Veterinary Research Communications

Safety and efficacy of an E2 glycoprotein subunit vaccine produced in mammalian cells to prevent experimental infection with bovine viral diarrhoea virus in cattle
--Manuscript Draft--

Manuscript Number:	VERC2968R1
Full Title:	Safety and efficacy of an E2 glycoprotein subunit vaccine produced in mammalian cells to prevent experimental infection with bovine viral diarrhoea virus in cattle
Article Type:	Original Article
Keywords:	BVDV; subunit vaccine; vaccine potency
Corresponding Author:	Andres Wigdorovitz, Ph.D INTA Hurlingham , Pcia Buenos Aires ARGENTINA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	INTA
Corresponding Author's Secondary Institution:	
First Author:	Andrea Pecora
First Author Secondary Information:	
Order of Authors:	Andrea Pecora
	Maria Sol Pérez Aguirreburualde
	Alejandra Aguirreburualde
	Maria Rosa Leunda
	Anselmo Odeon, PhD
	Sebastián Chiavenna, PhD
	Diego Bochoeyer, PhD
	Marcelo Spitteler
	Jorge L Filippi
	Maria José Dus Santos, PhD
	Susana M Levy, PhD
	Andres Wigdorovitz, Ph.D
Order of Authors Secondary Information:	
Abstract:	Bovine viral diarrhea (BVD) infection caused by bovine viral diarrhea virus (BVDV), a Pestivirus of the Flaviviridae family, is an important cause of morbidity, mortality and economical losses in cattle worldwide. E2 protein is the major glycoprotein of BVDV envelope and the main target for neutralising antibodies (NAbs). Different studies on protection against BVDV infection have focused on E2, supporting its putative use in subunit vaccines. A truncated version of type 1a BVDV E2 (tE2) expressed in mammalian cells was used to formulate an experimental oleous monovalent vaccine. Immunogenicity was studied through immunisation of guinea pigs and followed by trials in cattle. Calves of 8-12 months were vaccinated, twice with a 4 week interval, with either a tE2 subunit vaccine (n= 8), a whole virus inactivated vaccine (n= 8) or left untreated as negative control group (n= 8). Four weeks after the last immunisation the animals were experimentally challenged intranasally with a non-cytophatic BVDV strain. Following challenge, BVDV was isolated from all unvaccinated animals, while 6 out of 8 animals vaccinated with tE2 showed complete virological protection indicating that the tE2 vaccine presented a

	similar performance to a satisfactory whole virus inactivated vaccine.

Safety and efficacy of an E2 glycoprotein subunit vaccine produced in mammalian cells to prevent experimental infection with bovine viral diarrhoea virus in cattle

Pecora, Andrea[1]; Pérez Aguirreburualde, María Sol[1]; Aguirreburualde, Alejandra[2]; Leunda, Maria Rosa[3]; Odeon, Anselmo[3]; Chiavenna Sebastián[1]; Bochoeyer, Diego[2] Spitteler, Marcelo [2.]; Filippi, Jorge L.[2]; Dus Santos, Maria J.[1]; Levy, M. Susana[2]; Wigdorovitz, Andrés[1#].

(1) Instituto de Virología, CICVyA, INTA-Castelar Buenos Aires, Argentina.

(2) Biogénesis-Bago SA, Buenos Aires, Argentina.

(3) EEA Balcarce INTA, Argentina.

corresponding author. Tel: +5411 4621 1676; fax: +5411 4621 1743

E-mail address: awigdorovitz@cnia.inta.gov.ar

Abstract

Bovine viral diarrhea (BVD) infection caused by bovine viral diarrhea virus (BVDV), a Pestivirus of the *Flaviviridae* family, is an important cause of morbidity, mortality and economical losses in cattle worldwide. E2 protein is the major glycoprotein of BVDV envelope and the main target for neutralising antibodies (NAbs). Different studies on protection against BVDV infection have focused on E2, supporting its putative use in subunit vaccines. A truncated version of type 1a BVDV E2 (tE2) expressed in mammalian cells was used to formulate an experimental oleous monovalent vaccine. Immunogenicity was studied through immunisation of guinea pigs and followed by trials in cattle. Calves of 8-12 months were vaccinated, twice with a 4 week interval, with either a tE2 subunit vaccine (n= 8), a whole virus inactivated vaccine (n= 8) or left untreated as negative control group (n= 8). Four weeks after the last immunisation the animals were experimentally challenged intranasally with a non-cythopathic BVDV strain. Following challenge, BVDV was isolated from all unvaccinated animals, while 6 out of 8 animals vaccinated with tE2 showed complete virological protection indicating that the tE2 vaccine presented a similar performance to a satisfactory whole virus inactivated vaccine.

Key words: BVDV, E2, subunit vaccine, vaccine potency

Introduction

Bovine viral diarrhea (BVD) infection caused by bovine viral diarrhea virus (BVDV), a Pestivirus of the *Flaviviridae* family, is an important cause of morbidity, mortality and economical losses in cattle worldwide. Seroepidemiological studies have shown that most of the cattle population is infected with BVDV during their lifetime (Bolin, 1995). BVDV infections are associated with fertility problems, immunosuppression, diarrhea,

1

thrombocytopenia, and, frequently, unapparent courses (Thiel, 1996). Transplacental infection can lead to abortion, stillbirth, malformation, and persistent infection of the calves. Persistently infected (PI) calves may develop fatal mucosal disease when superinfected with a cytopathic biotype of the virus. Because they shed large amounts of virus, PI animals are the main viral reservoir and source of infection in cattle herds. Moreover, BVDV is a contaminant of bovine products and subproducts such as commercial fetal calf serum preparations; and susceptible mammalian cell lines are often inadvertently infected (Bolin et al., 1994).

