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Duration of protection and humoral immunity induced by an adenovirus-vectored subunit vaccine for foot-and-mouth disease (FMD) in Holstein steers

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious viral infection of cloven hooved animals that continues to cause economic disruption in both endemic countries or when introduced into a formally FMD free country. Vaccines that protect against clinical disease and virus shedding are critical to control FMD. The replication deficient human adenovirus serotype 5 (Ad5) vaccine vector expressing empty FMD virus (FMDV) capsid, AdtFMD, is a promising new vaccine platform. With no shedding or spreading of viral vector detected in field trials, this vaccine is very safe to manufacture, as there is no requirement for high containment faciitites. Here, we describe three studies assessing the proportion of animals protected from clinical vesicular disease (foot lesions) following live-FMDV challenge by intradermolingual inoculation at 6 or 9 months following a single vaccination with the commercial AdtFMD vaccine, provisionally licensed for cattle in the United States. Further, we tested the effect of vaccination route (transdermal, intramuscular, subcutaneous) on clinical outcome and humoral immunity. Results demonstrate that a single dose vaccination in cattle with the commercial vaccine vector expressing capsid proteins of the FMDV strain A24 Cruzeiro (Adt.A24), induced protection against clinical FMD at 6 months (100% transdermal, 80% intramuscular, and 60% subcutaneous) that waned by 9 months post-vaccination (33% transdermal and 20% intramuscular). Post-vaccination serum from immunized cattle (all studies) generally contained FMDV specific neutralizing antibodies by day 14. Anti-FMDV antibody secreting cells are detected in peripheral blood early following vaccination, but are absent after 28 days post-vaccination. Thus, the decay in antibody mediated immunity over time is likely a function of FMDV-specific antibody half-life. These data reveal the short time span of anti-FMDV antibody secreting cells (ASCs) and important performance characteristics of needle-free vaccination with a recombinant vectored subunit vaccine for FMDV.

Abbreviations: Ad5, human replication deficient adenovirus serotype 5; AdtFMD, commercial replication-deficient human adenovirus vectored FMD empty capsid; Adt.24, commercial replication-deficient human adenovirus vectored FMD expressing the capsid proteins from strain A24 Cruzeiro; ASCs, antibody secreting cells; BID_{50}, BID_{50}, bovine infectious dose 50%; DIVA, differentiate infected from vaccinated animals; dpc, days post-challenge; dpv, days post-vaccination; FFB, final formulation buffer; FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; IDL, intradermolingual; IM, intramuscular; mpv, months post-vaccination; MT, mean VNT titer in log_{10} scale; OIE, Office International des Epizooties (now World Organisation for Animal Health); PBMC, Peripheral blood mononuclear cells; PIADC, Plum Island Animal Disease Center's; SC, subcutaneous; TD, transdermal; UVM MREC, University of Vermont, Paul Miller Research and Educational Center; VNT, virus neutralization titer.

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1. Introduction

Foot-and-mouth disease (FMD) is caused by an *Aphthovirus* in the *Picornaviridae* Family, foot-and-mouth disease virus (FMDV). Clinical FMD, affecting cloven hooved animals, results in fever and epidermal vesicular lesions of the oro-nasal cavity, nasal planum, and coronary bands of hooves [1]. FMD is highly contagious and easily transmitted via aerosol, fomites, ingestion, or direct contact [1,2]. In countries where FMDV is endemic, the disease has significant economic impacts causing billions of dollars (USD) in losses per year due to animal morbidity, decreased animal productivity, restrictions of animal and animal product trade, as well disruption of tourism due to quarantine [1,3–5]. Introduction of FMD into disease-free countries can be economically disastrous, affecting food security, export markets, and associated collateral industries [1,4,5]. For example, in 2017, the United States, currently FMD-free, exported more than 2.8 billion pounds of beef and veal and 5.6 billion pounds of pork [6]. One estimate suggested an outbreak in California alone would result in a loss between $3 and $69 billion USD (with costs increasing daily due to delays in disease detection) [7].

Many countries remain reliant on FMD vaccines that have changed relatively little over the last 40–50 years [8]. To date, vaccination with chemically-inactivated vaccines has been relatively successful in controlling disease and for use in disease eradication, though semi-annual vaccine boosts are necessary [8,9–12]. Vaccination against FMD in endemic developing countries is costly and eradication via slaughter is not ideal in these areas due to dependency of the low income populace on livestock [8,12,13]. An efficacious, long acting vaccine is critical to control FMD [8].

One new, promising vaccine technology is the human replication deficient adenovirus 5 (Ad5) empty capsid FMDV platform (AdtFMD) [14–18]. The first of these vaccines conditionally licensed by the USDA is Adt.A24. This construct expresses the empty capsid of FMDV strain A24, Cruzeiro and is "the first molecular-based FMD vaccine that allows differentiation of infected from vaccinated animals (DIVA), is safe for manufacture, and is safe for use in meat and milk-producing livestock" [14]. This vaccine is formulated with a lipid/polymer adjuvant [16,17], is protective from homologous challenge 7 days post-vaccination, and does not shed the vaccine virus vector to other closely housed animals or into the environment [15,16].

Here we report on the first long-term studies assessing duration of protection induced by the Adt.A24 vaccine in Holstein steers at 6 and 9 months post single dose vaccination via three different vaccination routes. These intervals were chosen based on the product profile of current inactivated FMD vaccines applied in endemic regions. The performance of this vectored vaccine differed relative to the route of vaccination.

2. Materials and methods

2.1. Study design

Three independent, randomized, and controlled experimental challenge studies were conducted comparing duration of protection following vaccination of Holstein steers. Two studies compared the proportion of animals protected from clinical vesicular disease (foot lesions) following live-virus challenge by intradermolingual (IDL) inoculation at either 6 or 9 months following a single vaccination. The effect of route of vaccination (intramuscular (IM), subcutaneous (SC) or transdermal (TD)) was compared, and placebo-immunized control groups were included. Steers were randomized into treatment groups. Clinical outcomes following challenge was done blinded to identity of the treatment groups. Steers were challenged 6 and 9 months post-vaccination for studies 1 (2013–2014) and 2 (2014–2015), respectively. Study 3 (2016) was a planned 6 month post-vaccination challenge study that was cancelled and became a serology only study.

All vaccinations were conducted at the University of Vermont (UVM) Paul Miller Research and Educational Center (MREC) and all FMDV challenges were carried out at Plum Island Animal Disease Center's (PIADC) biosafety level 3 agriculture (BSL-3 Ag) facility. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of UVM and PIADC.

2.2. Study animals

Healthy Holstein steers obtained from commercial Vermont dairy farms were housed at the UVM MREC at least 2 weeks prior to vaccination and transported to PIADC (studies 1 and 2), at least 2 weeks prior to FMDV challenge. At the time of vaccination, steers were 4.5–7.4 months, 5.5–9.2 months, and 3.2–4 months of age in studies 1, 2, and 3, respectively.

All steers were in good health prior to vaccination, tested negative for bovine viral diarrhea virus (BVDV) and received doses of BoviShield Gold FP™ (Zoetis), Imrab (Merial), Dectomax™ (Zoetis), One Shot™, and Ultrachoice 7™ (Zoetis) as per manufacturer's recommendations prior to study initiation. Prior to transport to PIADC, animals received 6.6 mg/kg Excede™ (Zoetis) subcutaneously injected at the base of the ear, as prophylactic treatment against bovine respiratory disease (BRD) complex.

