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A DNA vaccination regime including protein boost and electroporation protects cattle against foot-and-mouth disease

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ABSTRACT

Protection against foot-and-mouth disease (FMD) using DNA technology has been documented for sheep and pigs but not for the highly susceptible species of cattle.

Twenty-five Holstein Friesian cross-bred cattle were vaccinated twice, 21 days apart, with a DNA vaccine containing the capsid coding region (P1) along with the non-structural proteins 2A, 3C and 3D (pcDNA3.1/P1-2A3C3D) of O_1 Kaufbeuren alone or coated onto PLG (D,L-lactide-co-glycolide) microparticles. In some pcDNA3.1/P1-2A3C3D was also combined with an adjuvant plasmid expressing bovine granulocyte macrophage colony stimulating factor (GM-CSF). DNA vaccinations were administered intramuscularly with, or without, the use of electroporation and at 42 days post primary vaccination cattle received a protein boost of 146S FMD virus (FMDV) antigen and non-structural protein 3D. For comparison, four cattle were vaccinated with a conventional FMD vaccine and two more included as unvaccinated controls. Apart from those immunised with PLG microparticles all cattle were challenged with 10^5 $TCID_{50}$ cattle adapted O_1 Lausanne FMDV virus at day 93 post primary vaccination.

All DNA vaccinated cattle regardless of regime developed good humoral and cell mediated responses prior to challenge. The best overall virus neutralising antibody, IFN-γ and clinical protection (75%) were seen in the cattle whereby the DNA was delivered by electroporation. In contrast, only 25% of cattle vaccinated with the DNA vaccine without electroporation were clinically protected. The addition of GM-CSF in combination with electroporation further improved the efficacy of the vaccine, as demonstrated from the reduction of clinical disease and virus excretions in nasal swabs.

We thus demonstrate for the first time that cattle can be clinically protected against FMDV challenge following a DNA prime-protein boost strategy, and particularly when DNA vaccine is combined with GM-CSF and delivered by electroporation.

1. Introduction

Foot-and-mouth disease (FMD) is an acute systemic disease of cloven-hoofed animals of which the causal agent is foot-and-mouth disease virus (FMDV). The disease is ranked first in the L'Office International des Epizooties (OIE, World Organisation for Animal Health) list of notifiable diseases, which by definition, means that it has the potential for rapid and extensive spread within and between countries. In addition, the virus can cause significant weight loss and a decrease in milk production in cattle (Alexandersen et al., 2003) and is thus one of the most economically important diseases of livestock worldwide.

European Union (EU) policy on FMD control recognises vaccination as a principal measure, and strategic vaccine reserves have been set up by the EU and many member countries for emergency purpose. Indeed, vaccination has moved more to the forefront of EU control policy, making it important to improve on the current chemically inactivated virus antigen vaccines which, although effective at preventing clinical signs of the disease, have several significant limitations (Doel, 2003). These include the requirement of high disease secure containment facilities for manufacture, poor thermostability and thus a relatively short shelf life, lack of induction of sterile immunity resulting in the occurrence of carrier animals and limited antigenic spectrum of protection.

A vaccine, or vaccination regime, which addresses many of these shortcomings would provide a more powerful prophylactic for the control of this disease. DNA vaccines have potential for use in very young animals and offer the vaccine manufacturers negligible risk

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of contamination from adventitious agents of animal origin in the final product. Other practical advantages of this approach include (i) a considerable reduction in the cost of production by negating the need for high containment facilities to produce this non infectious vaccine product, (ii) good thermo-stability properties reducing the need for a cold-chain and (iii) the ease of manipulation (Babiuk et al., 2000; Gurunathan et al., 2000; Cichutek, 2000) allowing incorporation of marker genes, the co-expression of multiple antigens, and ability to rapidly cover newly emerging field isolates. Underlining this, such new generation vaccines are beginning to find a niche in the veterinary products portfolio with the recent granting of licences for DNA based products including West Nile virus in horses (West Nile Innovator, Fort Dodge) (Davidson et al., 2005), haematopoietic necrosis virus in salmon (Apex-IHN, Novartis) (Garver, 2005), melanoma in dogs (Canine Melanoma Vaccine, Merial) (Bergman et al., 2006) and growth hormone releasing hormone (GHRH) (LifeTide SW5, VGX Animal Health) (Thacker et al., 2006).

It has already been shown that a DNA plasmid (pCDNA3.1) encoding the precursor (P1) of empty capsid and non-structural proteins 2A, 3C and 3D of FMDV in combination with an adjuvant plasmid expressing granulocyte macrophage colony stimulating factor (GM-CSF) induced strong immune responses to FMDV and protected pigs against live virus challenge (Cedillo-Barron et al., 2001).

Further optimisation of this vaccine strategy dramatically improved the neutralising antibody response and induced an early cellular immune response (Li et al., 2006). It has also been demonstrated that the same DNA (Cedillo-Barron et al., 2001) coated onto poly-D,L-lactide-co-glycolide (PLG) particles promoted specific antibody and cellular immune responses that fully protected sheep against viraemia and local virus replication in the oropharynx at, or before, 28 days post challenge (Niborski et al., 2006). However, even more dramatic improvement in the immune responses has been reported when such vaccines are used in combination with FMD inactivated homologous virus antigen and 3D recombinant protein as a final protein boost in pigs (Li et al., 2008).

Cattle are economically and agriculturally important, have very high susceptibility to FMDV, and can be long-term viral carriers after infection. However, reports on the efficacy of DNA vaccines in cattle are limited. Indeed, there is only one key paper cited, involving a two dose DNA prime-protein boost regime, or vice versa, which was sufficient to stimulate enhanced immunity against FMDV in cattle (Jin et al., 2005). In contrast to other studies however, this experiment involved DNA constructs and a protein boost that only represented part of the virus and the ability of this vaccination regime to protect against challenge was not evaluated.

Utilising the FMD DNA vaccine pcDNA3.1/P1-2A3C3D combined with an adjuvant plasmid expressing GM-CSF we evaluated different DNA prime and protein boost vaccination regimes in cattle, and based on the previous observations in pigs and sheep, examined how effective these varying immunisation regimes are at eliciting protective immunity and inhibiting FMDV replication up to 14 days post infection in this target.