Inactivated and modified-live vaccines (MLV) are used to protect cattle from BVDV infection. However, both types of vaccines have significant shortcomings. Production of BVDV inactivated vaccines is usually limited by the difficulties to obtaining enough amounts of viral antigen to induce high levels of neutralising antibodies (NAbs). In the case of MLV, the virus is a potential source of *in utero* infection and/or cause of immunosuppression (Liess et al., 1984; Roth and Kaeberle, 1983). Moreover, the efficacy of both types of vaccines can be interfered by maternal antibodies (Ellis et al., 2001).

On the other hand, subunit vaccines provide an opportunity to develop safe and efficacious vaccines, with the possibility of differentiating between vaccinated and infected animals, referred as "DIVA" vaccines (Differentiating Infected from Vaccinated Animals). However, the challenge is to produce a vaccine capable of eliciting an efficient immune response at an affordable cost for veterinary applications.

E2 is the major glycoprotein of BVDV envelope and the most immunogenic protein of this virus. NAbs induced in infected animals are mainly directed against E2. Sera from animals vaccinated with E2 neutralized several BVDV strains within a genogroup (Bolin et al., 1988). Moreover, it was demonstrated that NAbs raised against E2 prevented from infection with BVDV (Bolin, 1995; Toth et al., 1999). Thus, the use of immunodominant BVDV protein (E2) as a subunit vaccine may be a useful tool for the development of efficacious strategies to control the virus. Glycoprotein E2 has been successfully expressed in heterologous systems for vaccination purposes, such as baculovirus (Chimeno Zoth et al, 2007; Ferrer et al., 2007; Kweon et al., 1997), vesicular stomatitis virus (rVSV) (Kohl et al., 2007); Equine Herpesvirus type 1 (EHV-1) (Rosas et al., 2007); bovine herpesvirus (Donofrio et al., 2008; Schmitt et al., 1999), fowlpox vectors (Elahi et al., 1999), as well as mammalian cells (Thomas et al., 2009). The E2 gene has also been used in DNA immunisations (Couvreur et al., 2007; Liang et al., 2007; Liang et al., 2008).

In this work we evaluated the potency of a vaccine containing a truncated version of E2 (tE2), which lacks the 32 aminoacids of the transmembrane region and was developed previously in the laboratory, as the active ingredient. Results obtained indicated that titres of BVDV seroneutralising antibodies elicited by the subunit tE2 vaccine were comparable to those required for a satisfactory BVDV killed vaccine according to CFR (Code of Federal Regulations) 113.215. Furthermore, the vaccine also gave satisfactory results after a virus challenge test with a virulent strain of BVDV.

Materials and Methods

Vaccines and virus

2

Subunit tE2 vaccines were formulated to contain different amounts of recombinant protein -obtained from cultures of a CHO cell-line expressing a secreted version of BVDV type 1a E2- (Pecora et.al., 2009). BVDV inactivated virus vaccines used in trial 2 contained 2 μg/ml of E2 as measured by ELISA (see below). All vaccines were emulsified with ISA 50 (Biogénesis-Bagó S.A.) in 40:60 proportion. Vaccines were applied by the intramuscular route (im) in a dose of 0.6 ml for guinea-pigs and 3 ml for cattle.

The challenge virus, a noncytophatic BVDV 98/204 (isolated from INTA-Balcarce Experimental Unit), was propagated by infecting confluent monolayers of Madin-Darby Bovine Kidney (MDBK) cells, which were grown in MEM supplemented with 2% FBS. Titres were expressed as the inverse of the highest dilution able to inhibit $100TCID_{50}$.

Animals

Guinea pigs strain SSI:AL, 8-12 weeks old, were obtained from the animal care facilities of the CICVyA, INTA. Animals were checked for the absence of BVDV-specific antibodies by seroneutralisation (SN).

Aberdeen Angus bovines (1-3 years old) were obtained from the CICVyA experimental field units, INTA to carry out Trial 1. While, Aberdeen Angus calves, 8-12 month old and 160-200 kg weight were obtained from a farm in Buenos Aires province. All animals were checked for the absence of BVDV-specific antibodies by seroneutralisation test and BVDV antigen by RT-PCR.

ELISA for antigen quantification

A sandwich ELISA was used to detect and quantify the antigens (Pecora et al., 2009). Briefly, U-bottom polystyrene microplates (Maxisorp, NUNC) were coated with an anti-E2 mouse monoclonal antibody (Marzocca et al., 2007) and incubated at 4°C ON. After blocking the plates in PBS-T, 1% skimmed milk for 1 h at 37°C, samples were added at the corresponding dilution and incubated for 1 h at 37°C. As primary detection antibody, rabbit sera against tE2 (1:2000) were used and incubated for 1 h at 37°C. Thereafter, peroxidase-conjugated goat anti-rabbit IgG (1:1000) was used as secondary antibody, and incubated for 1 h at 37°C. Finally, plates were revealed using ABTS (Sigma) and optical densities (OD) were measured at 405 nm.

The standard curve was derived from CHO-K1-tE2 supernatant serially diluted in blocking buffer in order to obtain the following tE2 concentrations: 100, 50, 25, 12.5 and 6.25 ηg/ml. Calibrating samples were used to estimate the concentration of unknown samples.