2.3. Vaccination and post- vaccination clinical observations

Adt.A24 (Antelope Valley Bios, USDA Establishment No. 419, Lincoln, NE), is a molecular subunit vaccine derived from a replication deficient human adenovirus vector. Adt.A24 was used in all studies [15–17] and was prepared according to the Outline of Production (USDA product code 1FM1.R0; conditionally licensed), and suspended in final formulation buffer (FFB; Lonza, Walkersville, MD). The vaccine was mixed 1 part adjuvant, ENABL®-5X stock (VaxLiant, Lincoln, NE), and 4 parts Adt.A24/FFB. The adenovirus vaccine vector stock was stored at −80 °C, thawed, diluted in FFB and adjuvant within 2 h of vaccination, and held on ice prior to administration. The Adt.A24 target dose was 7.2 log10 $TCID_{50}$/2mL. The same vaccine lot (Lot # AV-001) was used for all 3 studies. Adt.A24 titers were tested by Antelope Valley Bios (USA) and results were within the assay range of the original titer results.

Steers in study 1 were vaccinated IM (n = 5), TD (n = 5) or SC (n = 5). Steers in studies 2 and 3 were vaccinated IM (n = 10) or TD (n = 10). Steer (#117) previously TD vaccinated (study 1) received an IM boost vaccination and served as a positive vaccine control in study 2. Three steers per study were placebo-vaccinated with endotoxin free, sterile water for injection, USP (Phoenix™, Clipper Distributing Co., LLC, MO). IM and SC vaccinations were given as a single 2 mL dose in the neck by syringe and 18G, 1.5 in. needle. TD vaccination was administered in two 1 mL inoculations (for a full vaccine dose) on one or both sides of the neck using the Pulse 250 Needle-free Transdermal Vaccination System (Pulse Needle-Free Systems, USA) at 50–60 PSI. TD inoculation success was measured as presence of a palpable dermal bleb at the vaccination site within 10 s post-vaccination.

Steers were monitored for adverse clinical reactions post-vaccination. Rectal temperatures and vaccination site reactions were monitored daily through 7 days post-vaccination (dpv) (Fig. 1). A fever was defined as a rectal temperature >102.8 °F

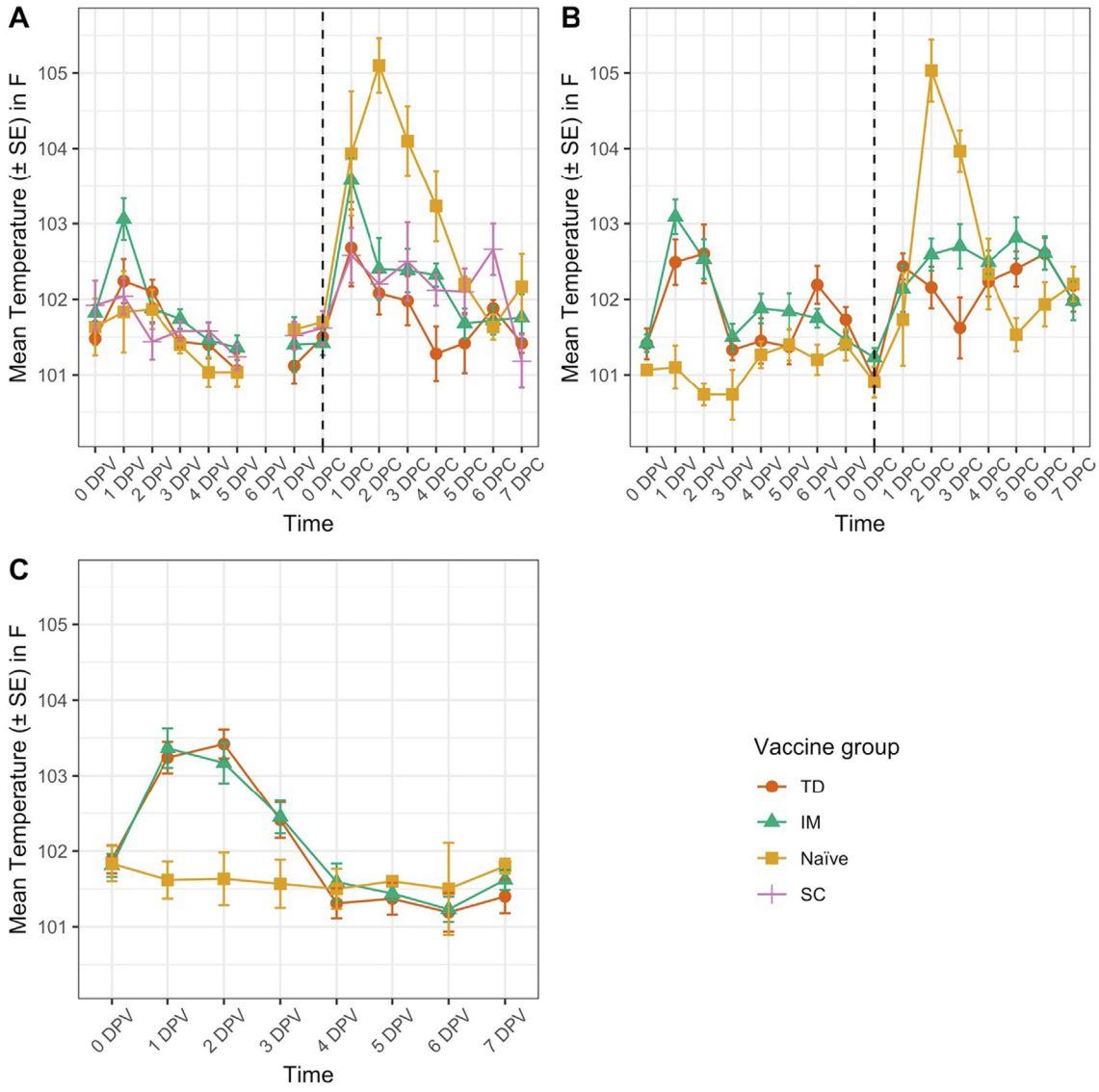

Fig. 1. Mean of temperatures ± standard error measured for each vaccine group pre- and post- vaccination and challenge. An animal with a temperature over 102.8°F was considered to have a fever. **A** Study 1, day of challenge = 6 months post vaccination. Temperatures were not measured on day 6 post vaccination. **B** Study 2, day of challenge = 9 months post vaccination. **C** Study 3, not challenged. DPV = days post vaccination. DPC = days post challenge. n = 5 for each vaccinated group in study 1, n = 10 in studies 2 and 3. All studies included 3 naïve steer. No temperatures were taken on day 6 in study 1.

(39.33 °C). Vaccine site swelling was measured in millimeters using a digital caliper.

2.4. FMDV challenge and post-challenge clinical observations

Following transport to PIADC, steers were acclimatized for 2 weeks prior to FMDV challenge. At time of challenge, steers were 10.5–13.4 months and 1.2–1.5 years of age for studies 1 and 2, respectively. Steers were challenged within a few days of 6 (study 1) and 9 (study 2) months post-vaccination (mpv). Animal health and condition was assessed daily.

FMDV A24 Cruzeiro challenge was conducted using the same challenge stock as previously described [15–17]. Briefly, steers were sedated with 0.22 mg/kg of xylazine IM in the hindquarter and challenged via the intradermolingual (IDL) route. The target challenge dose per animal was 1×10^4 bovine infectious dose 50% (BID_{50})/0.4 mL. 0.1 mL per site were inoculated into four sites on the dorsal surface of the tongue of each steer. FMDV titer was 3.2×10^4 $TCID_{50}$/mL (Study 1, 2014) and 1.6×10^4 $TCID_{50}$/mL (Study 2, 2015) based on back titration of challenge inoculum on LFBK $\alpha_v\beta_6$ cells. Sedation was reversed by administering 2–4 mg/kg of tolazoline intravenously.