2. Materials and methods

2.1. Plasmid DNA

Plasmids pcDNA3.1/P1-2A3C3D containing FMDV O_1 Kaufbeuren precursor P1 with self cleaving peptide 2A and viral protease 3C, to assemble icosahedral particles, and non-structural protein 3D, to provide additional T-cell epitopes, were prepared as previously described (Li et al., 2008). In order to insert the bovine GM-CSF gene into the same backbone plasmid as the FMDV DNA, pC1-neobGMCSF (provided by Dr. Geraldine Taylor, Compton Laboratory, Institute for Animal Health) was restriction enzyme digested with *Xho I* and *Not I* to release the GM-CSF gene that was then subsequently cloned into the multiple cloning region of pcDNA3.1 using the same restriction enzyme sites (pcDNA3.1/bGM-CSF). Endotoxin free plasmid DNA's were prepared and supplied commercially by Aldevron (USA). Recombinant 3D protein was prepared as per Li et al. (2008).

2.2. Preparation of PLG microparticles

PLG microparticles were prepared as (Lawlor et al., 2011) with the following modifications. The PLGA polymer (D,L-lactide-co-glycolide) was RG503 (Boehringer-Ingelheim, Germany), which has a copolymer ratio of 50/50 and a molecular mass of 34 kDa (manufacturer's data). Briefly, the 50 mg of microparticles were prepared by dissolving PLGA polymers in 2 ml dichloromethane by probe sonication for 8 s at 4 W creating the oil phase. One millilitre of 2.5% poly(vinyl) alcohol was added to the oil phase creating water in oil primary emulsion. The water in oil (W/O) phase was then remotely sonicated for 16 s at 4 W. The W/O emulsion was added in a drop wise manner to 20 ml of a 1% solution of cetyltrimethylammonium bromide (CTAB) and homogenised at 6500 rpm. Once all the W/O emulsion was added the speed of the homogeniser was increased to 17,500 rpm for 2 min. Following homogenisation, the solution was stirred overnight at 200 rpm in a fume hood to allow the DCM to evaporate and the spherical particles to form. Particles were then washed twice in deionised water and centrifuged at 8000 rpm for 8 min. Particles were then sized using a Malvern Mastersizer 200 and the electrokinetical potential was determined by zetasizer. The suspension was then flash frozen in liquid nitrogen before being freeze dried for 24 h.

DNA (pcDNA3.1/P1-2A3C3D) was adsorbed onto the microparticles by adding 10 ml of 5.1 mg/ml DNA to 90 ml TE (Tris/EDTA) buffer and incubated with 600 mg (2% w/w) cationic microparticles at 4 °C for 16 h. The microparticles were then separated by centrifugation at 8000 rpm, the pellet was washed three times with Tris–EDTA buffer, and the microparticles freeze-dried. The wash fluid was quantified using a nanodrop to determine DNA loss. PLGA DNA microparticles were re-suspended to 4 mg/ml.

2.3. Vaccination and sampling of cattle pre-challenge

Thirty-two Holstein Friesian cross-bred cattle of 6–7 months of age were housed separately in six groups of five (vaccinated animals) and one group of two (un-vaccinated controls) within the isolation units at the Institute for Animal Health, Pirbright. The six groups of vaccinates were immunised as follows:

Group 1: 2 mg PLG-pcDNA3.1/P12A3C3D + 0.4 mg pcDNA3.1/bGM-CSF (DNA + PLG + GMCSF)
Group 2: 2 mg PLG-pcDNA3.1/P12A3C3D + 0.4 mg pcDNA3.1/bGM-CSF + electroporation (DNA + PLG + GMCSF + elec)
Group 3: 2 mg pcDNA3.1/P12A3C3D + 0.4 mg pcDNA3.1/bGM-CSF (DNA + GMCSF)
Group 4: 2 mg pcDNA3.1/P12A3C3D + 0.4 mg pcDNA3.1/bGM-CSF + electroporation (DNA + GMCSF + elec)
Group 5: 2 mg pcDNA3.1/P12A3C3D + electroporation (DNA + elec)
Group 6: Conventional vaccine (7.5 μg, BEI-inactivated O_1 Lausanne, sucrose density gradient purified 146S FMDV antigen with Montanide ISA 2006 (Seppic) as the adjuvant)

Cattle in groups 1–5 received a repeat of the appropriate DNA boost at 21 days post first vaccination which was followed by a protein boost, consisting of 7.5 μg, BEI-inactivated, sucrose density

gradient purified 146S FMDV antigen, without adjuvant and 200 μg non structural protein 3D, 21 days later.

Cattle in groups 2, 4 and 5 received their DNA vaccine intramuscularly in combination with electroporation using an integrated administration apparatus as previously described (Drunen Littel-van den et al., 2008). Briefly, the electroporation device comprised an array of electrodes affixed to an automatic injection device. The electrode arrays consisted of four stainless steel electrodes of 35 mm length positioned at the summits of two adjoining equilateral triangles comprising a diamond configuration. The triangle summits were symmetrically positioned around a central injection port and spaced by 8 mm. The electrode array was inserted percutaneously into the muscle gluteus of the proximal hind limb. Activation of the device initiated the device insertion of a 0.8×32 mm injection needle through the central injection port to a depth of approximately 30 mm followed by distribution of the DNA within the local muscle tissue. Approximately 10 s after initiation of the injection, an electrical field of 250 V/cm amplitude was applied for a total duration of 400 ms at a 10% duty cycle (i.e. voltage is actively applied for a total of 40 ms of the 400 ms duration). Once the electroporation procedure was complete, the electrode array and injection needle were removed from the tissue.

Clotted and heparinised blood were collected on days 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 post primary vaccination. Nasal and Saliva swabs were collected on days 0, 21, 42 and 63 post primary vaccination.

Vaccination and sampling was carried out in accordance with EU Directive 2010/63/EU with the husbandry and care of the animals post vaccination ensured by institutional guidelines.

2.4. Challenge and sampling of cattle post-challenge

On day 133 post primary vaccination, four cattle in each of vaccination groups 3–6 only were randomly selected, together with two un-vaccinated controls and intradermolingually challenged in two sites with 10^5 $TCID_{50}$ (equivalent to the OIE approved dose of 10,000 BID [bovine infectious doses]$_{50}$), 0.1 ml per site, of cattle adapted O_1 Lausanne FMDV, which is antigenically indistinguishable to O_1 Kaufbeuren. Cattle were challenged at this time point since this was the earliest opportunity post vaccination whereby our high containment animal facilities were licensed to conduct live viral challenge. Cattle numbers taken to challenge were reduced from 5 to 4 per group in accordance with Home Office regulations relating to the large size and husbandry requirements of these animals. Clotted and heparinised blood, saliva, nasal and oropharyngeal fluid (OPF) samples were collected on days 0, 1, 2, 5, 7 and 14 days post challenge. Clinical signs were scored separately for foot vesicles, nasal vesicles, rectal temperatures and nasal discharge. In the case of the feet, a score was only recorded upon the development of a clear vesicle (maximum of 4/animal). Nasal lesions were scored as healing vesicle = 1, presence of vesicle = 2. Rectal temperatures were scored as follows: <39.5 °C = 0, ⩾39.5 °C to <40 °C = 1, ⩾40 °C = 2. Nasal discharge was scored as follows: serous = 1, sero-necrotic = 2, necrotic = 3. The experimental challenge and sampling was carried out in accordance with EU Directive 2010/63/EU with the husbandry and care of the animals post challenge ensured by institutional guidelines.