Seroneutralisation assay

Serum NAbs from guinea pigs and cattle were detected by virus neutralisation assay. Briefly, $100 TCID_{50}$ of BVDV (NADL strain) were co-incubated for 1 h at 37°C with 75 µl of log_4 dilutions of inactivated serum samples. Then, the mixture was added onto 3×10^4 MDBK cells/well. Plates were incubated for 72 h at 37°C under 5% CO_2. Control wells without virus were used for each serum sample in order to discard toxicity.
. A BVDV positive serum from an experimentally infected bovine was used as positive control, and a BVDV negative serum from a reference animal was considered as negative control. Differences in NAb titres among

groups were evaluated by ANOVA under a model of repeated measures throughout time, followed by a general contrast post-ANOVA test.

Statistical significance was assessed at $p < 0.05$ for all comparisons.

Vaccination of Guinea Pigs

Six groups of guinea pigs (n=7) were immunised with i) 1 µg of concentrated tE2 (c-tE2), ii) 0.5 µg of c-tE2, iii 0.2 µg of c-tE2, iv) CHO-K1 tE2 supernatant containing 0.08 µg of tE2 (tE2SN), v) 1 µg of purified tE2 (p-tE2) and vi) immunize, a vaccine containing 10^7 $TCID_{50/ml}$ BVDV, NADL strain, as a positive control group. Two negative control groups (n=7) were inoculated with 1 µg of Lac Z and mock-infected MDBK cells supernatant, respectively. All groups were immunised with 2 doses of 0.6 ml each, on days 0 and 21. Sera were sampled on days 0, 30 and 60 days post inoculation (dpi).

Guinea pig handling, inoculation, and sample collection were done by trained personnel under the supervision of a veterinarian and in accordance to protocols approved by the INTA's ethical committee of animal welfare (CICUAE).

Vaccination of Cattle

Trial 1: Vaccines were formulated as described before using doses of 5, 1 and 0.2 µg of tE2 per animal. Groups of 3 BVDV-free bovines were immunised with 3 ml of each vaccine on days 0 and 30. Sera from all animals were weekly sampled until 60 dpv. A positive control group was immunised with a vaccine containing 10^7 $TCID_{50/ml}$ BVDV, NADL strain, and a negative control group was immunised with 5 µg of LacZ.

Trial 2: Groups of 8 BVDV-free bovines were immunised with 3 ml of each vaccine tE2; BVDV inactivated or LacZ . tE2 vaccine was formulated by Biogénesis-Bagó S.A. and contained 2 µg per dose. Positive control group was immunised as described in trial 1. Negative control group was immunised with 2 µg of LacZ.

Challenge of Cattle

The animals were firstly evaluated for the presence of BVDV specific NAbs, viral genome (by RT-PCR) and viral antigen using a commercial kit, BioK 258 (Bioxx). The trial was carried out blinded for personnel in the field and in the laboratory. Cattle management, inoculation, and sample collection were conducted by trained personnel under the supervision of a veterinarian and following the protocols from the INTA's ethical committee of animal welfare (CICUAE).

The immunised calves were housed under strict isolation conditions at an animal research facility (INTA Balcarce) and fed with standard diet. The viral inoculum consisted of 25 ml of 10^9 $TCID_{50}$ of BVDV strain 98/204 (type 1b), and was administered by inhalation with a nebulizer at 55 dpi. This time point was defined as day 0.

 Clinical observation.

 Animals were observed every day and the following parameters were recorded: rectal temperature, alertness, appetite, respiratory rate, body condition, and presence and characteristics of diarrhea.

4

Haematological analysis

This assay was carried out using an automated haematological analyzer at days 0, 2, 4 and 14 post-challenge. Total white blood cells as well as differential leukocyte counts were determined.

Viral isolation assays

The isolation of the virus was carried out on buffy coat samples collected at days 0, 2, 4, 7 and 14 post-challenge. After four passages in MDBK cells, viral antigens were detected by immunofluorescence assay using an anti-BVDV polyclonal antibody conjugated with FITC (VMRD). The results were confirmed by RT-PCR, using IA and IB oligonucleotides (IA: 5' GAGGCTAG CCATGCCCTTAGT 3', IB: 5' TCAACTCCA TGTGCCATGTACAGCA 3'), which amplify the fragment comprised between positions 98 and 402 of the non-coding 5' region reported for the NADL sequence (Pellerin et al., 1994).

Results

Serological Response to tE2 protein

Guinea pig´s response

Immunogenicity of the tE2 was firstly evaluated in a guinea pig model based on the association found between this experimental model and the natural host (Fernandez et al., 2009). Different amounts of tE2 (1, 0.5 and 0.2 µg) were used to formulate experimental vaccines. Following primoinoculation, the animals produced NAbs against BVDV NADL strain, and after the second dose the NAb titres reached > 1:128 in all the experimental doses evaluated (Figure 1). Results showed that animals immunised with only 0.5 µg of tE2 produced an antibody response which did not differ from that evoked by an inactivated BVDV vaccine. The negative control group inoculated with a non-related protein, LacZ, did not produce NAbs against BVDV.

Cattle´s response

Based on the results obtained in the experimental model, the efficacy of the tE2 subunit vaccine was evaluated in BVDV's natural host. In that regard, the optimal dose of antigen was assessed by immunising 3 groups of 3 bovines each with vaccines containing 5 µg, 1 µg and 0.2 µg of tE2 per dose. Animals were boosted 30 dpi. None of the vaccinated animals showed local reactions or adverse effects throughout the trial. Sera were evaluated for the presence of NAbs (figure 2). After primoimmunisation, calves vaccinated with 5 µg tE2 and an inactivated BVDV vaccine showed seroconvertion. At 60 dpi bovines vaccinated with 1 µg of tE2 showed mean antibody titres of 1:128, while animals vaccinated with either 5 µg of tE2 or inactivated BVDV developed NAb titres higher than 1:512.

