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

Christopher Schutta, José Barrera, Melia Pisano, Laszlo Zsak, Marvin J. Grubman, Gregory A. Mayr, Mauro P. Moraes, Barbara J. Kamicker, David A. Brake, Damodar Ettyreddy, Douglas E. Brough, Bryan T. Butman, John G. Neilan

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**A B S T R A C T**

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 $1.0 \times 10^8$ to $2.1 \times 10^{11}$ 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 intradermal 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 vaccines 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.

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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 polyprotein 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 FMDV 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 FMDV 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-3a 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 (Cipheron 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 CFA, 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 cleidocapitale 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 × 10⁶ 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 contain 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 α₁β₂ [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 × 10⁴ 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 RT-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.

<table>
<thead>
<tr>
<th>Study no.</th>
<th>AdtA24 dose (PU/steer) and purification method</th>
<th>N</th>
<th>% Protection from clinical disease</th>
<th>% Protection against viremia</th>
<th>FMDV mean VNT (day of challenge)</th>
<th>Adt mean VNT titer (log_{10}) ± S.D. (day of challenge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FFB 2.1 × 10^11 CsCl</td>
<td>7</td>
<td>100%</td>
<td>0%</td>
<td>0</td>
<td>0.6 ± 0.0 ND</td>
</tr>
<tr>
<td>2</td>
<td>FFB 1.0 × 10^11 CsCl</td>
<td>7</td>
<td>100%</td>
<td>0%</td>
<td>0.6 ± 0.0 ND</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>FFB 1.0 × 10^11 CsCl</td>
<td>7</td>
<td>100%</td>
<td>0%</td>
<td>1.7 ± 0.3 ND</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>FFB 4.2 × 10^10 CsCl</td>
<td>5</td>
<td>80%</td>
<td>80%</td>
<td>0.6 ± 0.0 ND</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>5.0 × 10^10 D5</td>
<td>5</td>
<td>80%</td>
<td>80%</td>
<td>0.6 ± 0.0 ND</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>6</td>
<td>5.0 × 10^10 D5</td>
<td>5</td>
<td>80%</td>
<td>80%</td>
<td>0.6 ± 0.0 ND</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>7</td>
<td>FFB 2.0 × 10^10 Adt.Null</td>
<td>10</td>
<td>90%</td>
<td>90%</td>
<td>0.6 ± 0.0 ND</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>8</td>
<td>5.7 × 10^10 D5</td>
<td>20</td>
<td>55%</td>
<td>55%</td>
<td>0.6 ± 0.0 ND</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>100%</td>
<td>100%</td>
<td>0.6 ± 0.0 ND</td>
<td>0.6 ± 0.0</td>
</tr>
</tbody>
</table>

Different from the control group within a study:

* P-value < 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 (BPD_{50} or BPD_{80}) using the Spearman/Kärber method with endpoint generalized lesion data obtained on 14 dpc [16].

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 ≥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 μl) 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 dpc, compared to 7 dpc. Despite the absence of detectable VNT to FMDV A24 Cruzeiro, protection against FMDV generalized disease was 60% in both the 4 and 7 dpc groups (Table 1; data not shown for 4 dpc group).

7.4. DSS 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, DSS 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 DSS dose in terms of VNT titer and protection, the calculated DSS BPD_{50} (6.3 × 10^{8} PU) and DS7 BPD_{50} (3.5 × 10^{9} PU) were similar enough that we chose to conduct further studies on the less purified preparation, DSS, 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 DSS 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 DSS calculated BPD_{50} was 7.1 × 10^{9} PU, similar to the calculated BPD_{50} in Efficacy Study 4. In Efficacy Study 6, we tested an AdtA24 DSS vaccine produced under an experimental outline of production at an immunizing dose that was slightly below the DSS BPD_{50} value (7.1 × 10^{9} PU) determined in Efficacy Study 5 (Table 1). All vaccines were FMDV seropositive at 7 dpv, and 64% of these had VNT titers ≥ 1.5 log_{10}. 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 (± standard deviation) and percent VNT positive were at 14 dpv (1.3 ± 0.5 log_{10}; 80%) compared to 7 dpv (1.0 ± 0.4 log_{10}; 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 BPD_{50} to the DS5 and DS7 vaccines used in Efficacy Study 3 (Table 1). Although the DS3 preparation had a lower BPD_{50} (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].
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 × 10⁸ to 2.1 × 10¹¹ PU/steer dose range tested, 69% (103/150) of all AdtA24 vaccines 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 × 10¹⁰ PU/steer and 5.6 × 10¹⁰ 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 × 10⁸ PU/steer and 3.0 × 10¹⁰ 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 vaccines with VNT titers ≥1.2 log₁₀, and 87% (81/93) of vaccines with VNTs ≥0.9 log₁₀ were protected from clinical disease (Fig. 4). Among vaccinated, protected animals, the correlation between individual VNT titers and cumulative protection from clinical 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 log₁₀ and 1.9 log₁₀, 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 × 10⁹ PU
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 vaccines 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 of ≥5.0×10^{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 BPD_{50} and BPD_{60} 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. Ettedyredy, D. Brough, and B. Butman were employed by GenVec Inc. while the studies were performed.

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