2.5. Virus neutralising antibody test

Serum samples collected at intervals up to day 63 post primary vaccination were examined for anti-FMDV neutralising antibodies (Rweyemamu, 1984). The neutralising antibody titres were calculated as the $\log_{10}$ of the reciprocal antibody dilution required for 50% neutralisation of $100TCID_{50}$ virus. Cell culture adapted O_1 Lausanne FMDV was used to assess the homologous neutralising antibody response, whilst Asia 1 Shamir, C Noville, A22 Iraq, SAT1/05, SAT2 Eritrea and SAT3/09 FMDV were used to assess the heterologous virus neutralising response. Individual cattle sera were used to determine the homologous virus neutralising antibody response, whilst pooled cattle sera from each vaccination group were used to assess the heterologous virus neutralising response.

2.6. Virus isolation (VI)

Heparinised blood, nasal, saliva and OPF samples were examined for the presence of live virus by primary BTY cell culture inoculation and a confirmatory antigen ELISA as previously described (Fowler et al., 2008).

2.7. Real time PCR

One-step real-time RT-PCR (rRT-PCR) was carried out on heparinised blood, nasal, saliva and OPF samples according to the procedure described by Shaw et al. (2007) and Fowler et al. (2008). The TaqMan® primers (SA-IR-219-246F, SA-IR-315-293R) and probe (SAmulti2-P-IR-292-269R) used in the assay, targeted conserved sequences in the internal ribosomal entry site within the 5′ untranslated region (5′ UTR) of the FMDV genome (Reid et al., 2002).

2.8. PBMC proliferation assay

Heparinised blood was diluted with phosphate-buffered saline (PBS) (Invitrogen, Paisley, UK) and centrifuged after under laying with Histopaque-1077 (Sigma). Peripheral blood mononuclear cells (PBMCs) were collected from the interface and washed three times with cold PBS. PBMCs (2×10^5 per well) in proliferation medium {RPMI 1640 supplemented with 10% BVDV-free FCS, 5% non-essential amino acids (Invitrogen), 5 mM sodium pyruvate (VWR, Leicestershire, UK), 50 μg/ml gentamycin and 0.5 μM 2-mercaptoethanol} were incubated with a range of antigens; control and test antigens, [medium alone, pokeweed mitogen (PWM, 2.5 μg/ml, Sigma) and FMDV O_1 Manisa antigen diluted 1/1000] were added to each well at previously optimised concentrations (Windsor and Carr, 2011). The cultures were incubated for 5 days before 37Bq [^{3}H] Thymidine (Amersham, Buckinghamshire, UK) was added to each well. After a further overnight incubation, plates were harvested onto filter mats and the incorporated radioactivity was measured using a 1450 Microbeta counter (Perkin Elmer, USA).

2.9. Non-structural protein (NSP) ELISA

Pre and post challenge serum samples were assessed for the presence of antibodies against FMDV non-structural proteins (NSP) using the commercially available Prionics® FMDV-3ABC NS Elisa kit (Brocchi et al., 2006; Goris et al., 2007). Samples were considered positive if the % of inhibition was ⩾50.

2.10. Mucosal antibody (IgA)

An indirect ELISA for the detection of IgA antibodies to FMDV structural proteins was performed as previously described (Parida et al., 2006a) using samples taken at 0, 1, 2, 5, 7 and 14 days post challenge. An optical density (OD) cut-off value of 0.6 was applied to determine positive from negative samples.

2.11. Whole blood interferon-γ (IFN-γ)

The level of IFN-γ production from 200 μl of whole blood was assessed by re-stimulating white blood cells from vaccinated cattle

with inactivated FMD vaccine antigen (2 μg/ml O_1 Manisa/50 μl/well) for 24 h followed by a capture ELISA (Invitrogen, UK) as described previously (Parida et al., 2006b).

2.12. Statistical analysis

One way analysis of variance (ANOVA) with Tukey's Multiple Comparison post test was used to determine any significant difference in the homologous virus neutralising antibody levels between vaccination groups. A linear mixed model with 'day' and 'vaccine group' as factors and 'animal' as random effect, was used to determine any significant difference in the PBMC response between group and time. Statistical calculations were performed using Graphpad Prism 5 (ANOVA) or R 2.12.2 statistical package (Linear Mixed Model).

3. Results

3.1. Homologous neutralising antibody response

All DNA vaccinated cattle generated a detectable neutralising antibody response at 21 days post 1st vaccination (Fig. 1). The virus neutralising antibody response of conventionally vaccinated cattle at this time point was significantly higher when compared to all groups of DNA vaccinated cattle (one way-ANOVA, $p < 0.001$). Between day 21 and 42 the virus neutralising antibody response dropped following 2nd DNA vaccination in three groups (DNA + GMCSF, DNA + PLG + GMCSF and DNA + GMCSF + elec), whilst in the other two groups (DNA + PLG + GMCSF and DNA + elec) titres were maintained. However, all virus neutralising antibody titres from DNA vaccinated cattle were still significantly lower than conventionally vaccinated cattle at 42 days post vaccination (one way-ANOVA, $p < 0.001$).