Efficacy of tE2 in host animals

In the second cattle trial, which included a challenge test, seronegative calves were selected from a herd which had been monitored for the presence of BVDV since 2003 with negative results. Experimental groups included 8 bovines each that were immunised with 2 µg of tE2 per dose, following the same vaccination schedule of the previous experiments.

5

All assays included a positive control group that was immunised with a vaccine containing 10^7 TCID 50/ml of BVDV (NADL strain) and a negative control group was immunised with 2 µg of LacZ. At 15 dpi, early signs of a specific NAbs response were observed only in the group immunised with inactivated BVDV. However, by 30 dpv all vaccinated calves had seroconverted. Following the second vaccination, NAb titres increased to very high levels both in BVDV and tE2 immunised calves. At the time of challenge (t55) vaccinated animals presented specific NAb titres which did not significantly differ one from another (figure 3). In contrast, no NAbs were detected in the control group and remained so until 15 days post-challenge.

It is important to mention that at 35 dpi, all animals from vaccinated groups had developed SN antibody titres higher than 50 percent endpoint titre of 1:16, fulfilling the CFR requirement that at least 80% of vaccinated animals must present a titre of 1:16 or greater for a vaccine series to be considered satisfactory.

After challenge, NAb titres continued to rise in the vaccinated groups, while the negative control group showed seroconvertion. At the end of the trial, 30 days post challenge, all animals displayed high NAb titres.

Leukopenia

Post challenge, total leukocyte count fell sharply in all groups between days 1 and 3. Counts remained suppressed in all groups and recovered almost pre-challenge values by day 14. No statistical differences were apparent between the groups over time. Lymphocyte, neutrophil and monocyte count profiles showed similar patterns to those described for total leukocytes counts (figure 4).

Clinical disease

None of the animals developed respiratory or clinical signs of bovine virus diarrhea. By contrast, there was evidence of hyperthermia in all groups. In the control group, the mean temperature was moderately elevated on days 5-9, presenting average values over 40ºC. On the other hand, no animals from vaccinated groups showed rectal temperature over 40ºC for more than 2 days. Taken together these data indicate that the challenge test was valid and the vaccines evaluated can be considered satisfactory according to the CFR requirements (figure 3).

Vireamia

Virus was first isolated in the control group on day 2 post-challenge and was detected in all the control animals on at least one occasion until the day 9 post challenge. By contrast, BVDV was only detected in 2 out of eight calves vaccinated with tE2 and 1 out of eight animals immunised with the BVDV vaccine (Table 1). Results showed there was a reduction in viraemia as a result of vaccination.

Discussion

BVDV has a high detrimental effect in the livestock industry worldwide as it infects bovines during the whole productive period, causing a reduced milk production, reduced reproductive performance, and growth retardation, increased occurrence of other diseases, unthriftiness, early culling and increased mortality among

6

young stock. Economic losses due to BVDV infection can be prevented through vaccination programs accompanying measures to eliminate persistently infected immunotolerant animals. Current vaccines against BVDV are attenuated or inactivated and most of them are combination vaccines, containing BVDV and other viral agents. Attenuated or modified-live vaccines (MLV) are considered efficacious, but their safety is a matter of controversy. On the other hand, the available inactivated vaccines are safer than MLV, but the immune response induced by them is weak and short. At industry scale there is also a limitation on the production of inactivated vaccines related to obtaining enough viral antigen. Thus, there is a need for developing a new generation efficacious and safe vaccines. A subunit vaccine is safe in terms of horizontal and vertical transmission, and allows discrimination between vaccinated and infected animals. In this regard, we evaluated the tolerance and efficacy of a subunit vaccine based on glycoprotein E2, the most immunogenic structural protein of BVDV and the main target of NAbs (Yu et al, 1996).

The guinea pig model was used for preliminary evaluation of experimental vaccines containing tE2, since the comparison of the immune responses induced in bovines and guinea pigs when evaluating vaccines containing inactivated BVDV showed a high correlation (Fernandez et al., 2009).

The level of NAbs induced by vaccination is important for evaluating vaccine efficacy since it has been reported that titres of NAbs higher than or equal to 2 are critical for protection (Bolin, 1995).

At the beginning of the second trial, all animals were seronegative for BVDV. Vaccinated calves seroconverted after the first immunisation and showed high titres of NAbs at the time of challenge. Post challenge, NAb titres continued to increase in all vaccinated animals. In contrast, calves from the control group remained seronegative until 15 days post challenge.

A few BVDV recombinant subunit vaccines have been previously evaluated by others. Vaccination with a DNA vaccine encoding the full length E2 or a C-terminally truncated E2 was able to elicit moderate Ab responses and partial protection from BVDV challenge (Harpin et al., 1999; Nobiron et al., 2003) . Partial protection against BVDV was obtained when 3 doses of either 5 μg of E2 expressed in mammalian cells or 100 μg of E2 expressed in the baculovirus expression system were used to immunize cattle (Thomas et al., 2009). Similarly, another subunit E2 vaccine developed in SF9 cells failed to confer total protection in cattle after two immunizations with 50 μg of recombinant protein (Chimeno Zoth et al, 2007).

In another study, a DNA prime – protein boost strategy in calves was evaluated in calves and showed protection by in terms of weight loss, viral excretion and lymphopenia (Liang et al., 2007) .

In this work, leukopenia was evident in all groups, however a trend for the maintenance of higher number of lymphocytes, monocytes and neutrophils could be observed in vaccinated animals. This effect was also observed in the febrile response.

A reduction in PMBC-associated viraemia was observed in calves immunised with either tE2 or inactivated BVDV. 2 calves from tE2 group and 1 calf from the BVDV group shed detectable virus, while virus could be isolated from all animals of the unvaccinated group. Prevention of viraemia is a critical point when testing BVDV vaccine efficacy since viraemia in a pregnant animal is likely to result in vertical transmission of virus to the foetus.

