Enhanced neutralising antibody response to Bovine Virus Diarrhoea Virus (BVDV) inducedΔ by DNA Vaccine in Calves.

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Abstract:

DNA vaccination is effective in inducing potent immunity in mice; however it appears to be less so in large animals. Increasing the dose of DNA plasmid to activate innate immunity has been shown to improve DNA vaccine adaptive immunity. Retinoic acid-inducible gene I (RIG-I) is a critical cytoplasmic double-stranded RNA pattern receptor required for innate immune activation in response to viral infection. RIG-I recognise viral RNA and trigger antiviral response, resulting in type I interferon (IFN) and inflammatory cytokine production. In an attempt to enhance the antibody response induced by BVDV DNA in cattle, we expressed BVDV truncated E2 (E2t) and NS3 codon optimised antigens from antibiotic free-plasmid vectors expressing a RIG-I agonist and designated either NTC E2t(co) and NTC NS3(co). To evaluate vaccine efficacy, groups of five BVDV-free calves were intramuscularly injected three times with NTC E2t(co) and NTC NS3(co) vaccine plasmids individually or in combination. Animals vaccinated with our (previously published) conventional DNA vaccines pSecTag/E2 and pTriExNS3 and plasmids expressing RIG-I agonist only presented both the positive and mock-vaccine groups. Our results showed that vaccines coexpressing E2t with a RIG-I agonist induced significantly higher E2 antigen specific antibody response ($p < 0.05$). Additionally, E2t augmented the immune response to NS3 when the two vaccines were delivered in combination. Despite the lack of complete protection, on challenge day 4/5 calves vaccinated with NTC E2t(co) alone or NTC E2t(co) plus NTC NS3(co) had neutralising antibody titres exceeding 1/240 compared to 1/5 in the mock vaccine control group. Based on our results we conclude that co-expression of a RIG-I agonist with viral antigen could enhance DNA vaccine potency in cattle.

Keywords: Plasmid DNA vaccine, BVDV marker vaccine, RIG-I agonist

Introduction:

BVDV is endemic throughout much of the world. Even a clinically mild infection can significantly impact animal productivity [1]. Infection of the foetus during the first trimester may result in abortion or calves that are born persistently infected (PI) and continuously shed high levels of virus [1]. Strategies to control BVDV infection include culling of PI animals, enhanced biosecurity and the use of vaccines. Currently modified live and inactivated vaccines are used to protect cattle from BVDV. A major shortcoming of existing vaccines is the lack of a marker status to differentiate between vaccinated and naturally-infected animals.

The positive ssRNA BVDV genome encodes structural (core, Erns, E1 & E2) and non-structural proteins (Npro, NS2, NS3, NS4a/b, NS5a/b) [2], [3]. The major envelope glycoprotein (E2) and the non-structural protein 3 (NS3) have been studied as candidates for subunit vaccine [4], [5], [6], [7]. Cattle vaccinated with inactivated BVDV elicit high levels of neutralising anti-E2 antibody and are protected from viraemia and nasopharyngeal shedding [8]. NS3 is conserved across strains [3], [9] and it may assist in induction of cross-strain immunity. Our previous study demonstrated partial protection in cattle vaccinated with NS3 DNA vaccine [7]. These facts supported the design of BVDV marker subunit vaccine based on both E2 and NS3.

DNA vaccination emerged as an efficient means of eliciting cell mediated and humoral responses in small animal models against a number of antigens from parasites, bacteria and viruses. Despite the approval of four DNA vaccines in fish, horses, dogs and pigs in veterinary medicine [10], [11], [12], [13], DNA vaccine potency in large animals has not been encouraging. It remains an immunological problem that has to be overcome to enhance DNA vaccine potency.

Innate immune cells recognise specific pathogen associated molecular patterns via their pattern recognition receptors and thereby activate the adaptive immunity [14]. Thus, increasing innate immunity through inclusion of novel backbone-encoded innate immunity agonists can improve DNA vaccine induced immune responses. RIG-I is a cytoplasmic dsRNA pattern receptor required for innate immune activation in response to viral infection. Following RNA virus infection, RIG-I become activated through binding to its ligand (uncapped 5′-PPP-containing short, blunt dsRNA); this leads to type I IFN and inflammatory cytokines production. RNA polymerase III (Pol III) promoters are small, expressed at high levels and have the potential to retain 5′-PPP (J. A. Williams, world patent application

WO2006078979). Integration of RNA pol III promoter expressed RIG-I ligand into DNA vaccine backbone acts as a RIG-I-agonist resulting in enhanced plasmid immunostimulation and improved adaptive immune responses in mice [15], [16], [17].

To examine the effect of incorporation of RIG-I agonist on DNA vaccine potency in large animals, we compared the potency of DNA marker vaccine expressing E2t or NS3 in presence of RIG-I agonist in terms of viral protein-specific and neutralising antibody responses and protection efficacy in cattle.

2. Materials and methods

2.1. Cells and viruses

BVDV-free MDBK cells were cultured in Minimum Essential Medium (MEM) containing 10% BVDV-free foetal bovine serum (FBS) (Sigma-Aldrich, UK), 1% glutamine and 1% antibiotic/antimycotic (Life Technologies, UK). The non-cytopathic BVDV type-1a strain Ky1203, the cytopathic NADL-1a strain [2] and non-cytopathic 456497-1a were propagated in MDBK cells. COS-7 cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% FBS, 2 mM l-glutamine, and 1% antibiotic/antimycotic.