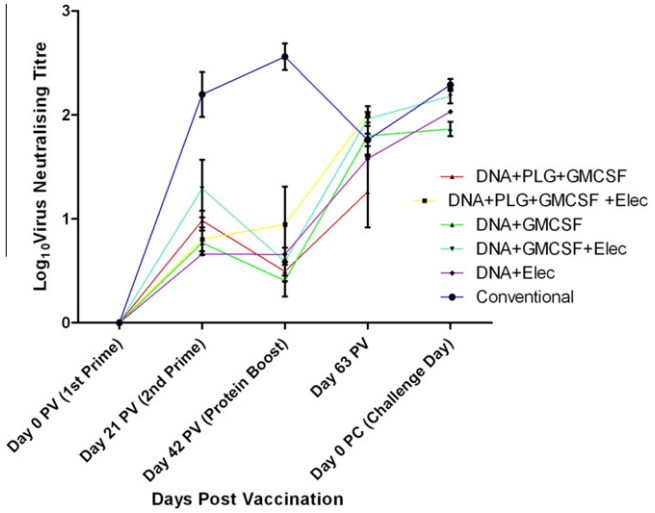

Fig. 1. Homologous virus neutralising antibody responses of cattle serum following vaccination. Cattle were either vaccinated with group 1: 2 mg PLG-pcDNA3.1-P12A3C3D + 0.4 mg bGMCSF vaccine; group 2: 2 mg PLG-pcDNA3.1P12A3C3D + 0.4 mg bGMCSF vaccine + electroporation; group 3: 2 mg pcDNA3.1P12A3C3D + 0.4 mg bGMCSF vaccine; group 4: 2 mg pcDNA3.1P12A3C3D + 0.4 mg bGMCSF vaccine + electroporation; group 5: 2 mg pcDNA3.1P12A3C3D + electroporation; group 6: Conventional vaccine (7.5 μg, BEI-inactivated, sucrose density gradient purified 146S FMDV antigen with Montanide ISA 206 (Seppic) as the adjuvant). DNA vaccinated cattle in groups 1–5 received two DNA primes, 21 days apart, followed by a protein boost at day 42 post primary vaccination with 7.5 μg, BEI-inactivated, sucrose density gradient purified 146S FMDV antigen, without adjuvant and 200 μg non structural protein 3D Error bars represent 1 standard error (SE) above and below the mean.

Between day 42 and day 63 post vaccination the virus neutralising antibody titres in the conventionally vaccinated cattle rapidly declined to a significantly lower titre (mean ± SEM: 1.8 ± 0.06, one way-ANOVA, $p = 0.01$) to that observed at day 42 post vaccination (mean ± SEM: 2.6 ± 0.13). The virus neutralising antibody titres then appeared to increase, however this increase was not significant when compared to the titres on day 63 post vaccination (one way-ANOVA, $p > 0.05$).

Following protein boost on day 42 post 1st DNA vaccination, all DNA vaccinated animals, regardless of vaccination strategy, produced high virus neutralising antibody titres which had increased to similar levels at day 63 (21 days post protein boost). At this time point the titres between DNA vaccinated cattle and conventionally vaccinated cattle were not statistically different (one way-ANOVA, $p > 0.05$). At day 63 it was decided that cattle in the two PLG groups would not be followed to challenge, based upon the fact that these cattle had statistically similar antibody titres to the other DNA vaccination regimes that were less complicated in preparation. On day of challenge DNA vaccinated cattle had high levels of virus neutralising antibody which were similar to conventionally vaccinated cattle (mean ± SEM: DNA + GMCSF: 1.9 ± 0.07, DNA + GMCSF + elec: 2.2 ± 0.07, DNA + elec: 2.0 ± 0.12 and conventional: 2.3 ± 0.06).

3.2. Heterologous neutralising antibody response

A neutralisation antibody response at 100 $TCID_{50}$ was noted in three DNA vaccination groups (DNA + GMCSF + PLG, DNA + GMCSF and DNA + GMCSF + elec) against Asia 1 Shamir at day 63 post vaccination, but was not apparent against other serotypes (Table 1). In contrast, conventionally vaccinated cattle did not demonstrate any heterologous neutralising antibody reactivity at this same 100 $TCID_{50}$.

3.3. FMDV specific PBMC proliferation

PBMC's from all cattle, at every sampling point, responded to PWM (data not presented). The mean FMDV specific PBMC proliferation responses detected in vaccinated cattle at 0, 21, 42 and 63 days post vaccination are presented in Fig. 2. No FMDV specific proliferative response could be noted at day 0 post vaccination in any vaccination group. At 21 days post primary vaccination all cattle, regardless of vaccination group, had FMDV specific PBMC proliferation response, moreover three of the DNA vaccination groups (DNA + GMCSF, DNA + GMCSF + elec and DNA + elec) demonstrated a greater (Mean ± SE: 78,304 ± 14,452, 71,460 ± 4022, 80,203 ± 9173 respectively) PBMC proliferative response to that of cattle receiving a single conventional vaccination (Mean ± SE: 39,302 ± 4898). Comparison of all DNA vaccination groups by day 63 (21 days post protein boost) highlighted similar PBMC proliferations which was mirrored by the conventionally vaccinated cattle. Statistical analysis determined that there was a significant increase in proliferative response in all vaccination groups over time ($p < 0.001$) until day 42 post vaccination, however, this significant response did not vary between vaccination group ($p = 0.06$).

3.4. Protection from challenge

Both unvaccinated controls developed generalised disease with visible vesicles on all four feet by day 2. Conventionally vaccinated cattle were fully protected from experimental challenge, as determined by absence of generalised disease (Table 2). In addition 6 of the 8 DNA vaccinated cattle in groups 4 and 5 (DNA + GMCSF + elec and DNA + elec) were fully protected despite these DNA vaccinated animals being challenged 91 days after the protein boost. This was in contrast to the DNA vaccinated cattle in group 3 (DNA + GMCSF) where only one vaccinated animal was protected. Total clinical

Table 1
Heterologous virus neutralising response 21 days post boost (day 63 post vaccination). Cattle were either vaccinated with group 1: 2 mg PLG-pcDNA3.1P12A3C3D + 0.4 mg bGMCSF vaccine; group 2: 2 mg PLG-pcDNA3.1P12A3C3D + 0.4 mg bGMCSF vaccine + electroporation; group 3: 2 mg pcDNA3.1P12A3C3D + 0.4 mg bGMCSF vaccine; group 4: 2 mg pcDNA3.1P12A3C3D + 0.4 mg bGMCSF vaccine + electroporation; group 5: 2 mg pcDNA3.1P12A3C3D + electroporation; group 6: Conventional vaccine (7.5 μg, BEI-inactivated, sucrose density gradient purified 146S FMDV antigen with Montanide ISA 206 (Seppic) as the adjuvant). DNA vaccinated cattle in groups 1–5 received two DNA primes, 21 days apart, followed by a protein boost at day 42 post primary vaccination with 7.5 μg, BEI-inactivated, sucrose density gradient purified 146S FMDV antigen, without adjuvant and 200 μg non structural protein 3D. Shading indicates where a detectable neutralising response was detected.