Steers were monitored daily post-challenge for clinical signs of FMD and sedated for vesicular lesion assessment on days 4, 7, 11, 21 and 28 post-challenge (dpc) (study 1) and 4 and 7 dpc (study 2). Clinical scores were determined by the number of feet presenting FMD lesions (0 = no lesions on any foot, 1 = lesions on one foot, 2 = lesions on two feet, 3 = lesions on 3 feet, 4 (maximum score) = lesions on all feet) as previously described and according to the World Organisation for Animal Health (OIE) guidelines [15–17,19]. Oral lesions were not used in clinical score outcome due to intradermolingual challenge route. Rectal temperatures were monitored up to 14 dpc. Unanticipated wellbeing issues related to clinical lameness and extended recumbency required the euthanasia of some steers in study 2 prior to 21 dpc.

2.5. Sample collection and processing

All peripheral blood samples were collected via venipuncture from the jugular or tail vein into serum and sodium heparin

vacutainers® (Becton, Dickinson and Company, USA). In study 1, pre-challenge samples were collected on day 0, weekly to day 28 and monthly until 6 mpv. In studies 2 and 3, pre challenge samples were collected on day 0, every two days to day 10, weekly to day 28 and then monthly until either 9 (study 2) or 6 (study 3) mpv. Samples were kept in wet ice until transport to the laboratory and serum samples (only) were left at 4 °C overnight for processing the following day. Post-challenge samples were collected in the same manner on days of vesicular lesion assessment. Serum samples were aliquoted and stored at either −20 °C or −80 °C until use in virus neutralization assays.

Peripheral blood mononuclear cells (PBMCs) were isolated via Histopaque 1083 (Sigma) (studies 1 and 2) or UNI-SEP$_{MAXI}$ tubes (Novamed, Israel) (study 3). Briefly, blood was diluted with 1x Dulbecco's phosphate-buffered saline (DPBS). Histopaque was underlaid and the samples centrifuged at 1800x g for 25 min at 25 °C, with no centrifuge brake engaged. Alternatively, PBMC isolation from DPBS diluted blood samples was conducted using UNI-SEP$_{MAXI}$ tubes according to manufacturer's instructions. Cells were isolated, washed and re-suspended in ELISpot media (see 2.7 *ELISpot*). Remaining PBMCs were cryopreserved in a media/FBS/10% DMSO cocktail for future analyses.

2.6. Virus neutralization titers (VNTs)

Heat-inactivated serum samples (56 °C, 30 min) were used to measure VNTs to human adenovirus serotype 5 (Ad5) and to FMDV A24 Cruzeiro as previously described [15–17]. The mean of the VNTs (MT, $\log_{10}$ scale) ± standard error (SE) for each treatment were determined. Ad5 and A24 VNTs of $\geq$1.2 $\log_{10}$ and $\geq$ 0.9 $\log_{10}$, respectively, were considered positive [15–17], and the upper assay detection limit was 3.0 $\log_{10}$. Assignment of a 0.6 $\log_{10}$ value for any sample below the assay limit of detection allowed a mean titer for each treatment group to be determined.

2.7. Elispot

The detection of FMDV-specific, antibody secreting B cells of all bovine isotypes (IgG_1, IgG_2, IgA, IgM) was conducted using the ELISpot assay we previously described [20]. Coating antibody (monoclonal mouse anti-bovine IgG_1, anti-IgG_2, anti-IgA, and anti-IgM, Green Mountain Antibodies, Burlington, VT) was diluted in carbonate coating buffer (Sigma), added to ELISpot plates (HA, 0.45 μm, surfactant-free, mixed cellulose ester membrane, Millipore, USA), wrapped in aluminum foil and kept at 4 °C until use (maximum one week).

On the day of sampling, coating antibody was removed and plates were washed using ELISpot media (RPMI-1640 plus supplements [antibiotics, antimycotics], Life Technologies). ELISpot media was added to plates and incubated at 37 °C for 20–40 min, removed and PBMC dilutions added to the plates. In study 2, cells were plated in duplicate at 1×10^6, 5×10^5 and 2.5×10^5 PBMCs/well for samples up to 2 months post-vaccination. The remaining samples were plated in duplicate at 1×10^6 and 5×10^5 cells per well. In study 3, cells were plated in duplicate at 5×10^5 and 2.5×10^5 PBMCs/well through the entire study. Plates were incubated at 37 °C, 5% CO_2, for approximately 18 h, washed with 1x PBS/ 0.05% Tween (PBST) and cells were lysed using deionized water, and plates dried at RT. Plates were kept in a cool, dry, dark location until shipment to PIADC.

Upon arrival at PIADC, plates were processed as previously described, with minor modifications [20]. All incubations were conducted at RT, protected from light, for 1 h. Briefly, plates were hydrated with Milli-Q water and washed with PBST. 2 μg/mL of biotinylated FMDV A24 Cruzeiro virus in PBST was added to each plate and incubated. Plates were washed with Milli-Q water and incubated with filtered NeutrAvidin®-Horseradish peroxidase conjugate (Thermofisher, USA) in PBST. Plates were washed and developed with True Blue Substrate (Seracare, USA); reaction was stopped with Milli-Q water, and dried overnight. An ImmunoSpot® reader (CTL, USA) was used to detect antibody secreting cells (ASCs) and data analyzed using ImmunoSpot® Software (CTL, USA). ASCs were calculated per 1×10^6 cells. The average ASCs per duplicate wells was determined, with a negative threshold of 2 ASCs (samples with less than 2 ASCs were counted as negative). In study 2, only data from wells plated with 5×10^5 cells/well were analyzed due to technical problems with reading 1×10^6 cells/well.

2.8. Statistical analysis

Logistic regression models were fitted by maximum likelihood to evaluate potential associations between route of vaccination or study number and post-vaccination mean differences in proportion of steers developing fevers and either Ad-5 or FMDV VNTs, where each of these outcomes was treated as a binary response (1 = yes or 0 = no). Similarly, logistic regression models were applied to explore associations between pre- and post-viral challenge health status and clinical disease protection for the cohort of steers that experienced the unanticipated well-being issues in study 2. Mixed models accounting for repeated measures were fitted by restricted maximum likelihood (REML) to evaluate the effect of route of vaccination and study on differences in the mean VNTs (in $\log_{10}$ scale) and differences in the mean ASCs for each isotype with steer included as a random effect. ELISpot observations were used to compare numbers of ASCs between treatment groups (IM, TD and naïve negative controls) by conducting Kruskal-Wallis non-parametric tests at each time point. The resulting test p-values were adjusted to control for false discovery rate across multiple comparisons over time points using the Benjamini-Hochberg's method [21]. The distributions of the data were visualized using ordinary Tukey's box and whiskers plots, which included the identification of potential outliers (observations more than 1.5 times the interquartile range above or below the upper and lower quartiles respectively). The relationship between FMDV VNT and clinical outcome following virus challenge (characterized as a binary outcome, clinical score 0 or $\geq$1) was evaluated using a repeated measures logistic mixed model fitted by REML with route of vaccination and study as fixed effects and steer as a random effect. Similarly, we evaluated FMDV VNTs on day of challenge as a predictor of clinical outcome in separate mixed models, either using all data from studies 1 and 2 or analyzing data from each of these studies (1 and 2) individually. Because steers in study 3 were not challenged, only data from studies 1 and 2 could be included in the analysis of correlates of protection. Positive control steer #117 (study 2) was not included in the statistical analyses.

The statistical analyses were conducted on JMP®Pro (version 14.0.0) and the R system for statistical computing v3.4 [22]. Statistical significance was determined at the usual 5% significance level (p-value $\leq$0.05).