2.2. Plasmids cloning

The antibiotic-free selection NTC838 series expression vectors utilised herein contain a 150 bp RNA-based selectable marker (RNA-OUT) that confers sucrose resistance onto Escherichia coli strain NTC4862 (DH5α-derived with chromosomally integrated RNA-IN-SacB) by suppression of SacB (levansucrase) translation. This enabled antibiotic-free plasmid selection in sucrose containing media [18]. The codon-optimised (co) E2t and NS3 genes were designed using highly expressed bovine gene codon usage and synthesised by GeneArt. The synthetic genes were cloned into the eRNA41H RIG-I agonist-encoding NTC8382-eRNA41H VA2 vector [15] to make the NTC E2t(co) and NTC NS3(co) vectors (Fig. 1). A tissue plasminogen activator (tPA) signal sequence was included in the E2t vector, to provide a signal sequence for secretory E2t expression. The NS3 gene was expressed without a secretory signal since it is an internal protein. The parent NTC8382-eRNA41H VA2 vector without transgene was used as a mock vaccine. The NTC8382-eRNA41H VA2 derived vectors were produced in NTC4862 using antibiotic-free, sucrose media selection. All inserts and vaccine clones were confirmed by sequencing. For DNA vaccination, 10–20 mg of

endotoxin-free plasmid DNA of each clone was produced and formulated in sterile water. Plasmids expressing native viral E2 truncated (pSecTag/E2) and NS3 (pTriExNS3) are described previously [4], [5], [6], [7].

2.3. Transfection and immunostaining

COS-7 cell were seeded in a 24-well plate (5 × 105 cells/ml), and grown to 70–80% confluence prior to transfection. Plasmid DNA expressing NTC E2t(co), NTC NS3(co), pSecTag/E2 or pTriExNS3 were transfected using lipofectamine LTX (Life Technologies) as described before [19]. Cells were immunostained for BVDV as detailed previously [19].

2.4. Experimental design

2.4.1. DNA vaccination

All animal procedures were approved by the local ethical review process and met all those requirements of the Home Office (Scientific procedures) Act 1986 under their code of practice. Twenty five mixed breed calves (5–8 months old) were sourced and confirmed BVDV antibody- and antigen-free prior to the study. Calves were housed in a purpose built barn with biosecurity measures in place to avoid possible exposure to adventitious pathogens. Calves were allocated to five treatment groups according to random block design based on age. All vaccines were administered intramuscularly at a dose of 500 μg in 4 ml PBS. Treatment groups were as in Table 1. Animals received 3 vaccinations (V1,V2,V3) at 21 days intervals.

Treatment group	**Vaccine formula**	**Vaccine type**
Group 1	NTC NS3(co)	Candidate vaccine
Group 2	NTC NS3(co) + NTC E2t(co)*	Candidate vaccine
Group 3	NTC E2t(co)	Candidate vaccine
Group 4	pTriExNS3 + pSecTag/E2*	Positive control
Group 5	Parent NTC8382-eRNA41H VA2	Mock vaccine (vector without transgene)

* “+” Indicates co-administration of two vaccine formulas.

2.4.2. Virus challenge and sampling

Pre-challenge (baseline) measurements of rectal temperatures and haematological profiles commenced on study day -2, 1 and on challenge day (day 0). On day 0, all animal were challenged with 10 ml dose containing 2.39 x10^6 $TCID_{50}$ of early passage of field isolate 456497 (BVDV type 1a strain) which was subsequently back titrated after challenge (1 x 10^6/ml). Challenge inoculum was instilled into the nares upon inspiration. Thereafter, measurements of all parameters were made daily until study day 14 (except day 1 and 2), with a final time point on day 21. Rectal temperatures were taken daily, until day 10, and EDTA blood samples for haematology until day 14. EDTA blood samples were processed using an automated haematological analyser (Cell Dyn, Abbot) to determine total and differential leucocyte counts. Lymphocytes, neutrophils and monocytes were all quantified in the differential count. Nasal swabs and additional blood samples were taken on each occasion for virus isolation and for serology on day 0, 3, 5, 6, 7, 10, 14 and 21. All calves were weighed and euthanised on study day 28 post challenge.

2.5. Virus isolation from buffy coat samples and nasopharyngeal swabs

All EDTA blood samples taken for virology from day 0 onwards were prepared by extracting and washing the total white blood cell pellet. The presence of virus was then determined in these samples after dilution in 1 ml maintenance medium. Briefly samples were inoculated in duplicate onto freshly prepared FBL cells in 24-well plates and incubated for 5 days. After freeze thaw and dilution of material the inoculum was passed once more onto cells seeded on sterile glass coverslips and incubated for a further 5 days. Presence of virus was detected via immunofluorescence staining using a hyperimmune polyclonal bovine anti-BVDV serum and a Cy-3-conjugated anti-bovine antibody (Stratech, UK). Scoring was performed using a fluorescence microscope and appropriate filter sets. To detect BVD virus in nasopharyngeal swabs, the samples were diluted (1:5) in maintenance medium (Medium Essential Medium containing 2% foetal calf serum and antibiotic/antimycotic solution) and then inoculated, in duplicate, onto freshly prepared FBL cells in 24-well plates and incubated for 5 days at 37 ºC. After one round of freeze-thaw and dilution of material, the inoculum was passed once more onto fresh FBLs and incubated for a further 5 days. Presence of virus was detected via immunofluorescence staining as described previously [20].

2.6. Serology

Levels of neutralising antibody to homologous (Ky1203nc) and heterologous (NADL) were established as described before [20]. The results are expressed as the reciprocal of the serum dilution at which 50% of the virus was neutralised. Detection of antibody response to whole BVDV virus, in serum was performed using indirect ELISA (23) with some modification. The E2 specific antibody ELISA, using purified E2 protein, was performed as described previously [19]. Levels of antibody to E2 protein were determined as ELISA optical density values (OD) where cut off was considered 0.5 (mean OD of mock vaccine sera at day 0 + 3SD). Anti-NS3 specific antibody response was performed using the PrioCHECK commercial Kit following the manufacturer protocol where results are expressed as percentage of inhibition, inhibition values of $\geq$ 50 were considered positive.

2.7. Statistical analysis

Antibody responses were compared between groups using analysis of variance (ANOVA) techniques and between two groups using t test. All tests were carried out using GraphPad™ Software. Statistical significance was claimed at $p < 0.05$.