	Serotype						
	O_1 Lausanne	Asia 1 Shamir	A22 Iraq	C Noville	SAT2 Eritrea	SAT3/09	SAT1/05
Vaccination group	100 $TCID_{50}$	100 $TCID_{50}$	100 $TCID_{50}$	100 $TCID_{50}$	100 $TCID_{50}$	100 $TCID_{50}$	100 $TCID_{50}$
Conventional	1.8	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
DNA + elec	1.6	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
DNA + PLG + GMCSF	1.3	0.6	<0.3	<0.3	<0.3	<0.3	<0.3
DNA + PLG + GMCSF + elec	2.0	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
DNA + GMCSF	1.8	0.3	<0.3	<0.3	<0.3	<0.3	<0.3
DNA + GMCSF + elec	2.0	0.7	<0.3	<0.3	<0.3	<0.3	<0.3

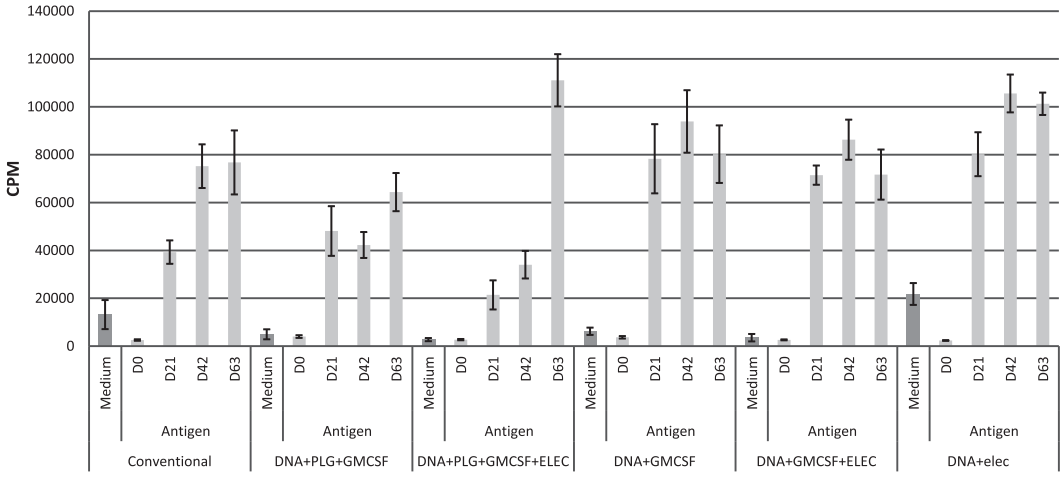

Fig. 2. Mean day 0–63 PBMC proliferation responses after stimulation with O_1 Manisa antigen. Error bars represent standard error (SE) above and below the mean. CPM: 3H Thymidine incorporation 401 – counts per minute. Medium control represents mean CPM from days 0–63.

Table 2
Generalised vesicle development post challenge (PC). The number represents how many feet were affected with vesicles. Animals were euthanised at the point of which three or more feet developed vesicles.

	Conventional				DNA + ele				DNA + GMCSF				DNA + GMCSF + elec				Unvaccinated	
	3497	3458	3508	3507	3454	3494	3516	3471	3480	3521	3519	3487	3511	3539	3515	3549	Control 1	Control 2
Day 1PC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Day 2PC	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	4	4
Day 3PC	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0		
Day 4PC	0	0	0	0	2	0	0	0	0	2	3	4	3	0	0	0		
Day 5PC	0	0	0	0	4	0	0	0	0	2				0	0	0		
Day 6PC	0	0	0	0		0	0	0	0	3				0	0	0		
Day 7PC	0	0	0	0		0	0	0	0					0	0	0		
Day 8PC	0	0	0	0		0	0	0	0					0	0	0		
Day 9PC	0	0	0	0		0	0	0	0					0	0	0		
Day 10PC	0	0	0	0		0	0	0	0					0	0	0		

scores generated from rectal temperatures (°C), nasal vesicles and nasal discharge also mirrored the generalised disease results (Table 3).

3.5. Real time PCR and virus isolation

Live FMDV was recovered from saliva and OPF samples from all animals with the majority of positive samples at day 2 post challenge (Table 4). In the case of nasal samples, virus was only recovered from six individual animals sporadically over the course of 5 days (Table 4). Interestingly, and comparable to conventionally vaccinated cattle, virus was not recovered in nasal samples from cattle in vaccination group 4 (DNA + GMCSF + elec). In addition, virus was only recovered from blood taken from the two control animals on days 1 and 2 post challenge (data not presented). The FMDV RNA detected by quantitative rRT-PCR largely mirrored the samples which were positive for virus isolation (Table 5) and in addition confirmed the absence of FMDV genome in the nasal samples collected from cattle in groups 4 (DNA + GMCSF + elec) and 6 (conventional vaccine).

Table 3
Total clinical scores generated from rectal temperatures (°C), nasal lesions and nasal discharge following challenge on day 93 post final vaccination. Nasal vesicles were scored as healing vesicle = 1, presence of vesicle = 2. Rectal temperatures were scored as follows: <39.5 °C = 0, ⩾39.5 °C to <40 °C = 1, ⩾40 °C = 2. Nasal discharge was scored as follows: serous = 1, sero-necrotic = 2, necrotic = 3. Shading indicates where clinical scores were recorded.

	Conventional				DNA + ele				DNA + GMCSF				DNA + GMCSF + elec				Unvaccinated	
	3497	3458	3508	3507	3454	3494	3516	3471	3480	3521	3519	3487	3511	3539	3515	3549	Control 1	Control 2
Day 1PC	1	2	2	2	2	2	2	2	1	2	2	2	2	1	2	2	1	2
Day 2PC	3	3	2	1	5	1	2	3	1	3	3	3	3	3	3	3	3	3
Day 3PC	2	2	1	1	5	2	2	2	1	3	4	2	2	2	2	0		
Day 4PC	1	1	1	1	2	1	1	2	1	3	2	2	1	2	1	1		
Day 5PC	1	0	1	0	1	1	1	1	0	2				0	0	0		
Day 6PC	1	1	0	0		1	0	1	0	1				0	0	0		
Day 7PC	1	1	0	0		0	1	1	0					0	0	0		
Day 8PC	0	0	0	0		0	0	1	1					0	0	0		
Day 9PC	0	0	0	0		0	0	0	0					0	0	0		
Day 10PC	0	0	0	0		0	0	0	0					0	0	0		
Total	10	10	7	5	16	8	9	13	5	14	11	9	8	8	8	6	4	5

Table 4
Virus isolation (VI) results for saliva, oro-pharyngeal (OPF) and nasal samples taken between days 1 and 5 post challenge (PC). All animals were negative in all samples on days 0, 7 and 14PC and therefore data is not presented. Shading indicates positive animals and hatched shading indicates time points at which animals had been euthanised.