Mechanisms underlying protection against BVDV infection are not completely understood, however, humoral immunity is considered important in controlling BVDV infection (Bolin and Ridpath, 1995). Free virus in

blood and lymph can be neutralised by NAb titres as low as of 1:2. Thus, the presence of NAbs and the effective priming of humoral memory appeared to be important factors in preventing and controlling BVDV infection.

Our results show that an E2 oleous subunit vaccine, applied in a 2 dose regime is able to elicit protective NAb titres. Furthermore, this subunit vaccine probed to be satisfactory according CFR requirements for BVDV killed virus vaccines. Further studies are required to establish the performance of the vaccine under field conditions and its use as a DIVA vaccine in the context of control and eradication programs.

Figure legends

Figure 1.. Dose response in guinea pigs.
Experimental groups (n=7) were immunised with oleous vaccines containing i) 1 µg ii) 0.5 µg iii) 0.2 µg of tE2 . At 30 and 60 days post inoculation (dpi) animals were bled and sera were evaluated by SN. A positive control group was immunised with a vaccine containing 10^7 TCID 50/ml BVDV, NADL strain. As negative controls 2 groups were immunised with mock or lacZ.

Figure 2. Dose response in cattle (trial 1)
Groups of bovines (n=3) were immunised with 5, 1 or 0.2 µg of tE2. At 30 and 60 dpi animals were bled and sera were evaluated by seroneutralisation assay. A positive control group was immunised with a vaccine containing 10^7 TCID 50/ml BVDV, NADL strain, and a negative control group was immunised with 5 µg of LacZ.

Figure 3. Efficacy trial with viral challenge (trial 2). .
Groups of bovines (n=8) were immunised following the same scheme as trial 1 and 2. At 55 dpi animals were challenged with type 1b BVDV as described in Materials and Methods. Solid arrows indicate times of vaccination and the dotted arrow indicates the day of challenge. Dotted line indicates the cut off established by CFR to consider a BVDV inactivated vaccine satisfactory.

Figure 4: Temperature and leukocyte fluctuations after viral challenge
Rectal temperatures were recorded and blood samples were taken on days 2, 4, 7 and 14. An automated haematological analyzer was used to determine total, lymphocyte, neutrophils and monocyte counts. (A) Rectal temperature. (B) Total number of leukocytes. (C) Number of lymphocytes. (D) Number of neutrophils. (E) Number of monocytes.

Ethical standards: the text must contain a declaration that the experiment comply with the current laws of the country in which they were performed

Conflict of interest
The authors declare that they have no conflict of interest.

References

Bolin, S., Moennig, V., Kelso Gourley, N.E., Ridpath, J., 1988, Monoclonal antibodies with neutralising activity segregate isolates of bovine viral diarrhea virus into groups. Brief report. Archives of virology 99, 117-123.

Bolin, S.R., 1995, Control of bovine viral diarrhea infection by use of vaccination. The Veterinary clinics of North America 11, 615-625.

Bolin, S.R., Ridpath, J.F., 1995, Assessment of protection from systemic infection or disease afforded by low to intermediate titres of passively acquired neutralising antibody against bovine viral diarrhea virus in calves. American journal of veterinary research 56, 755-759.

Bolin, S.R., Ridpath, J.F., Black, J., Macy, M., Roblin, R., 1994, Survey of cell lines in the American Type Culture Collection for bovine viral diarrhea virus. Journal of virological methods 48, 211-221.

Chimeno Zoth, S., Leunda, M R., Odeón, A., Taboga, O., 2007. Recombinant E2 glycoprotein of bovine viral diarrhea virus induces a solid humoral neutralizing immune response but fails to confer total protection in cattle. Brazilian Journal of Medical and Biological Research 40: 813-818

Couvreur, B., Letellier, C., Olivier, F., Dehan, P., Elouahabi, A., Vandenbranden, M., Ruysschaert, J.M., Hamers, C., Pastoret, P.P., Kerkhofs, P., 2007, Sequence-optimised E2 constructs from BVDV-1b and BVDV-2 for DNA immunisation in cattle. Veterinary research 38, 819-834.

Donofrio, G., Sartori, C., Franceschi, V., Capocefalo, A., Cavirani, S., Taddei, S., Flammini, C.F., 2008, Double immunisation strategy with a BoHV-4-vectorialized secreted chimeric peptide BVDV-E2/BoHV-1-gD. Vaccine 26, 6031-6042.

Elahi, S.M., Shen, S.H., Talbot, B.G., Massie, B., Harpin, S., Elazhary, Y., 1999, Recombinant adenoviruses expressing the E2 protein of bovine viral diarrhea virus induce humoral and cellular immune responses. FEMS microbiology letters 177, 159-166.

Ellis, J., West, K., Cortese, V., Konoby, C., Weigel, D., 2001, Effect of maternal antibodies on induction and persistence of vaccine-induced immune responses against bovine viral diarrhea virus type II in young calves. Journal of the American Veterinary Medical Association 219, 351-356.

Fernandez, F., Costantini, V., Barrandeguy, M., Parreno, V., Schiappacassi, G., Maliandi, F., Leunda, M., Odeon, A., 2009, Evaluation of experimental vaccines for bovine viral diarrhea in bovines, ovines and guinea pigs. Revista Argentina de microbiologia 41, 86-91.

Ferrer, F., Zoth, S.C., Calamante, G., Taboga, O., 2007, Induction of virus-neutralising antibodies by immunisation with Rachiplusia nu per os infected with a recombinant baculovirus expressing the E2 glycoprotein of bovine viral diarrhea virus. Journal of virological methods 146, 424-427.