3. Results

3.1. Post-vaccination clinical observations

No severe adverse clinical reactions to the vaccine were observed. In all studies, no placebo-immunized steers demonstrated a clinical response to placebo vaccination, whereas the clinical response in vaccinated steers included transient fever and localized swelling at the site of vaccine administration (TD). Increase in rectal temperature ranging from 0.1 to 2.5°F above

the normal cut-off was detected 1–2 dpv in vaccinated steers in all studies, returning to a normal range by 2–4 dpv (Fig. 1). The proportion of vaccinated steers that developed fever post-vaccination in study 3 (80%) was statistically significantly higher than in study 1 (33%, $p = 0.026$) but not significantly different from study 2 (70%, $p = 0.42$). Analysis of the effect of route of vaccination was not a statistically significant predictor of the probability of developing a fever post-vaccination ($p = 0.073$) in the study. No statistically significant differences in the peak ($p = 0.313$) or mean ($p = 0.289$) FMDV VNTs were observed between 7 and 56 dpv among steers that developed a fever post-vaccination and those that did not.

Palpable cutaneous nodules in TD vaccinated steers (all studies) or subcutaneous swelling in SC vaccinated steers (study 1) were noted at the vaccination sites, which generally resolved by 7 dpv and completely resolved by 21 dpv for all steers. (Supplemental Table 1a–c). Steers vaccinated IM only occasionally exhibited palpable intramuscular swelling at the inoculation site.

3.2. Ad5 VNT

Prior to vaccination, all steers were seronegative for Ad5 neutralizing antibodies, and all placebo-immunized steers remained seronegative throughout each study (Fig. 2). Early post-vaccination, Ad5 VNTs were variable among steers across all studies (Fig. 2). In study 3 there was a statistically significantly lower proportion of steers (5/20, 25%) with positive Ad5 titers by 7–8 dpv compared to study 1, 14/15 (93%, $p < 0.001$) or study 2, 13/20 (65%, $p = 0.009$). By 14 dpv, 100% of steers in studies 1 and 2 and 18/20 (90%) in study 3 were positive; the remaining two steers were seropositive by 28 dpv. In study 1, all but one vaccinated steer (SC), (14/15, 93.3%) remained seropositive through 6 mpv. In study 2, 14/20 (70%) and 9/20 (45%) of vaccinated steers were seropositive at 6 and 9 mpv, respectively. Only one protected steer, #153 (TD), remained seropositive throughout study 2, the other three protected steers did not. The positive control animal (boosted) showed the highest titers compared to all single

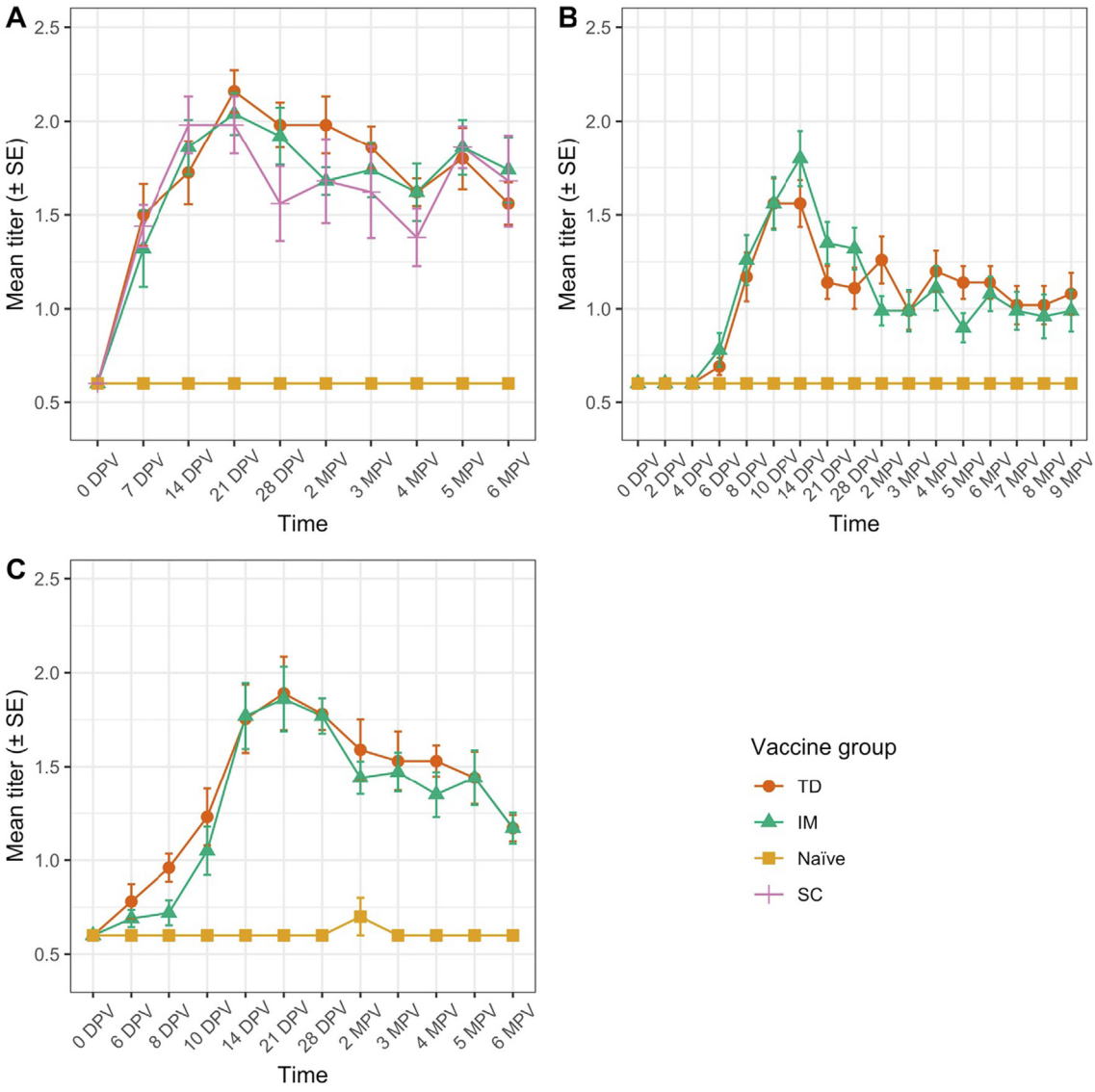

Fig. 2. Ad5 virus neutralization titers. Mean of titers ± standard error measured for each vaccine group pre- and post- vaccination and challenge. A titer of ≥1.2 is positive. A titer ≤0.6 is considered negative. **A** Study 1, day of challenge = 6 months post vaccination. **B** Study 2, day of challenge = 9 months post vaccination. **C** Study 3, not challenged. DPV = days post vaccination. DPC = days post challenge. n = 5 for each vaccinated group in study 1, n = 10 in studies 2 and 3. All studies included 3 naïve steers. Titers were not measured for naïve steers in study 1 until the day of challenge.

vaccinated animals in study 2, beginning at 6 days post boost (data not shown). Ad5 VNTs were not measured post-challenge.

3.3. FMDV VNT

Prior to vaccination, all steers were seronegative for FMDV specific neutralizing antibodies, and all placebo-immunized steers remained seronegative throughout each study prior to challenge (Fig. 3). Post-vaccination, serum from vaccinated steers (all studies) contained anti-FMDV antibody by day 14, with one exception (#161 (IM) in study 2 which was positive by day 21). On average, vaccinated steers produced higher FMDV VNTs post-vaccination in studies 1 ($p < 0.001$) and 3 ($p < 0.001$) compared to study 2. All steers in studies 1 and 3 remained seropositive through 6 mpv (day of challenge for study 1). In study 2, similar to anti-Ad5 results, anti-FMDV titers were variable and inconsistent. At 6 mpv overall seroconversion in vaccinated steers was 70% (9/10 TD, 5/10 IM) and only 50% (7 TD, 3 IM) of vaccinated animals were seropositive at all time points. On the day of challenge (9 mpv), 85% (9/10 TD and 8/10 IM) of vaccinated steers were seropositive, only 35% (4 TD, 3 IM) of which were seropositive at all time points. Following challenge, all steers had increased FMDV VNTs by 7 dpc (Fig. 3, panels A and B). Placebo-immunized controls seroconverted by 14 dpc.

