disease virus. *J Clin Microbiol* 2013;51(6 (Jun)):1714–20 [PubMed PMID: 23515553. Pubmed Central PMCID: PMC3716081. Epub 2013/03/22. eng.].
- [14] LaRocco M, Krug PW, Kramer E, Ahmed Z, Pacheco JM, Duque H, et al. Correction for LaRocco et al., A continuous bovine kidney cell line constitutively expressing bovine alphavbeta6 integrin has increased susceptibility to foot-and-mouth disease virus. *J Clin Microbiol* 2015;53(2 (Feb)):755 [PubMed PMID: 25617444. Pubmed Central PMCID: PMC4298512. Epub 2015/01/27. eng.].
- [15] OIE. World Organisation for Animal Health. Foot and mouth disease. Chapter 2.1.5. Available from (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.05.FMD.pdf); 2012 [accessed 23.11.15].
- [16] Doel TR. Potency assessment of inactivated viral vaccines. In: Mowat N, Rweyemamu M, editors. *Vaccine manual. The production and quality control of veterinary vaccines for use in developing countries*. Rome, Italy: Food and Agriculture Organization of the United Nations; 1997.
- [17] Mayr GA, O'Donnell V, Chinsangaram J, Mason PW, Grubman MJ. Immune responses and protection against foot-and-mouth disease virus (FMDV) challenge in swine vaccinated with adenovirus-FMDV constructs. *Vaccine* 2001;19(15–16):2152–62 [PubMed PMID: 11228388. Epub 2001/03/03. eng. Feb 28].
- [18] Sumida SM, Truitt DM, Lemckert AA, Vogels R, Custers JH, Addo MM, et al. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol (Baltimore MD: 1950)* 2005;174(11):7179–85 [PubMed PMID: 15905562. Epub 2005/05/21. eng. Jun 1].
- [19] Callahan JD, Brown F, Osorio FA, Sur JH, Kramer E, Long GW, et al. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J Am Vet Med Assoc* 2002;220(11):1636–42 [PubMed PMID: 12051502. Epub 2002/06/08. eng. Jun 1].