3. Results.

3.1. Plasmid cloning and in vitro expression of viral antigen proteins

Bovine codon optimized E2t (co) and NS3(co) synthetic genes were cloned into a RIG-I agonist encoding, antibiotic free selection vector NTC8382-eRNA41H-VA2 [15] **(Figure 1)**. Successful cloning of E2t(co) and NS3(co) was evident by in vitro expression in COS-7 cells. Diffuse fluorescence was observed in the cytoplasm in cells transfected with plasmids expressing E2t or NS3 when immunostained with the BVDV hyperimmune serum (Fig. 2). These results confirm that E2t and NS3 proteins were expressed.

3.2. Clinical and haematological observations

3.2.1. Rectal temperature

The pattern of pyrexia observed was characteristic of BVDV infection. The mean rectal temperatures (°C) of each vaccine group are presented in Fig. 3A. The pre-challenge (day −2 to 0) temperatures were similar for all groups. The mean group temperature values remained within the normal range (38.5–39.5 °C) throughout the study except for a transient rise recorded in all groups on day 8 with the highest mean temperature noted in vaccine group

(NTC E2t(co)). No significant difference was observed between vaccine groups and the mock group.

3.2.2. Haematology

A typical leucopenia was observed from day 3 post-challenge in all groups with no significant difference between vaccine groups and the mock group (Fig. 3B). The latter had 51.1% reduction against baseline leucocyte counts compared to a 38.1% reduction in NTC NS3(co) vaccine group, 38.3% and 36.6% in NTC NS3(co) + NTC E2t(co) and NTC E2t(co) vaccine groups, respectively. The positive control group however had 46.5% reduction in leucocytes counts. Following from day 3 post-challenge, leucocytes counts started increasing although a reduction was seen also on day 7 in NTC E2t(co) group. However by day 10, blood counts returned to approximately near pre-challenge values in all groups.

3.3. Antibody response

Specific antibody response to BVDV E2 and NS3 antigens following vaccination and challenge were determined by ELISA (Fig. 4).

3.3.1. Anti-NS3 antibody response

On day of challenge, sero-conversion to NS3 was evident in 3 calves in NTC NS3(co) + NTC E2t(co) group, but not in NTC NS3(co) group (Fig. 4A). However, by day 14 post-challenge anti-NS3 antibodies were detected in all calves in NTC NS3(co) group, 4 calves in NTC NS3(co) + NTC E2t(co), in one calf in NTC E2t(co) and pTriExNS3 + pSecTag/E2 compared to 2 calves in the mock group. NS3-seroconversion continued to rise to 100% by day 28 in all animals vaccinated with NTC NS3(co) or mock vaccine and to 80% in pTriExNS3 + pSecTag/E2 group.

3.3.2. Anti-E2 antibody response

On challenge day, 3 calves in each of vaccine groups NTC NS3(co) + NTC E2t(co) and NTC E2t(co) had significantly detectable level of anti-E2 antibody compared to other groups (p value < 0.05). By 7 days post-challenge, anti-E2 sero-conversion in these 2 groups increased to 4/5 and 5/5 calves, respectively. This level continued to rise in all 10 animals and sustained until end of the study (Fig. 4B).

Delay in antibodies to E2 was seen in pTriExNS3 + pSecTag/E2 group, where sero-conversion was detected 7 days post-challenge in 3/5 calves and in all calves by 14 days.

Anti-E2 antibody response was detected at low prevalence in NTC NS3(co) and mock groups by day 14 post-challenge and increased to 4 and 5 calves, respectively, albeit at a lower level, by 28 days.

3.3.3. Neutralising antibody

3.3.3.1. Homologous neutralising response

By challenge day, all calves vaccinated with NTC NS3(co) + NTC E2t(co) or NTC E2t(co) had generated neutralising titres (mean: >493) to the homologous virus compared to 4/5 calves in pTriExNS3 + pSecTag/E2 (mean: >285) (Tables 2 - 3). The levels of neutralising titres in these groups continued to rise throughout reaching high titres (≥10,240) in all animals. In NTC NS3(co) and mock groups however, neutralising antibodies were detected only at day 14 at lower titres (80–1280 and 80–905, respectively) and continued to rise to the end of the study reaching the highest titre of 5120 and 1810 in some of these animals, respectively.

3.3.3.2. Heterologous neutralising response

Heterologous neutralising antibody titres to NADL strain were also detected on challenge day in all E2-vaccinated calves except one calf in pTriEx/NS3 + pSecTag/E2 group (Tables 2-3). The titres were higher in the group vaccinated with NTC E2t(co) with 3 calves showing ≥640. These titres continued to rise throughout to >640 in all calves by day 14 post challenge. In NTC NS3(co) and mock groups however, heterologous neutralising titres were detected only 14 days post-challenge (40–≥640 and 20–≥640, respectively).

3.4. Virus isolation

Virus isolation from nasal swab and buffy coat samples was undertaken. All calves were negative for virus on the day 0. Virus was recovered from nasal swab samples between days 5–7. In the mock group, infectious virus was first isolated on day 5 from one calf and on day 7 from 4 calves. In the vaccinated groups however, infectious virus was isolated from the

nasal swabs on days 5–7 from only 1 or 2 calves including pTriExNS3 + pSecTag/E2 group, although in one calf vaccinated with NTC NS3(co) the virus was detected on day 3. The results from buffy coat samples showed that infectious virus was isolated only in one calf from the mock group on day 6. This animal remained viraemic on days 7 and 10. No virus was isolated from buffy coat samples from vaccine groups except from one calf (day 5) vaccinated with NTC E2t(co).