3.4. Elispot analysis of B cell responses to FMDV.

ELISpot assay protocols were finalized using samples from study 1 and conducted post-vaccination on isolated PBMCs from studies 2 and 3 to assess circulating ASCs (Fig. 4). Overall, ASCs producing IgM anti-FMDV were observed early following vaccination in both studies. In study 2 (Fig. 4a), the group inoculated transdermally showed the peak response on day 4, while there was one animal that never showed IgM anti-FMDV ASCs and four others that showed very few ($\leq$6) at any given time. In the group inoculated intramuscularly, the peak response was also on day 4. Again there was one animal with no IgM anti-FMDV ASCs detected and two animals with a very low level response ($\leq$8 at a given time point).

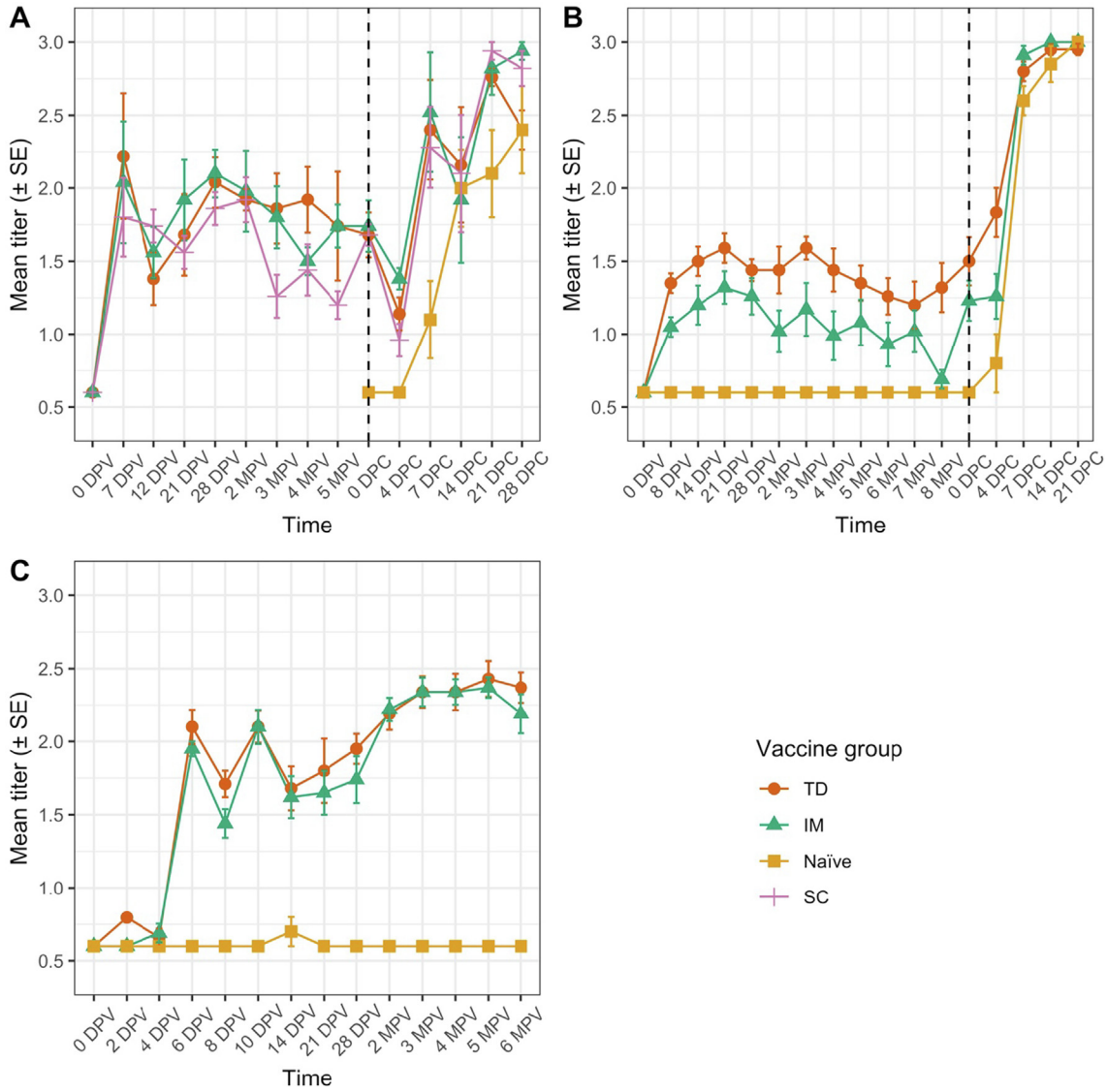

Fig. 3. FMDV virus neutralization titers. Mean of titers ± standard error measured for each vaccine group pre- and post- vaccination and challenge. A titer of $\geq$0.9 is positive. A titer $\leq$0.6 is considered negative. **A** Study 1, day of challenge = 6 months post vaccination. **B** Study 2, day of challenge = 9 months post vaccination. 4 DPC: n = 19 vaccinated, n = 3 naive, 7 DPC: n = 19 vaccinated, n = 3 naïve, 14 DPC: n = 8 vaccinated, n = 2 naïve, 21 DPC: 7 vaccinated, n = 1 naive, **C** Study 3, not challenged. DPV = days post vaccination. DPC = days post challenge. n = 5 for each vaccinated group in study 1, n = 10 in studies 2 and 3. All studies included 3 naïve steers. Titers were not measured for naïve steers in study 1 until the day of challenge.

In study 3, the animals getting TD inoculation of vaccine responded with IgM anti-FMDV on day 4 (peak 6 dpv) and though no animals were negative across the assay period in this study, 5 of 10 animals had low level responses (≤3 ASCs at any given time point) and the balance of the steers had lower responses than in Study 2 . The IM vaccinated animals had similar results, though, overall even less than TD vaccinated steers. Further, as in study 2, one steer was negative throughout the assay period.

B cells secreting IgG_1 anti-FMDV antibody dominated the B cell response in experiment 2. In the TD inoculated cattle, IgG_1 ASCs were observed early, between day 4 and day 14 following vaccination, fewer at day 21. With one exception, IgG_1 anti-FMDV ASC were absent after day 21. In the group vaccinated IM, the IgG_1 response was less consistent on day 4, but, as in the TD group, lasted through day 14. Similar to the TD vaccinated animals, no IgG_1 anti-FMDV ASCs were detected after 21 dpv. In the final experiment, study 3, responses showed detection of significant IgG_1 ASCs for anti-FMDV antibody at 6 dpv and lasted in the peripheral blood largely until 28 dpv.

Finally, there was no statistically significant detection of IgG_2 or IgA anti-FMDV ASCs in either experiment at any time point (Figs. 4a and b and Supplemental Table 2a and b). In study 2, IgG_2 anti-FMDV ASCs were detected only once each in a few animals in the assay period and those on different days. In experiment 3, though all vaccinated animals were positive at least once, ASC numbers were transient and very low (≤7). Remarkably, one steer showed the highest number of IgG_2 ASCs observed in these studies (196 ASC, at 8 DPV; data not shown). In conclusion, circulating IgG_2 and IgA anti-FMDV ASCs may not play a significant role following vaccination by either route, TD or IM. Overall, the anti-FMDV ASC response in study 2 was higher than study 3, but the IgG1 anti-FMDV ASC response was more prolonged in study 3 for both routes of inoculation.