Harpin, S., Hurley, D.J., Mbikay, M., Talbot, B., Elazhary, Y., 1999, Vaccination of cattle with a DNA plasmid encoding the bovine viral diarrhoea virus major glycoprotein E2. The Journal of general virology 80 (Pt 12), 3137-3144.

9

Kohl, W., Grone, A., Moennig, V., Herrler, G., 2007, Expression of the surface glycoprotein E2 of Bovine viral diarrhea virus by recombinant vesicular stomatitis virus. The Journal of general virology 88, 157-165.

Kweon, C.H., Yoon, Y.D., An, S.H., Lee, Y.S., 1997, Expression of envelope protein (E2) of bovine viral diarrhea virus in insect cells. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 59, 415-419.

Liang, R., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2007, Compatibility of plasmids encoding bovine viral diarrhea virus type 1 and type 2 E2 in a single DNA vaccine formulation. Vaccine 25, 5994-6006.

Liang, R., van den Hurk, J.V., Landi, A., Lawman, Z., Deregt, D., Townsend, H., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2008, DNA prime protein boost strategies protect cattle from bovine viral diarrhea virus type 2 challenge. The Journal of general virology 89, 453-466.

Liess, B., Orban, S., Frey, H.R., Trautwein, G., Wiefel, W., Blindow, H., 1984, Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus in cattle. II. Inoculation of pregnant cows without detectable neutralising antibodies to BVD virus 90-229 days before parturition (51st to 190th day of gestation). Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine 31, 669-681.

Nobiron, I., Thompson, I., Brownlie, J., Collins, M.E., 2003, DNA vaccination against bovine viral diarrhoea virus induces humoral and cellular responses in cattle with evidence for protection against viral challenge. Vaccine 21, 2082-2092.

Pecora, A., Perez Aguirreburualde, M.S., Rodriguez, D., Seki, C., Levy, M.S., Bochoeyer, D., Dus Santos, M.J., Wigdorovitz, A., 2009, Development and validation of an ELISA for quantitation of bovine viral diarrhea virus antigen in the critical stages of vaccine production. Journal of virological methods 162, 170-178.

Pellerin, C., van den Hurk, J., Lecomte, J., Tussen, P., 1994, Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260-268.

Rosas, C.T., Konig, P., Beer, M., Dubovi, E.J., Tischer, B.K., Osterrieder, N., 2007, Evaluation of the vaccine potential of an equine herpesvirus type 1 vector expressing bovine viral diarrhea virus structural proteins. The Journal of general virology 88, 748-757.

Roth, J.A., Kaeberle, M.L., 1983, Suppression of neutrophil and lymphocyte function induced by a vaccinal strain of bovine viral diarrhea virus with and without the administration of ACTH. American journal of veterinary research 44, 2366-2372.

Schmitt, J., Becher, P., Thiel, H.J., Keil, G.M., 1999, Expression of bovine viral diarrhoea virus glycoprotein E2 by bovine herpesvirus-1 from a synthetic ORF and incorporation of E2 into recombinant virions. The Journal of general virology 80 (Pt 11), 2839-2848.

Thiel, 1996, Pestiviruses, In: B.N. Fields, D.M.K., P.M. Howley, et al. (Ed.) Virology. Lippincott-Raven, Philadelphia, pp. 1059-1073.

Thomas, C., Young, N.J., Heaney, J., Collins, M.E., Brownlie, J., 2009, Evaluation of efficacy of mammalian and baculovirus expressed E2 subunit vaccine candidates to bovine viral diarrhoea virus. Vaccine 27, 2387-2393.

Toth, R.L., Nettleton, P.F., McCrae, M.A., 1999, Expression of the E2 envelope glycoprotein of bovine viral diarrhoea virus (BVDV) elicits virus-type specific neutralising antibodies. Veterinary microbiology 65, 87-101.

Table 1: Virus isolation from buffy coat following BVDV challenge

GROUP	Calf Nº	Viral Isolation*	VN t55**
Negative	075	P	<1:8
	190	P	<1:8
	194	P	<1:8
	197	P	<1:8
	408	P	<1:8
	161	P	<1:8
	422	P	<1:8
	452	P	<1:8
BVDV	078	N	1:512
	151	N	1:512
	163	N	1:512
	182	N	1:512
	403	N	1:128
	421	P	1:32
	444	N	1:128
	460	N	1:512
tE2	082	N	1:32
	119	N	1:128
	167	N	1:128
	175	N	1:128
	384	P	1:32
	397	N	1:128
	445	N	1:128
	459	P	1:8

(*) Virus isolation from buffy coats was carried by four passages on MBDK cells and it was further detected by immunofluorescent assay (**) VN test was performed at the time of challenge (55 dpi).