4. Discussion

Increasing levels of plasmid vector-mediated activation of innate immune signalling pathways is an approach to improve DNA vaccine-adaptive immunity. In this study we aimed to incorporate the RIG-1 agonist eRNA41H into plasmid vector expressing E2 or NS3 BVDV antigens as part of an ongoing effort to develop an effective marker DNA vaccine for BVDV. The incorporation of RIG-1 agonist was used to enhance the humoral immune response of an influenza HA DNA vaccine [15]. In other study, RIG-I agonist have been used as adjuvant in combination with influenza virus inactivated vaccine resulting in enhanced anti-influenza HA-specific IgG. This adjuvant was equally effective at increasing the efficiency of an influenza inactivated vaccine as a poly(I·C)- or a squalene-based adjuvant [21]. The adjuvant effect of RIG-I agonist was further demonstrated in a recent study in mice when co-administering with inactivated influenza vaccine led to a robust HA antibody response and protection against virus challenge [22]. In the present study, we generated DNA vaccines based on E2 and NS3 with a DNA backbone containing RIG-I agonist and designed in compliance with Food and Drug Administration (FDA) regulatory guidance's [16], [18], [23]. Additionally, taking into account the merit of codon optimisation to host sequence, all vaccines encoding proteins were codon optimised to Bos taurus where their immunogenicity and protective efficacy were assessed in calves.

The selection of E2 and NS3 proteins as antigen targets for a BVDV vaccine has been established previously. Our own studies and that of others tested the efficacy of E2-based vaccines and showed an encouraging level of partial protection from BVDV challenge [6], [20], [24], [25], [26], [27], [28], [29]. Vaccination of cattle with the DNA vaccine expressing the highly immunogenic E2 induces protective immunity [6]. We have previously shown that immunity to BVDV in cattle can be augmented by vaccination with the highly immunogenic NS3 protein [7].

Transient leucopenia and biphasic febrile response, with few overt signs of infection are typical feature observed in susceptible healthy cattle following challenge with BVDV-1 virus. The virus replicates in the nasal epithelium before spreading to the tonsils [30]. Thereafter, virus dissemination occurs through the blood and lymphatic system, with virus being isolated from the blood around 3 days post-challenge. A viraemic period of 7–10 days is normally observed.

In this study, a rise in rectal temperature above the normal range was recorded in all groups on day 8 post-challenge and returned to normal range by day 9. There was no difference in transient pyrexia between vaccinated animals and the mock group.

Transient leucopenia was observed by three days post-challenge in all vaccinated groups and mock group. In NTC E2t(co) vaccine group, leucopenia was evident on day 7 but returned to pre-challenge level by day 10 (Fig. 3B). In NTC NS3(co) + NTC E2t(co) and the positive control group, white blood counts returned to near pre-challenge values by day 10. In contrast, although not significant, leucopenia was more pronounced in mock vaccine group on day 3 post-challenge with no difference to vaccine groups on day 6–7, returning to near pre-challenge values by day 10.

Prevention of viraemia is a key parameter for a BVDV vaccine, since viraemia in a pregnant animal may result in vertical transmission of infectious virus to the foetus [31], [32]. In this study reduction in virus isolation observed in nasal swab samples from NTC NS3(co) + NTC E2t(co) group is promising where BVDV was isolated from 2/5 calves for one day only. These results were equivalent to that of the positive control group (ptriExNS3 + pSecTag/E2) and comparable to partial protection achieved in our previous studies [6], [7]. Similarly, in calves vaccinated with NTC E2t(co) alone, the virus was detected in 2 calves but for one and two days. In NTC NS3(co), however the virus was isolated from all calves for one or two days highlighting the importance of the role of the immune response to E2 in the control of BVDV infection. The virus was isolated from 4/5 calves in the mock group.

The results of virus isolation from buffy coat showed the virus can only be isolated on day 5 from one calf vaccinated with NTC E2t(co) whereas no virus was isolated in any of other animals in this group or from any of the other vaccine groups. However, the results of BVDV isolation from the mock group was rather disappointing where the virus was isolated from only one animal for three days (6, 7 and 10).

One of the key characteristic features of vaccine efficacy is the induction of sufficient humoral immune response to afford protection. As such, the levels of antibody response generated against BVDV proteins and virus neutralising antibody response were assessed. High levels of anti-NS3 antibodies were observed in all NS3-containing vaccines. Anti-NS3 antibody response was evident on challenge day when NTC NS3(co) vaccine was co administered with NTC E2t(co) and not in NTC NS3(co) group suggesting that E2 augment immune response to NS3. Overall vaccination with NTC NS3(co) in combination with or without E2t(co) induced higher anti-NS3 antibody response as compared to pTriExNS3 + pSecTag/E2 (Fig. 4A). These results highlight the effect of RIG-I agonist in enhancing the immune response to NS3. The effect of RIG-I agonist was also clear at the level of immune response to E2 protein. Animals vaccinated with NTC E2t(co) alone or co-administered with NTC NS3(co) induced anti-E2 antibody response on the challenge day whilst no response was seen in any of the positive or mock groups. The anti-E2 antibody level increased post-challenge including the positive control group but the response remained lower in the latter group. Overall, NTC NS3(co) and NTC E2t(co) vaccines primed the immune response to the challenge virus. We showed that immune response to NS3 was augmented when NTC NS3(co) was administered with NTC E2t(co) suggesting RIG-I agonist enhanced to the antibody response to BVDV.

Studies have shown that passive antibody provides protection against systemic BVDV infection [33], [34]. Virus neutralising antibodies have proven to be partially protective at a titre of 1/160 and fully protective at a titres of 1/240 [33]. The levels of neutralising antibody titres raised against homologous and heterologous strains were assessed. On challenge day, both vaccine groups (NTC E2t(co) and NTC NS3(co) + NTC E2t(co)) had neutralising titre >240 in 4/5 animals with the remaining animal >160. In contrast, only one calf had neutralising titre >240 in pTriEx NS3 + PSecTag/E2 vaccinated group. These results coincided with the high E2-antibody response in these two vaccine groups suggesting the importance of RIG-I agonist. The neutralising response continued to rise in both vaccine groups containing NTC E2t(co) and pTriExNS3 + pSecTag/E2 reaching >7.6 and >6.3 fold increase, respectively, to that induced in the mock vaccine 3 weeks post-challenge. In the NTC NS3(co) vaccine group, neutralising titre was 1.5 fold higher than in the mock group.