Vaccine	ID	Day 1PC VI			Day 2PC VI			Day 5PC VI		
		Saliva	OPF	Nasal	Saliva	OPF	Nasal	Saliva	OPF	Nasal
DNA + ele	3516	NEG	POS	NEG	POS	POS	NEG	NEG	NEG	NEG
	3471	NEG	POS	NEG	POS	POS	NEG	POS	POS	POS
	3494	NEG	POS	NEG	POS	POS	NEG	POS	NEG	POS
	3454	NEG	POS	NEG	POS	POS	POS	NEG	NEG	POS
Conventional	3497	NEG	POS	NEG	POS	POS	NEG	POS	NEG	NEG
	3458	POS	POS	NEG	POS	POS	NEG	POS	POS	NEG
	3508	POS	POS	NEG	POS	POS	NEG	NEG	POS	NEG
	3507	POS	POS	NEG	POS	POS	NEG	NEG	POS	NEG
DNA + GMCSF	3480	NEG	POS	NEG	POS	POS	NEG	POS	POS	NEG
	3519	POS	POS	POS	POS	POS	NEG			
	3487	POS	POS	NEG	POS	POS	POS			
	3521	POS	POS	NEG	POS	POS	POS	NEG	POS	NEG
DNA + GMCSF + Elec	3549	NEG	POS	NEG	POS	POS	NEG	NEG	NEG	NEG
	3539	NEG	POS	NEG	POS	POS	NEG	NEG	NEG	NEG
	3515	NEG	POS	NEG	POS	POS	NEG	POS	NEG	NEG
	3511	NEG	POS	NEG	POS	POS	NEG			
Un vacc	Crtl1	POS	POS	POS	POS	POS	POS			
	Crtl2	POS	POS	POS	POS	POS	POS			

3.6. NSP antibody

Antibodies against NSP's were detected at a level considered positive in ten of the thirteen surviving cattle on day 7 post challenge (Table 6). By day 14 post challenge antibodies against NSP's could be detected in all surviving cattle. Since the unvaccinated controls were euthanised on day 2 post challenge their serum was not tested for NSP specific antibody.

3.7. Evaluation of mucosal antibody (IgA)

No FMDV specific mucosal IgA was detected in the saliva of either conventionally or DNA vaccinated animals prior to challenge. FMDV specific IgA was detected in an animal on day 14 post challenge from groups 6 (conventional) and 4 (DNA + GMCSF + elec) (data not shown). Near positive IgA samples were detected in an additional three animals, two from group 6 (conventional) and one from group 3 (DNA + GMCSF) (data not shown).

3.8. IFN-γ response in re-stimulated whole blood

Mean IFN-γ responses from re-stimulated whole blood on day 63 post vaccination were calculated (mean ± SE) per vaccination group as follows, DNA + elec (0.28 ng/ml ± 0.19), DNA + GMCSF (0.09 ng/ml ± 0.26), DNA + GMCSF + elec (0.11 ng/ml ± 0.38) and conventional (0.11 ng/ml ± 0.03).

Whole blood IFN-γ responses were then categorised for animals in each vaccinated group which were either protected or unprotected post challenge. In the case of the DNA vaccinated cattle which were protected against challenge ($n = 7$), re-stimulated whole blood IFN-γ response was detected in all groups at 63DPV with the greatest response noted in vaccination groups 4 (DNA + GMCSF + elec) ($n = 3$ cattle protected, mean ± SE: 0.84 ng/ml ± 0.47) and 5 (DNA + elec) ($n = 3$ cattle protected, mean ± SE: 0.55 ng/ml ± 0.2) (Fig. 3), for which electroporation was used as the delivery method. This was in contrast to the conventionally vaccinated cattle whereby virtually no IFN-γ response could be detected at 63DPV ($n = 4$ cattle protected, mean ± SE: 0.03 ng/ml ± 0.03) (Fig. 3).

On the other hand, for the cattle which were not protected ($n = 5$), whole blood IFN-γ at 63DPV was lower compared to the cattle which were protected in vaccination groups 4 (DNA + GMCSF + elec) ($n = 1$ cattle unprotected, 0.011 ng/ml) and 5 (DNA + elec) (n = 1 cattle unprotected, 0.05 ng/ml). In the case of vaccination group 3 (DNA + GMCSF), the IFN-γ levels appear to be higher in the animals ($n = 3$) not protected (mean ± SE: 0.46 ng/ml ± 0.36)

Table 5
rRT-PCR results for saliva, oro-pharyngeal (OPF) and nasal samples taken between days 1 and 14 post challenge (PC). All animals were negative by rtPCR on day 0 PC and thus data is not presented. Shading indicates positive animals (ct-values of less than 35 were considered positive), hatched shading indicates time points at which animals had been euthanised.