Placebo-immunized animals showed very low transient numbers of ASCs for any given antibody isotype for at least one time point in both studies. The positive control steer #117 (study 2) showed a predominantly IgG_1 response to a second vaccination, 4–8 days post boost, though ASCs were present at one time point

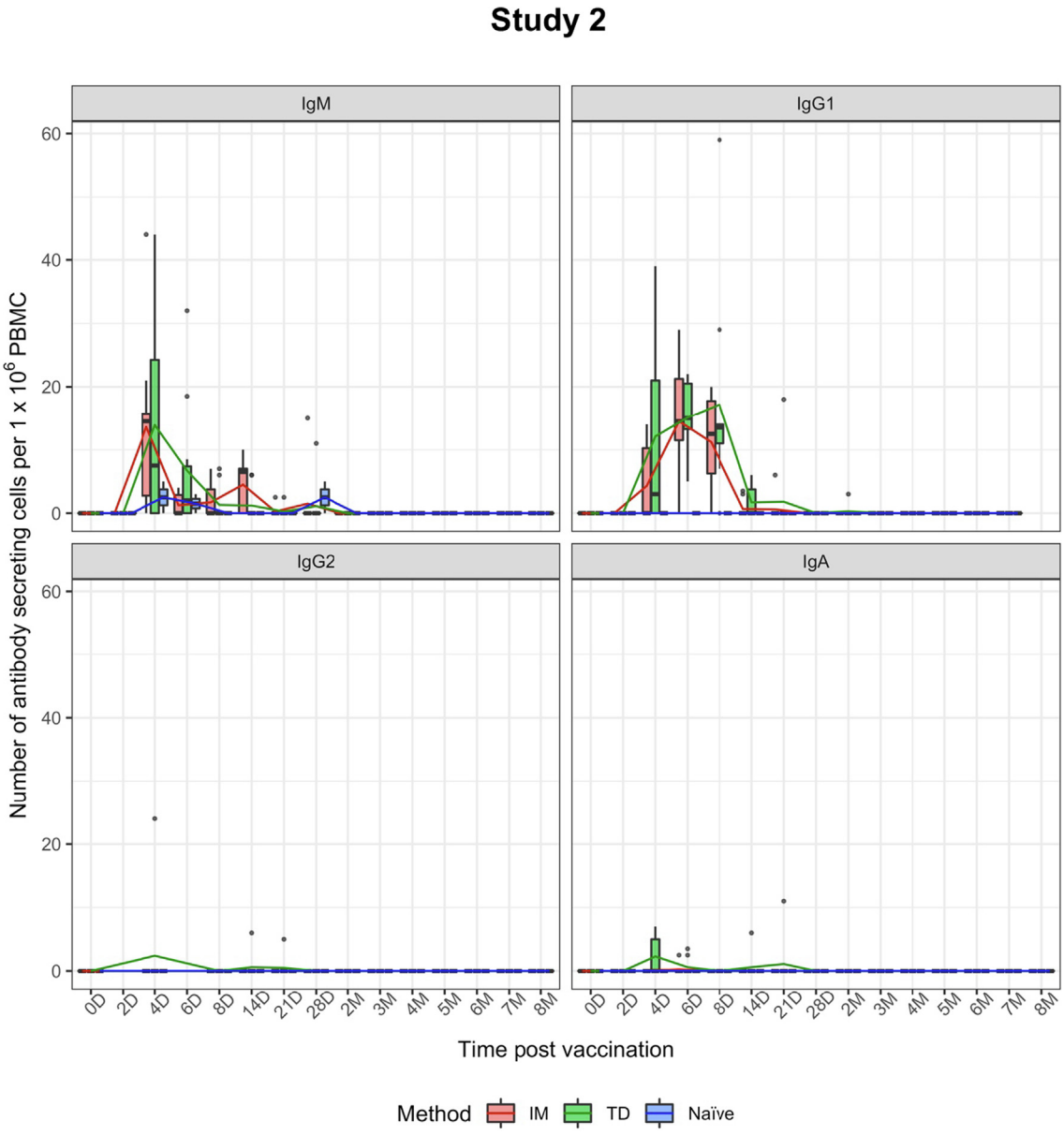

Fig. 4a. ELISpot results for study 2. X-axis: Time in days/months following vaccination. Y- axis: number of antibody secreting cells per 1×10^6 PBMC for each method (lines are average values, boxplots represent data distribution, horizontal segment within box is the median value, points refer to outliers).

each for IgM, IgG_1, and IgG_2 post boost. No IgA anti-FMDV ASCs were detected.

3.5. Efficacy results

In study 1, 100% TD, 80% IM and 60% SC vaccinated steers were protected from challenge 6 months after a single vaccination (Tables 1 and 2). In study 2, 33% TD and 20% IM vaccinated steers were protected from challenge 9 months after a single vaccination (Tables 1 and 2). Clinical evidence of FMDV infection in study 2 (i.e., presence of pedal vesicular lesions) was not recorded past 7 dpc due to animal welfare concerns associated with severe lameness; steers were humanely euthanized between 7 and 21 dpc. Twelve of 23 steers showed evidence of lameness prior to challenge, potentially associated with animal weight and room/floor surfaces during the acclimatization period, which may have influenced clinical severity post-challenge. However, lameness prior to challenge was apparently not statistically significantly associated with euthanasia prior to 21 days post-challenge ($p = 0.846$).

All placebo-immunized steers reached a maximum clinical score of 4 by 4 dpc (Tables 3 and 4). In study 1, of the 3 vaccinated steers which developed lesions (by 21 dpc), only one reached a clinical score of 4 (Table 3). In contrast in study 2, of the 14 vaccinated steers that developed lesions (by 7 dpc), 10 reached a maximum clinical score of 4 (Table 4). Post-challenge rectal body temperatures and onset of fever were variable (Fig. 1), with marginally statistically non-significant association between post-challenge fever and clinical disease protection ($p = 0.051$).

3.6. Correlations with protection

In a univariate analysis the odds of being protected from clinical disease post-challenge increased with an increasing FMDV VNT ($p < 0.001$). However, when the effect of study and route of vaccination were included as predictors of clinical outcome, FMDV