Heterologous neutralising antibody response was also evident in this study. On challenge day, 3/5 and 2/5 animals in NTC E2t(co) and NTC NS3(co) + NTC E2t(co) groups, respectively, had >240 titres compared to one calf at 160 titre in pTriEx NS3 + pSecTag/E2 group. The

heterologous response was primed 7 days post-challenge but remained higher in NTC E2t(co)-containing vaccines (mean titre: >427 and >493) than in pTriExNS3 + pSecTag/E2 group (mean titre: >288).

In conclusion, this study endorsed the importance of RIG-I agonist incorporation in DNA vaccine backbone to enhance vaccine potency in large animals including cattle. Investigation of the cytokine profiles induced by the DNA vaccine incorporating BVDV antigen and RIG-I agonist, vaccine dose and delivery routes warrant further study to achieve complete protection.

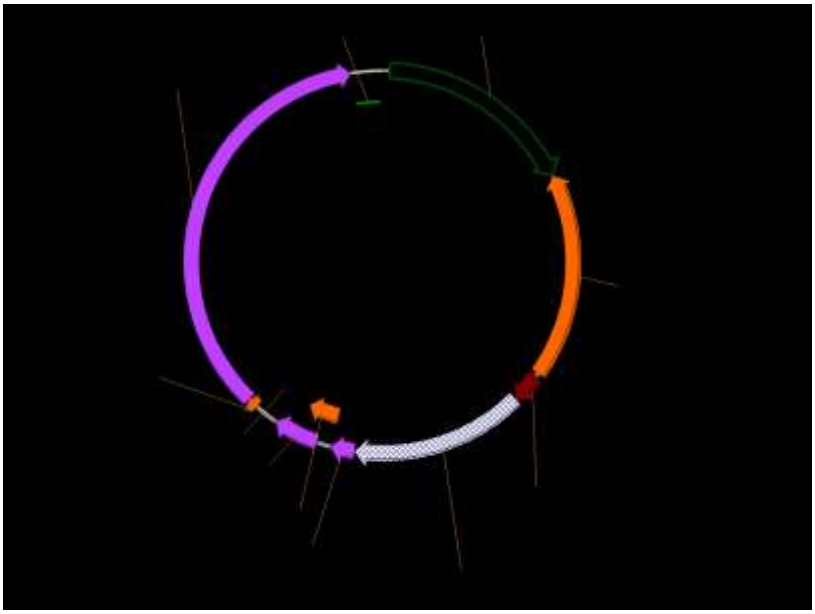
Acknowledgement

The authors gratefully acknowledge Novartis Animal Vaccines Limited for the financial support.

List of Figures

Figure 1. Antibiotic-free vectors co-expressing a RIG-I agonist (immunostimulatory RNA) with BVDV NS3 antigen (A) and BVDV E2t antigen (B)

(A)



(B)

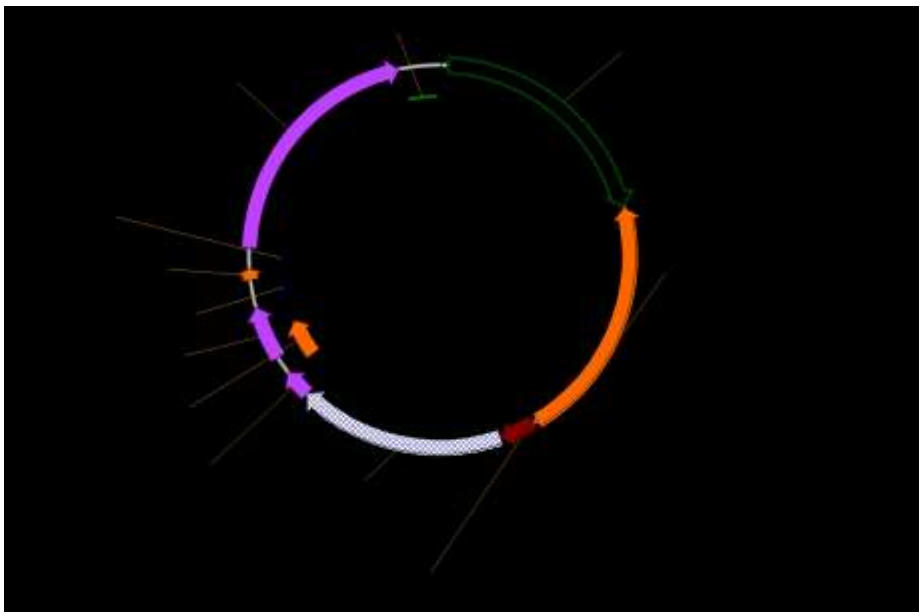


Figure 2. *In vitro* expression of E2 and NS3 viral antigens in COS-7 cells by different DNA vaccine vectors. Transfected cells were treated with BVDV hyperimmune polyclonal serum and indirect immunofluorescence staining. Diffuse cytoplasmic staining was observed in cells transfected with vaccines expressing codon and non-codon optimised E2t or NS3. Granular fluorescence staining was also observed in cells transfected with vaccine expressing E2t, suggesting an association of the expressed protein with the cell membranes.