line figure
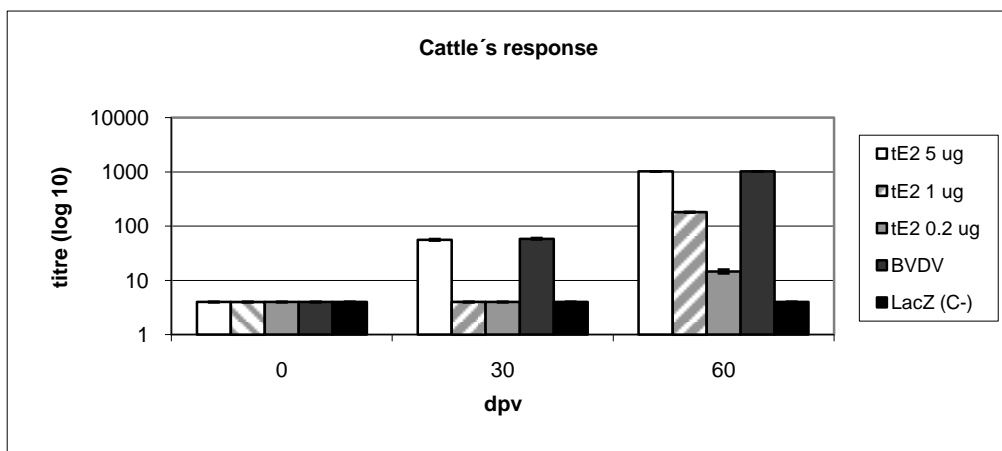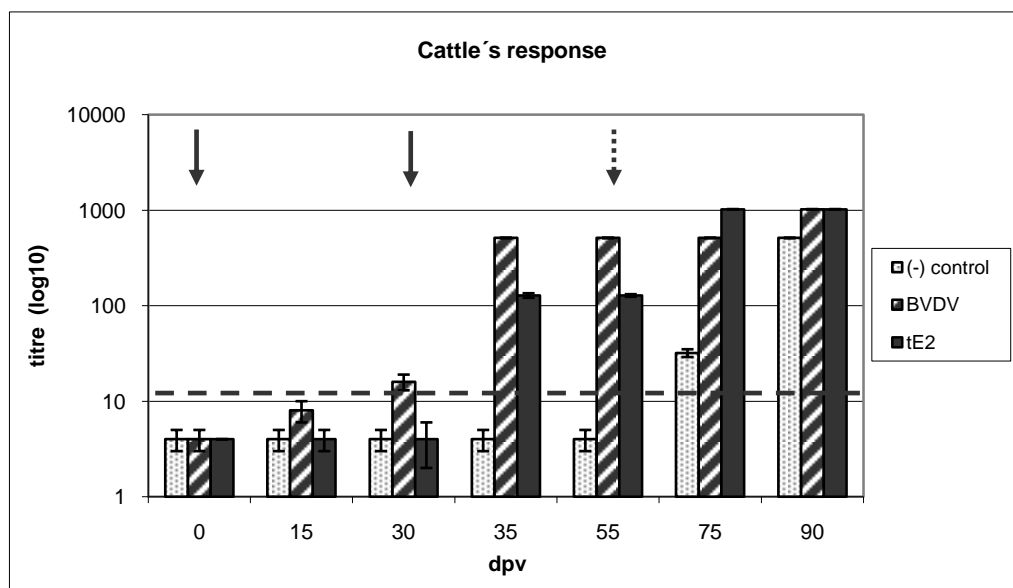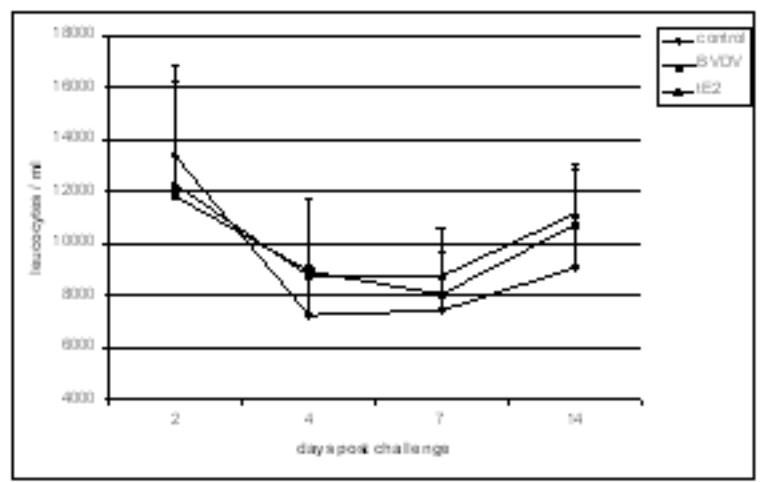Click here to download line figure: Figure 1.xls

Figure 1

line figure
Click here to download line figure: Figure 2.xls

Figure 2

Cattle´s response

titre (log 10)

	tE2 5 ug
	tE2 1 ug
	tE2 0.2 ug
	BVDV
	LacZ (C-)