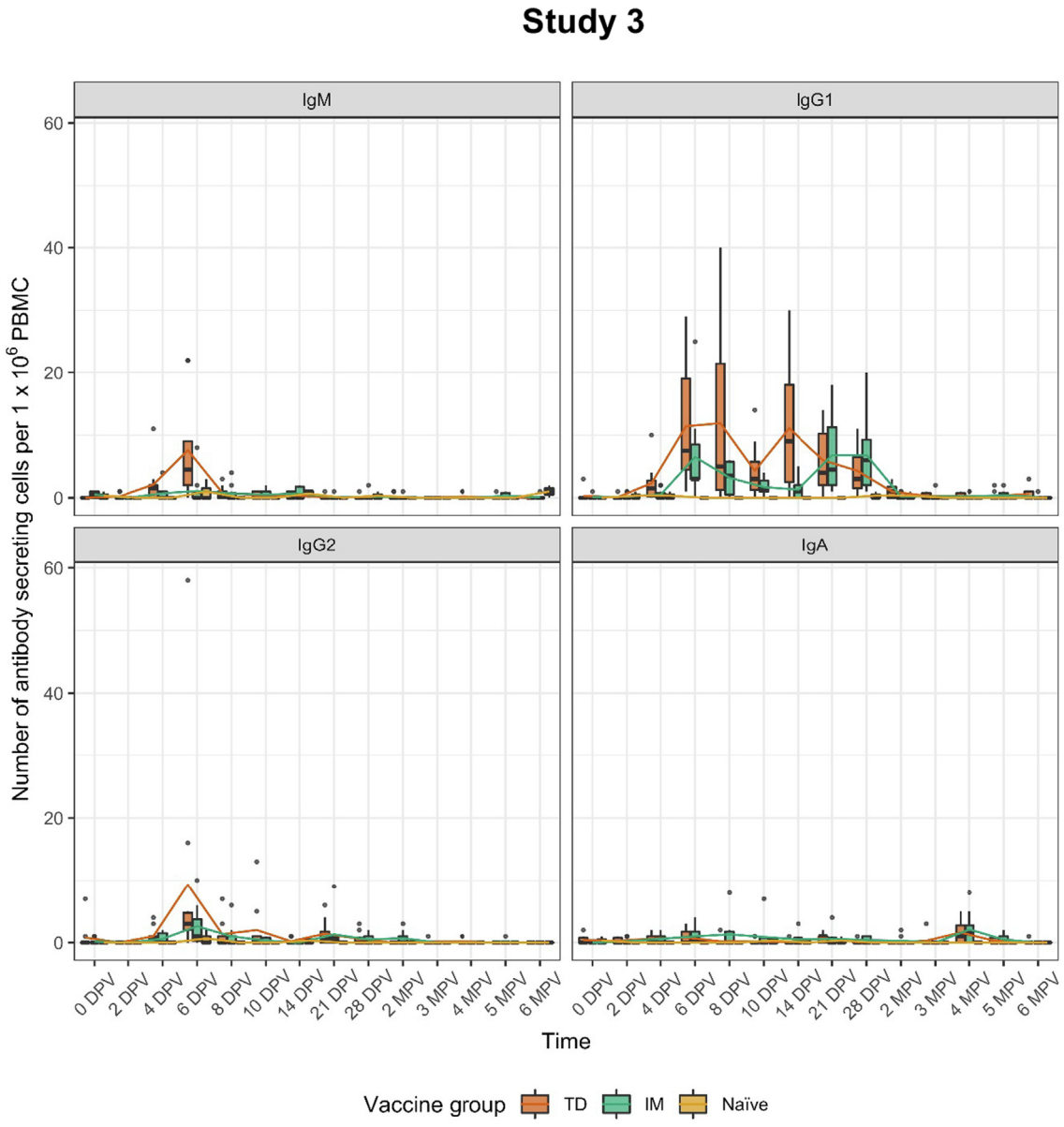

Fig. 4b. ELISpot results for study 3: X-axis: Time in days/months following vaccination. Y- axis: number of antibody secreting cells per 1×10^6 PBMC for each method (lines are average values, boxplots represent data distribution, horizontal segment within box is the median value, points refer to outliers; a very large outlier at 8D for IgG2 (#229, value = 196) was excluded to facilitate visualization).

Table 1
Mean log_{10} ± standard error (SE) of the virus neutralization titers (VNT) to FMDV and Ad5, with regards to challenge. [1]For study 3 this is the last sample taken at 6 months post vaccination (italicized numbers). [2]Last sample post challenge for study #1 was day 28 and day 7 for study #2. [3]Last UVM collected sample prior to challenge was 6 months and 8 months post vaccination for study 1 and 2, respectively and 6 months for study 3. [4]Study 3 animals were not challenged. DPC = days post challenge, TD = transdermal vaccination, IM = intramuscular vaccination, SC = subcutaneous vaccination, naïve = unvaccinated. Virus titers of 0.6 log_{10} are considered negative.

			FMDV VNT (mean log_{10} ± SE)					Ad5 VNT (mean log_{10} ± S.E.)	
Study number	Vaccine group	N	Day 0	Day of challenge[1]	Day 7 post challenge	Last sample post challenge[2]	% Protection from clinical disease	Day 0	Last UVM sample[3]
1	TD	5	0.6 ± 0.0	1.7 ± 0.2	2.4 ± 0.3	2.4 ± 0.1	100	0.6 ± 0.0	1.6 ± 0.1
	IM	5	0.6 ± 0.0	1.7 ± 0.2	2.5 ± 0.4	2.9 ± 0.1	80	0.6 ± 0.0	1.7 ± 0.2
	SC	5	0.6 ± 0.0	1.7 ± 0.1	2.3 ± 0.3	2.8 ± 0.1	60	0.6 ± 0.0	1.7 ± 0.2
	Naive	5	0.6 ± 0.0	0.6 ± 0.0	1.1 ± 0.3	2.4 ± 0.3	0	0.6 ± 0.0	0.6 ± 0.0
2	TD	10	0.6 ± 0.0	1.5 ± 0.2	2.8 ± 0.1	–	33	0.6 ± 0.0	1.1 ± 0.1
	IM	10	0.6 ± 0.0	1.2 ± 0.1	2.9 ± 0.1	–	20	0.6 ± 0.0	1.0 ± 0.1
	Naïve	3	0.6 ± 0.0	0.6 ± 0.0	2.6 ± 0.1	–	0	0.6 ± 0.0	0.6 ± 0.0
3[4]	TD	10	0.6 ± 0.0	*2.4 ± 0.1*	–	–	–	0.6 ± 0.0	1.2 ± 0.1
	IM	10	0.6 ± 0.0	*2.2 ± 0.1*	–	–	–	0.6 ± 0.0	1.2 ± 0.1
	Naive	3	0.6 ± 0.0	*0.6 ± 0.0*	–	–	–	0.6 ± 0.0	0.6 ± 0.0

VNT was no longer a statistically significant predictor. Clinical disease response was affected by study (lower odds of clinical FMDV foot lesions among study 1 steers) and route of vaccination (increased odds of clinical FMDV foot lesions among SC vaccinated steers compared to IM and TD vaccinated steers). Overall, and accounting for the effect of study differences, steers vaccinated by the SC route were 4 times more likely to have one or more foot lesions post-challenge compared to IM vaccinated animals ($p < 0.001$; OR 95% CI: 1.97 – 8.13), and 10 times more likely to have foot lesions following FMDV challenge compared to TD vaccinated steers ($p < 0.001$; OR 95% CI: 5.00 – 23.49). Animals vaccinated by the TD route were 2.7 times more likely to be protected following live virus challenge compared to IM vaccinated steers ($p < 0.001$; OR 95% CI: 1.65–4.47).

The odds of being protected following virus challenge increased with increasing FMDV VNT measured at 6 months post-vaccination ($p = 0.007$). However, when the effect of FMDV VNTs on the day of challenge (6 months vs 9 months) on clinical outcome was measured, VNTs were no longer predictive of protection ($p = 0.07$) and steers in study 1 had increased odds of protection compared to steers in study 2 ($p = 0.005$). Separate analysis of study 1 and 2 results showed no statistically significant association between virus neutralizing titer and protection.

4. Discussion

The ideal FMD vaccine would protect following a single dose, be DIVA compatible, not require a cold chain, would be economical to mass-produce, and affordable for the end user [13]. The conditionally licensed Adt.A24 FMD vaccine has been shown to attain some of these attributes, as it is DIVA compatible and can be manufactured without a requirement for high containment facilities [14,17,18].

Here, we report that Adt.A24/ENABL vaccine formulation conferred 100% and 80% protection against clinical disease at 6 months post single dose vaccination via the TD and IM routes, respectively. Barrera et al. previously reported 100% (10/10) of vaccinated steers and 97% (33/34) of vaccinated steers were protected from clinical experimental challenge at 7 and 14 days post IM vaccination, respectively, using a 1.2 log_{10} lower dose of the Adt.A24/ENABL vaccine formulation. Collectively, this suggests Adt.A24 vaccine provides a high level of protection early [16] , with a duration of protection of 6 months post-vaccination.

Lower levels of protection were observed for a second animal cohort, challenged 9 months post-vaccination. The difference in observed protection between 6 and 9 months is consistent with the waning of circulating anti-FMDV titers. In study 2, steers failed to maintain consistent anti-FMDV titers, though 89% were seropositive at the time of challenge 9 months post-vaccination. Notably, 3 of the 5 protected cattle in study 2 had no anti-FMDV neutralizing antibody detected at multiple time points during the study. There was a significant difference in VNTs on the day of challenge between the studies. Anti-Ad5 VNTs in these studies are consistent with those observed at 7 and 28 dpv reported elsewhere [15,17], with the additional aspect that the titers did not wane for the duration of the study, including the 9 month study. As a result, a boost at the indicated six months following initial vaccination, could be compromised by the anti-Ad5 titers and require a different virus vector. Such a protocol has been reported for other vaccine antigens [24,25].