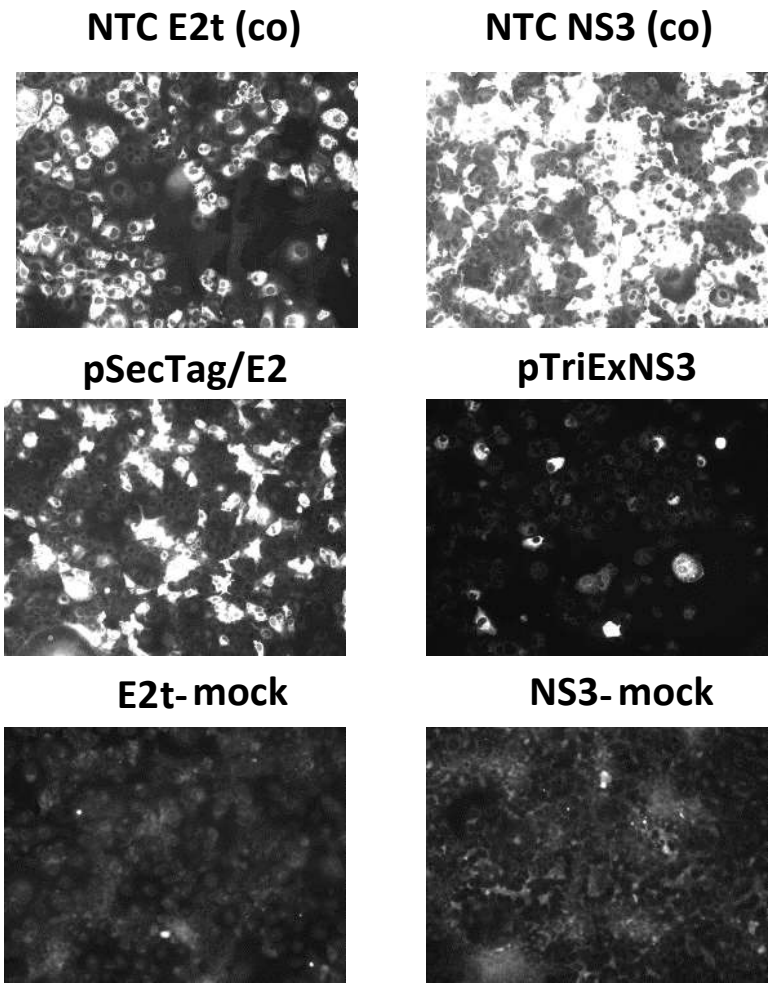
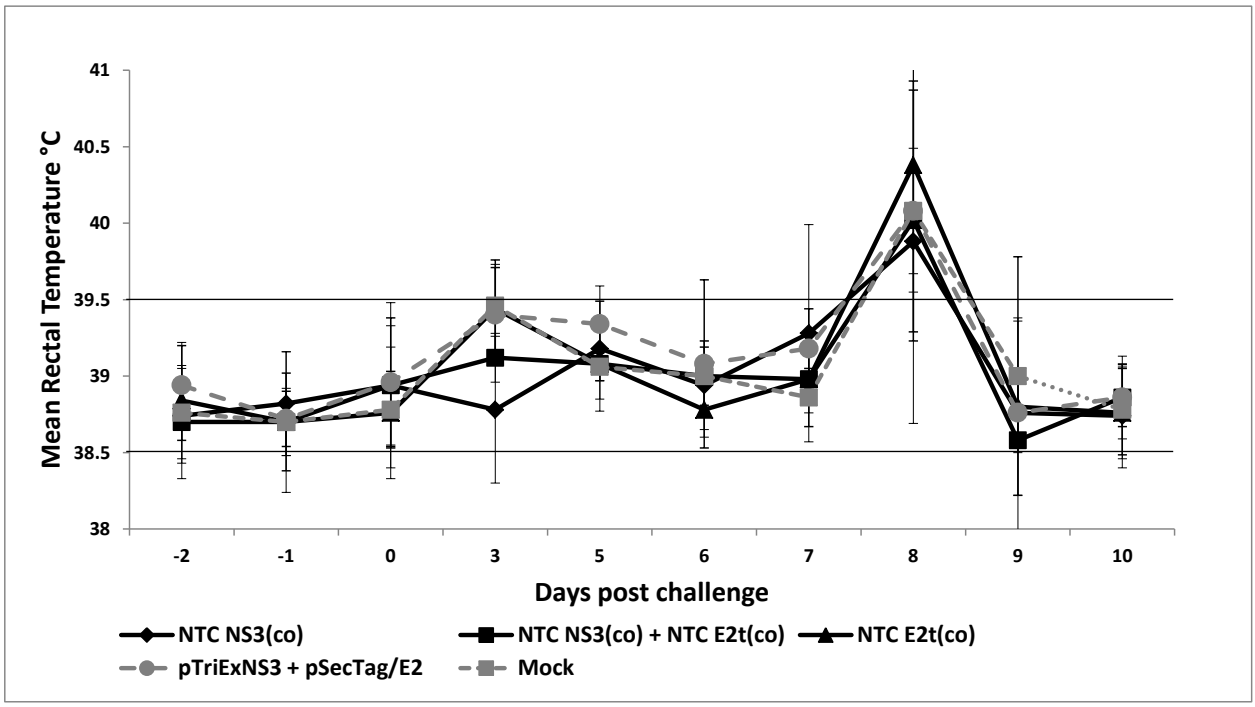


Figure 3. Clinical manifestation (rectal temperature and leucopenia fluctuation) following BVDV challenge (day 0) of calves vaccinated with NTC NS3(co), NTC NS3(co) + NTC E2t(co), NTC E2t(co), pTriExNS3 + pSecTag/E2 or mock vaccine. (A) Mean rectal temperature ± SD (B) Mean total leucocyte counts expressed as a percentage of pre-challenge values.