Vaccine	ID	Day 1PC rT-PCR			Day 2PC rT-PCR			Day 5PC rT-PCR			Day 7PC rT-PCR			Day 14PC rT-PCR		
		Saliva	OPF	Nasal	Saliva	OPF	Nasal	Saliva	OPF	Nasal	Saliva	OPF	Nasal	Saliva	OPF	Nasal
DNA + ele	3516	No Ct	16.94	48.13	28.04	18.71	No Ct	33.22	27.85	37.27	No Ct	33.85	42.23	No Ct	45.97	No Ct
	3471	No Ct	30.12	No Ct	32.52	20.39	No Ct	25.61	25.35	33.84	35.48	32.51	37.03	No Ct	41.48	40.19
	3494	No Ct	13.05	No Ct	32.22	15.39	No Ct	24.3	24.24	36.03	36.3	29.06	40.29	No Ct	No Ct	No Ct
	3454	No Ct	33.12	No Ct	30.56	21.01	28.19	31.16	30.43	28.97						
Conventional	3497	No Ct	23.06	No Ct	22.29	18.71	No Ct	30.56	21.9	No Ct	No Ct	35.59	40.99	No Ct	37.41	N/A
	3458	27.76	14.46	No Ct	23.75	17.79	No Ct	27.79	25.53	36.28	41.39	34.42	40.26	No Ct	33.27	N/A
	3508	36.05	14.42	No CT	23.04	18.73	No Ct	No Ct	28.64	No Ct	38.1	32.08	No Ct	No Ct	42.72	No Ct
	3507	26.35	16.1	49.24	27.89	16.49	No Ct	42.97	33.39	No Ct	No Ct	34.75	43.15	No Ct	38.89	No Ct
DNA + GMCSF	3480	No Ct	29.92	No Ct	24.64	16.39	No Ct	27.89	24	43.23	No Ct	31.67	No Ct	No Ct	40.55	No Ct
	3519	No Ct	16.89	No Ct	34.9	24.68	No Ct									
	3487	29.41	16.18	No Ct	27.59	20.48	No Ct									
	3521	43.37	16.69	No Ct	18.4	17.55	29.99	32.29	30.74	No Ct						
DNA + GMCSF + Elec	3549	No Ct	27.03	No Ct	29.08	21.88	No Ct	36.51	32.36	No Ct	No Ct	34.97	No Ct	No Ct	No Ct	No Ct
	3539	No Ct	No Ct	No Ct	29.47	15.4	No Ct	34.41	28.61	39.66	No Ct	36.6	No Ct	No Ct	41.81	42.46
	3515	No Ct	13.55	No Ct	29.55	20.15	46.79	28.14	29.41	No Ct	No Ct	33.77	No Ct	No Ct	48.01	No Ct
	3511	No Ct	44.28	No Ct	32.38	22.3	No Ct									
Un vacc	Crtl1	44.47	15.66	28.36	22.35	20.81	21.78									
	Crtl2	24.4	14.97	33.2	22.31	21.82	21.33									

than those protected ($n = 1$) (mean ± SE: 0.14 ng/ml). However, the individual values from the unprotected cattle were 0.09 ng/ml, 0.12 ng/ml and 1.1 ng/ml, with one animal disproportionally increasing the overall mean of this group. Therefore this animal was removed from the data set presented in Fig. 3.

Whole blood IFN-γ was also measured at 14DPC for those animals which were protected. In the case of vaccination group 6 (conventional vaccine), 3 (DNA + GMCSF) and 5 (DNA + elec) there was a measurable increase in IFN-γ response post challenge, with the greatest increase detected in group 3 (DNA + elec) ($n = 3$ cattle protected, mean ± SE, 1.54 ng/ml ± 0.32) (Fig. 3). There was no increase in IFN-γ levels following challenge in vaccination group 4 (DNA + GMCSF + elec).

4. Discussion

The first publications detailing the possibilities of using DNA technology as a vaccine against FMDV were published in the late 1980s (Brown, 1987) and early 1990s (Brown, 1992). These publications were shortly followed by the successful generation of the first DNA vaccine against FMDV (Wong et al., 2000). However, despite this early success, to date, there has only been four documented cases which have demonstrated full protective efficacy against FMDV in the naturally susceptible species sheep and pigs (Niborski et al., 2006; Li et al., 2008; Wong et al., 2000, 2002), along with another four where partial protection was observed (Cedillo-Barron et al., 2001; Ward et al., 1997; Benvenisti et al., 2001; Mingxiao et al., 2007; Borrego et al., 2011).

Here we have demonstrated for the first time that clinical protection against FMDV can also be achieved in cattle following the use of a DNA prime-protein boost strategy and that this protection was best achieved by use of an electroporation delivery device and the inclusion of the immune modulator bovine GM-CSF.

After priming with DNA vaccine, the titres of virus neutralising antibody were similar between the different vaccination groups, however, following protein boost, there was a trend for groups that included the use of the electroporation device (DNA + GMCSF + elec and DNA + elec), to elicit higher titres of virus neutralising antibody at the day of challenge than those that did not use this mode of delivery (DNA + GMCSF), however this was not statistically significant. Furthermore, inclusion of bovine GM-CSF in the vaccination regime with electroporation (DNA + GMCSF + elec) appeared to further enhance the virus neutralising antibody responses to closely match those of the conventionally vaccinated cattle, though again this observation is not statistically significant. Not surprisingly, DNA vaccinated groups which generated highest antibody titres (DNA + GMCSF + elec and DNA + elec) also had the highest number of clinically protected cattle following challenge. A surprising, and perhaps significant observation was that the 2nd DNA vaccination added little to the humoral response in terms of boosting immunity as had been previously demonstrated in pigs (Li et al., 2008) and cattle (Jin et al., 2005). The reason for this is unclear although this lack of a boost effect questions the value of a 2nd DNA vaccination and may simplify the vaccination strategies used in cattle and requires further investigation.

The levels of FMDV antigen specific IFN-γ production in re-stimulated blood were also higher at 63DPV in the protected cattle which employed electroporation than those protected in the non-electroporated and conventionally vaccinated groups. Similarly, the inclusion of bovine GM-CSF provided the greatest overall IFN-γ response when combined with electroporation. In all but one vaccination group (DNA + GMCSF + elec) the IFN-γ titres increased following challenge, and since Parida et al. (2006b) demonstrated a correlation between FMDV antigen specific IFN-γ production in re-stimulated blood of vaccinated animals and

Table 6
NSP serology from vaccinated animals for day 0, 5, 7 and 14 days post challenge (PC). Solid shading indicates positive samples (% inhibition of ±50% was considered positive), hatched shading indicates time points at which animals had been euthanised.

Vaccine	ID	Day 0PC	Day 5PC	Day 7PC	Day 14PC
DNA + GMCSF	3487	Negative			
	3480	Negative	Negative	46.9	86.2
	3521	Negative	Negative		
	3519	Negative			
DNA + GMCSF + elec	3549	Negative	Negative	71.3	88.6
	3515	Negative	Negative	65.1	91.7
	3511	Negative			
	3539	Negative	Negative	76.1	90.6
DNA + elec	3471	Negative	Negative	74.6	94.4
	3516	Negative	Negative	75.1	95.3
	3494	Negative	Negative	80.5	94.1
	3454	Negative	Negative		
Conventional	3458	Negative	Negative	84.6	90.4
	3549	Negative	Negative	71.3	88.6
	3508	Negative	Negative	81.7	90.9
	3507	Negative	Negative	55.7	77.3