The difference in responses between the animals in the two trials reported may have been due to the actual cohort itself, (e.g., source, lineage) although major histocompatibility complex (MHC) type was not associated with protection (data not shown). It is possible that pre-challenge clinical lameness observed at PIADC following their arrival from UVM confounded outcomes in the 9 month cohort (study 2). Although underestimating the number of FMD positive steers in the 9 month cohort was a possibility because animals were euthanized due to clinical lameness prior to the end of the 21 day observation period, 14 of 19 vaccinated steers were recorded with at least 1 vesicular lesion by day 7 post challenge. Additionally, it is unlikely that experienced observers at PIADC would conflate pedal vesicular lesions with those associated with other causes of lameness not uncommon in cattle (e.g., laminitis or sub-solar abscesses). Other factors, including changes in feed ration or foot-trimming prior to transport from Vermont to Plum Island may have contributed to the observed pre-challenge lameness. All those factors considered, protection did not extend to 9 months post-vaccination in this study. Additional studies are needed to confirm these observations.

Detection of circulating FMDV specific IgM secreting B cells was relatively short lived (peak at 4–6 dpv). Concomitantly, a rapid isotype switch to IgG_1 secreting B cells was detected while detection of IgG_2 and IgA FMDV-specific B cells induced by vaccination was inconsistent in both studies analysed and not statistically significant. ASCs in the peripheral blood were rarely detected after 2 mpv and the majority of animals were negative after 28 dpv. Preliminary data indicate no anti-FMDV ASCs are found in the draining lymph nodes or spleen of Adt.A24 vaccinated steers at 2 mpv either (Arzt and Kenney, unpublished).

To the best of our knowledge, this is the first study to demonstrate FMDV experimental challenge protection in cattle following TD (needle-free) vaccination with a recombinant vectored subunit vaccine. Despite the relatively small number of steers in these studies, the TD delivery system appeared to provide superior pro-

Table 2
Mean log_{10} ± standard error (SE) of the virus neutralization titers (VNT) to FMDV and Ad5 for protected and unprotected animals. [1]One steer was euthanized prior to challenge. [2]Last sample post challenge for study 1 was day 28 and day 7 for study 2. Last UVM collected sample prior to challenge was 6 months and 8 months post vaccination for study 1 and 2, respectively.

		Protected				Unprotected			
			FMDV VNT (mean log_{10} ± SE)		Ad5 VNT mean log_{10} ± SE)		FMDV VNT (mean log_{10} ± SE)		Ad5 VNT (mean log_{10} ± SE)
Study number	Vaccine group	N	Day of challenge	Last sample post challenge[2]	Last UVM sample	N	Day of challenge	Last sample post challenge[2]	Last UVM sample
1	TD	5	1.7 ± 0.1	2.4 ± 0.1	1.5 ± 0.1	0	–	–	–
	IM	4	1.9 ± 0.2	2.9 ± 0.1	1.9 ± 0.2	1	1.2 ± 0.0	3.0 ± 0.0	1.2 ± 0.0
	SC	3	1.7 ± 0.1	2.8 ± 0.2	1.3 ± 0.3	2	1.6 ± 0.1	2.8 ± 0.1	2.1 ± 0.3
	Naive	0	–	–	–	3	0.6 ± 0.0	2.4 ± 0.3	0.6 ± 0.0
2	TD	3	1.6 ± 0.5	2.7 ± 0.2	1.0 ± 0.3	6[1]	1.3 ± 0.2	2.9 ± 0.1	1.1 ± 0.2
	IM	2	1.5 ± 0.3	2.7 ± 0.3	0.9 ± 0.0	8	1.1 ± 0.1	3.0 ± 0.0	0.9 ± 0.1
	Naïve	0	–	–	–	3	0.6 ± 0.0	2.6 ± 0.1	0.6 ± 0.0

Table 3
Study 1. Clinical scores following challenge of cattle. Any vesicles on each foot are scored as 1 yielding a maximum clinical score of 4. There is no scoring for vesicles in the mouth or on the tongue, the inoculation site of the challenge virus. [1]Protected.

		Days post challenge			
Vaccine group	Steer	0	7	14	21
TD	109[1]	0	0	0	0
	126[1]	0	0	0	0
	129[1]	0	0	0	0
	135[1]	0	0	0	0
	140[1]	0	0	0	0
IM	118	0	3	4	4
	124[1]	0	0	0	0
	128[1]	0	0	0	0
	137[1]	0	0	0	0
	141[1]	0	0	0	0
SC	123[1]	0	0	0	0
	125[1]	0	0	0	0
	131	0	2	2	2
	132[1]	0	0	0	0
	139	0	1	3	3
Naïve	130	0	4	4	4
	138	0	4	4	4
	142	0	4	4	4

tection compared to IM or SC needle delivery of the same dose. A previous study also reported effective FMD protection can be enhanced following TD vaccination with killed virus vaccine using a needle-free delivery system [23]. Another study reported absence of an injection site and no gross physical reaction following IM vaccination using the same Adt.A24/ENABL vaccine formulation used here [16]. We observed minor vaccine associated changes (i.e., transient febrile responses) in steers vaccinated by all routes, and localized injection site reactions following SC and TD vaccination. Future studies might evaluate the potential for reducing AdA24 vaccine dose and volume for TD immunization.

Our experience utilizing a non-BSL-3Ag facility for the 6–9 month vaccination phase followed by transport to and challenge in a BSL-3 Ag research facility suggests such vaccine studies are feasible. However, additional animal husbandry and management criteria should be considered for housing steers that exceed 160–260 kg, which was the standard weight range series of recent studies conducted at PIADC [15–17].

In conclusion, a single dose of the conditionally licensed Adt. A24 vaccine in a controlled, experimental setting, was demonstrated to confer 6 months protection against clinical FMD. Additional vaccine challenge efficacy experiments are required to fully evaluate the duration of protection. These data elicit many questions about the bovine immune response following vaccination with AdtFMD vaccines. Experiments to more clearly understand these immune responses will be of great value in informing future AdtFMD vaccine design.

Author contributions

T.S., J.W.B., E.R., and W.T.G. wrote the manuscript. J.W.B. and W. T.G. designed the study and secured funding and regulatory approvals for conducting these studies. D.B. and J.W.B. arranged for procurement of the vaccine and determination of vaccine titers used in each study. J.W.B., T.S., K.E., M.P., and W.T.G. were involved in vaccination, sample acquisition, animal handling, and/or sample processing pre-challenge. M.K. and M.W. were involved in sample processing post-challenge. J.B., T.S., M.K., M.L. and K.E. conducted FMDV and Ad5 VNT pre- and post-challenge. J.P. and J.A. performed the live virus challenge and clinical assessments after challenge. J. P-A. T.S., and J.W.B. conducted the data management and analysis, J. P-A. produced the figures, and J.P-A. and J.W.B. authored the description of the statistical methods and results. All authors have approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 4
Study 2. Clinical scores following challenge of cattle. Any vesicles on each foot are scored as 1 yielding a maximum clinical score of 4. There is no scoring for vesicles in the mouth or on the tongue, the inoculation site of the challenge virus.Last day of observations was day 7. [1]Protected. [2]Steer was not challenged.

		Days post challenge	
Vaccine group	Steer	0	7
TD	153[1]	0	0
	157	0	4
	162	0	4
	164[2]	0	–
	165	0	2
	167	0	1
	168[1]	0	0
	172	0	4
	173	0	4
	179[1]	0	0
IM	155	0	3
	159	0	3
	160[1]	0	0
	161	0	4
	163[1]	0	0
	166	0	4
	169	0	4
	171	0	4
	174	0	4
	178	0	4
Naïve	170	0	4
	177	0	4
	180	0	4

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.08.017.

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