(A)



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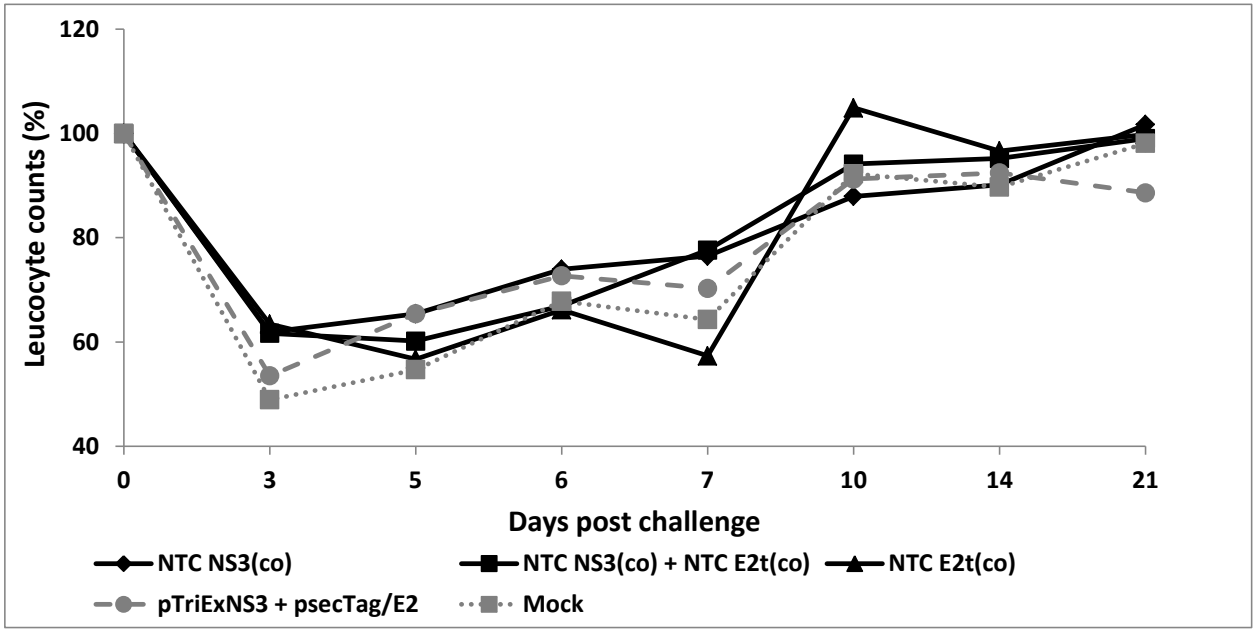
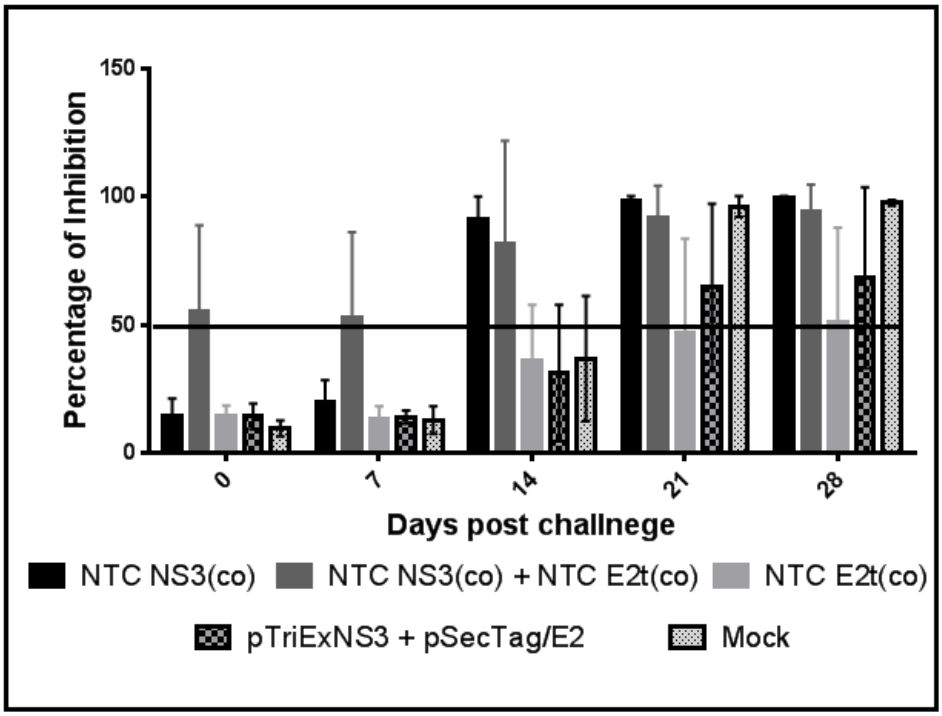


Figure 4. Serum antibody responses after challenge (day 0) in calves vaccinated three times; with NTC NS3(co), NTC NS3(co) +NTC E2t(co), NTC E2t(co), pTriExNS3 + pSecTag/E2 or mock vaccinated. Data shows (A) anti-BVDV NS3-antibody response presented as Percentage of Inhibition (PI), where PI ≥ 50 considered positive, (B) anti-E2 antibody response, where results are recorded as mean OD values at 1/500 (at $\log_{10}$ 2.69897) dilution. Bars represent ± SD.

(A)



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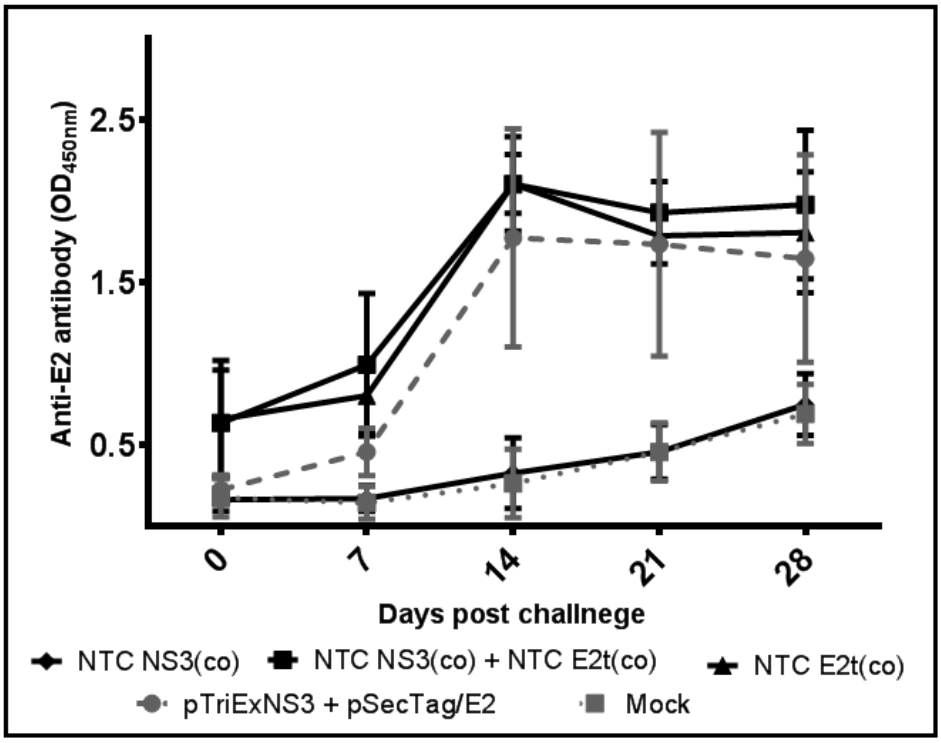