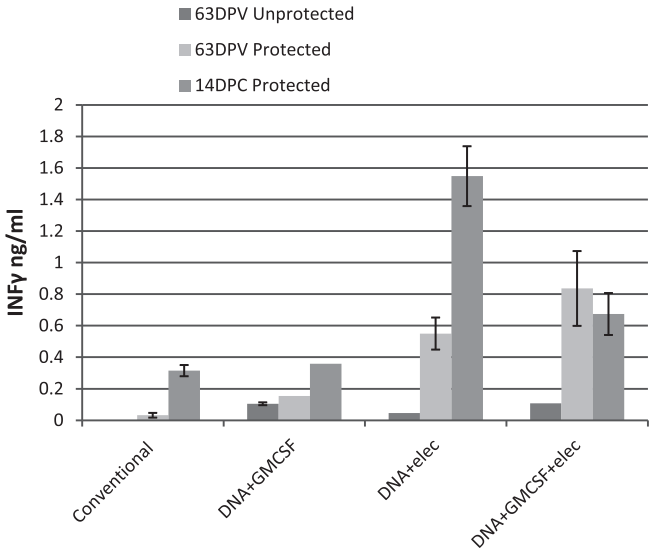

Fig. 3. IFN-γ responses in re-stimulated whole blood from different groups of vaccinated cattle at 63DPV and 14DPC following stimulation with 146S O_1 Manisa inactivated antigen. IFN-γ responses are presented for both protected (conventional $n = 4$, DNA + GMCSF n = 1, DNA + elec n = 3 and DNA + GMCSF + elec $n = 3$) and unprotected (conventional $n = 0$, DNA + GMCSF $n = 2$ (outlier animal removed), DNA + elec $n = 1$ and DNA + GMCSF + elec $n = 1$) cattle at 63DPV and from protected (conventional $n = 4$, DNA + GMCSF $n = 1$, DNA + elec $n = 3$ and DNA + GMCSF + elec $n = 3$) cattle at 14DPC. Error bars represent the standard error (SE) above and below the mean. In the case where only a single animal is presented error bars do not appear.

reduced persistent infection in cattle, these DNA vaccinated cattle might have been prevented from becoming carrier animals. Although, this can only remain speculative given the small sample size and length of time the cattle were kept post challenge. It should also be remembered that although the overall level of IFN-γ from DNA vaccinated cattle correlated with protection, these protected cattle also had high levels of neutralising antibody. As has been previously shown (Oh et al., 2006), a positive correlation was also found between clinical protection and IFN-γ and virus neutralisation antibody in both the conventionally and DNA vaccinated cattle. However, because of the limited number of animals used in this study we can only speculate the role of FMDV specific IFN-γ producing memory cellular responses in the conferred protection of DNA vaccinated animals.

There was not an obvious difference between the virus isolation, rRT-PCR and PBMC proliferation results from the different vaccination groups. All animals were positive on at least one day post challenge in saliva and OPF samples. Perhaps this is not surprising since all the cattle were challenged intradermolingually and thus virus might be expected to be isolated from the oral and pharyngeal areas 2–3 days post challenge given this approach. What is interesting is the trend for cattle vaccinated with DNA + GMCSF + elec to have a reduction in total number of nasal samples determined as positive when compared to the other DNA vaccination strategies.

All vaccinated, including conventionally vaccinated animals, produced an FMDV specific proliferation on, or before, 21DPV which was maintained until the last sample point. However, in comparison to previous published data (Li et al., 2008; Ward et al., 1997), the proliferative responses were not enhanced in all of the DNA vaccinated groups above that of the conventionally vaccinated nor after the 2nd DNA prime or indeed following the protein boost. This provides further argument for refinement of the DNA vaccination strategy and removal of the 2nd DNA vaccination step.

Although viral replication may not have been prevented in any vaccination group, including the conventional vaccine group, as indicated by NSP seroconversion, it is possible that the degree of persistence of viral replication may differ between vaccination groups. The mean NSP antibody level for the DNA + GMCSF + elec vaccinated group was seen to be the lowest on day 7 compared to all other DNA vaccination groups and conventionally vaccinated animals, and comparable to conventionally vaccinated cattle on day 14 post challenge.

Increased IgA levels post challenge have also been shown to be indicative of viral replication and persistence (Goris et al., 2007). In the case of this study, three out of the four conventionally vaccinated animals demonstrated positive or weak positive OD values at day 14 post challenge. In contrast, only two of the DNA vaccinated animals demonstrated positive samples at day 14 post challenge. However, it is very difficult to interpret if this is a true observation since five of the twelve DNA vaccinated animals were terminated by day 14 post challenge and thus we do not know the IgA values for all DNA vaccinated cattle at this specific time point post challenge. It would be interesting to determine these values in DNA vaccinated cattle beyond 28 days post challenge to see if these vaccination regimes reduce virus persistence.

There have been reports of DNA vaccination that induce humoral immune responses that are more broadly cross-reactive (Li et al.,

2008) and even cross protective (Ulmer et al., 1993; Lorenzen et al., 2002; Mohler et al., 2006). However, in this study we only observed limited cross reactivity to one serotype, Asia 1 Shamir, in three of our DNA vaccination groups at a virus titre of 100$TCID_{50}$. Nevertheless, the cross reactive antibody titres generated in two of the three groups (DNA + GMCSF + PLG and DNA + GMCSF + ele) would be considered to be at a level that could confer 44–79% protection in cattle (Brehm et al., 2008).

5. Conclusion

This study has demonstrated that DNA vaccinated cattle can be clinically protected 91 days post protein boost against FMDV challenge following a prime-boost strategy involving a DNA vaccine. In addition, conventionally vaccinated cattle were clinically protected *ca.* 4.5 months after vaccination. As is the case with conventional vaccination, protection in this study was correlated to virus neutralising antibody. Currently, the strategy which produced the least clinical outcomes, was a combination of a DNA plasmid encoding the FMDV capsid precursor (P1) and 3C protease with another plasmid encoding bovine GM-CSF that are both delivered with electroporation.

The favourable results obtained in these studies with a prototype electroporation device designed for administration in cattle provides a rational basis for further investigation of this method for FMDV DNA vaccine development. Clearly, the long term feasibility of this delivery method for field use in a given veterinary application will be contingent on a rigorous cost benefit analysis balancing the improved potency observed with electroporation based delivery with the cost and additional complexity associated with its administration. However, recent developments with the technology provide the basis for optimism that the technology could be adapted for veterinary field use if interim results warranted. These include the initiation of human clinical studies in the developing world (http://www.iavi.org/news-center/Pages/PressRelease.aspx?pubID=3214) as well as ongoing development of portable, battery operated devices for procedure administration.

Future studies should also investigate the possibilities of refining the vaccination strategy, with particular attention paid on delivery of a single DNA vaccination, use of an appropriate chemical or perhaps even simultaneous delivery with the protein itself.

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