Table 2. Homologous neutralisation titres

Vaccine	Calf ID	Days post challenge				
		Day V3	Day 0	Day 7	Day 14	Day 21
NTC NS3(co)	558	<10	<10	<40	226	1280
	575	<10	<10	<40	905	1810
	576	<10	<10	<40	160	1280
	590	<10	<10	<40	80	640
	606	20	<10	<40	1280	5120
NTC NS3(co) + NTC E2t(co)	564	>640	>640	>2560	>2560	>10240
	567	<10	320	1280	>2560	>10240
	579	<10	226	320	>2560	≥10239
	595	160	>640	>2560	>2560	≥10239
	605	113	≥640	>2560	>2560	>10240
NTC E2t(co)	560	<10	>640	1280	>2560	>10240
	570	<10	453	905	>2560	>10240
	583	320	>640	>2560	905	≥10239
	593	<10	226	1280	>2560	>10240
	602	453	≥640	2560	>2560	>10240
pTriExNS3+ pSecTag/E2	563	<10	226	226	>2560	≥10239
	568	<10	160	640	>2560	>10240
	581	<10	113	1280	>2560	>10240
	588	<10	<10	<40	453	1810
	607	453	≥640	905	>2560	>10240
Mock	565	<10	<10	<40	905	905
	566	<10	<10	<40	80	1810
	574	<10	<10	<40	113	453
	586	<10	<10	<40	160	1810
	596	<10	<10	<40	640	1810

Table 3. Heterologous neutralising titres

Vaccine	Calf ID	Days post challenge			
		Day V3	Day 0	Day 7	Day 14
NTC NS3(co)	558	<10	< 10	<10	320
	575	<10	<10	<10	80
	576	<10	<10	<10	>640
	590	<10	<10	<10	40
	606	<10	<10	<10	640
NTC NS3(co) + NTC E2t(co)	564	226	453	>640	>640
	567	10	80	453	>640
	579	<10	20	80	>640
	595	<10	453	640	>640
	605	40	113	320	>640
NTC E2t(co)	560	57	>640	>640	>640
	570	<10	160	227	>640
	583	56	>640	>640	>640
	593	10	113	320	>640
	602	113	≥640	>640	>640
pTriExNS3 + pSecTag/E2	563	<10	20	20	>640
	568	<10	40	453	>640
	581	<10	28	>640	>640
	588	<10	<10	<10	>640
	607	80	160	320	>640
Mock	565	<10	<10	<10	40
	566	<10	<10	<10	640
	574	<10	<10	<10	20
	586	<10	<10	<10	226
	596	<10	<10	<10	>640

Table 3. Virus isolation from buffy coat and nasal swabs following virus challenge (day 0) with 2.39 x10^6 $TCID_{50}$ of the BVDV field isolate 456497. (+) indicates virus isolation, (-) indicate no virus was isolated.

Vaccine	Calf ID	Buffy coat						Nasal swabs					
		Days post challenge						Days post Challenge					
		0	3	5	6	7	10	0	3	5	6	7	10
NTC NS3(co)	558	-	-	-	-	-	-	-	+	-	-	-	-
	575	-	-	-	-	-	-	-	-	-	+	-	-
	576	-	-	-	-	-	-	-	-	+	-	-	-
	590	-	-	-	-	-	-	-	-	-	+	+	-
	606	-	-	-	-	-	-	-	-	-	-	+	-
NTC NS3(co) + NTC E2t(co)	564	-	-	-	-	-	-	-	-	-	-	+	-
	567	-	-	-	-	-	-	-	-	-	-	-	-
	579	-	-	-	-	-	-	-	-	-	-	-	-
	595	-	-	-	-	-	-	-	-	-	+	-	-
	605	-	-	-	-	-	-	-	-	-	-	-	-
NTC E2t(co)	560	-	-	-	-	-	-	-	-	-	-	-	-
	570	-	-	-	-	-	-	-	-	-	+	+	-
	583	-	-	-	-	-	-	-	-	-	+	-	-
	593	-	-	-	-	-	-	-	-	-	-	-	-
	602	-	-	+	-	-	-	-	-	-	-	-	-
pTriExNS3 + pSecTag/E2	563	-	-	-	-	-	-	-	-	-	+	-	-
	568	-	-	-	-	-	-	-	-	-	-	-	-
	581	-	-	-	-	-	-	-	-	-	-	-	-
	588	-	-	-	-	-	-	-	-	+	-	-	-
	607	-	-	-	-	-	-	-	-	-	-	-	-
Mock	565	-	-	-	-	-	-	-	-	-	-	+	-
	566	-	-	-	-	-	-	-	-	-	-	-	-
	574	-	-	-	-	-	-	-	-	-	-	+	-
	586	-	-	-	-	-	-	-	-	-	-	+	-
	596	-	-	-	+	+	+	-	-	+	-	+	-

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