dpv
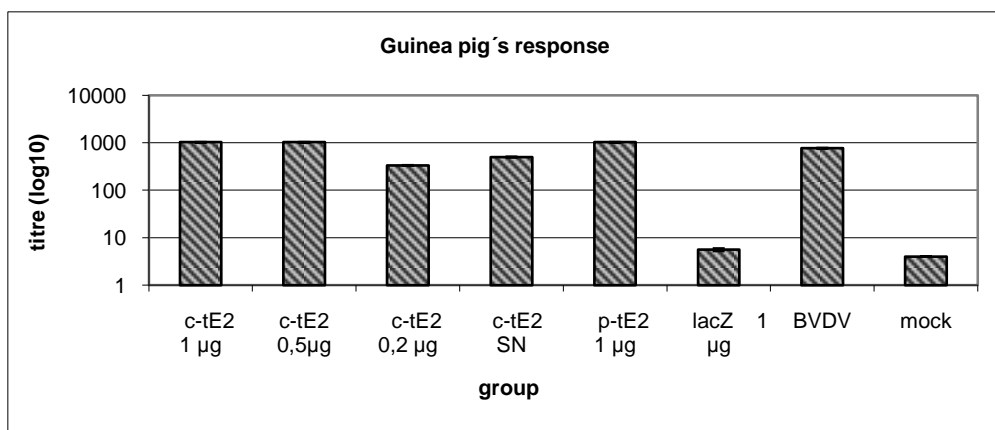
Figure 3

Figure 4

A

B

C

D
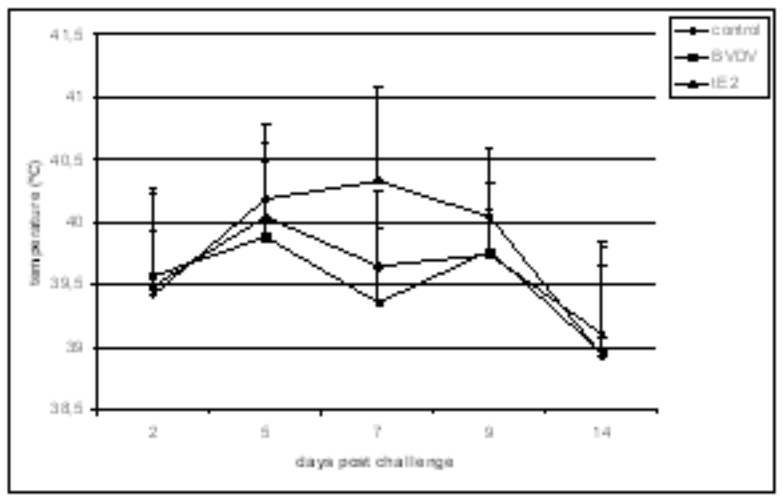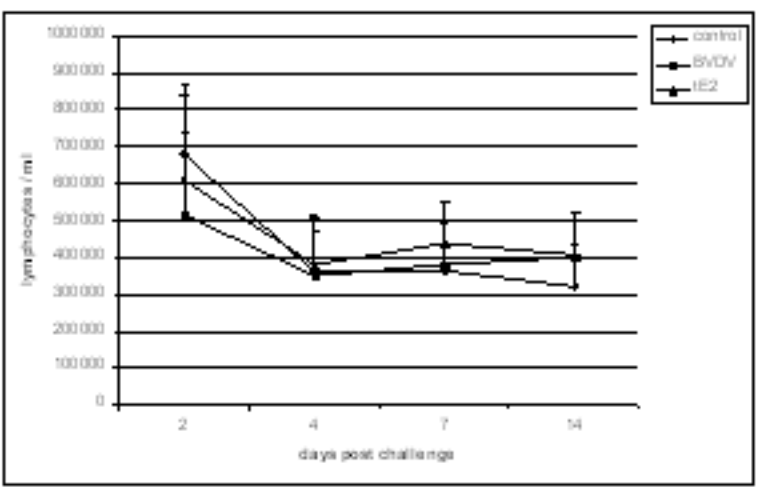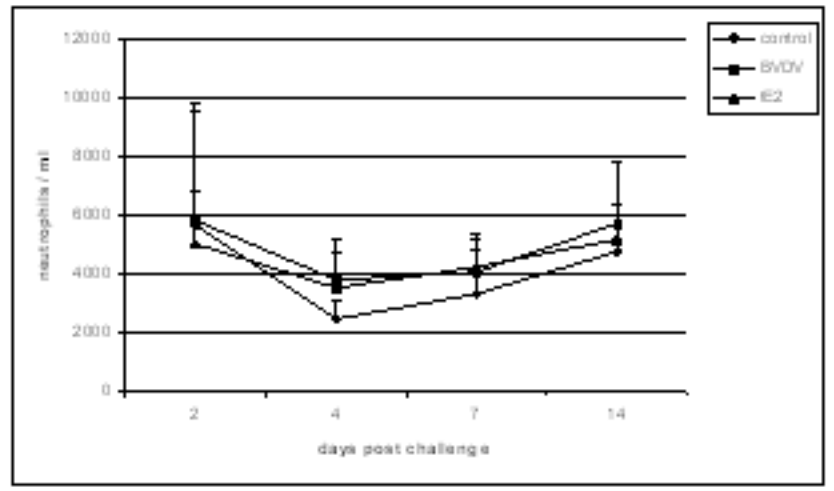
line figure
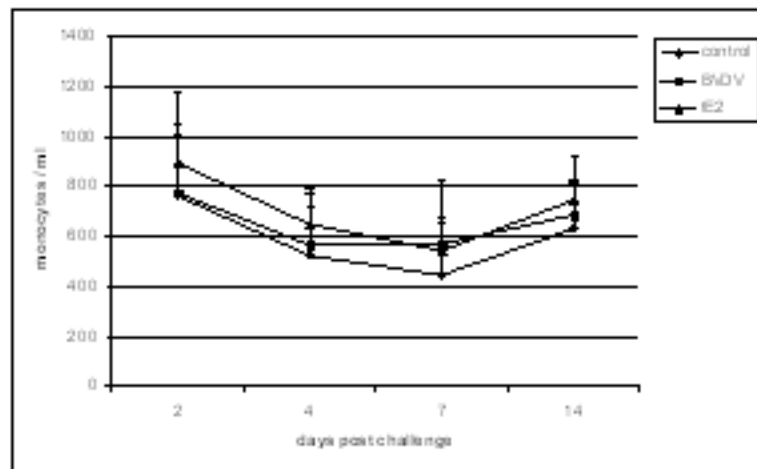
RESPONSE TO THE REVIEWERS

I want to thank the reviewers for their excellent revision of the manuscript. The comments and suggestions were very helpful.

The responses to reviewer Wenzhi Xue´s comments are specified below.

Sincerely,

Andrés Wigdorovitz

1- As suggested by the reviewer, the Abstract and Introduction were modified.

2 -The E2 secreted version was obtained from BVDV type 1a. That information was included in the M and M section: Virus and Vaccine
I have made a complete revision of the manuscript and I have changed "ug/ml and ng/ml " for "µg/ml".

3-The Vaccine dose size for guinea pigs was 0.6 ml. The value was modified in line 56, page 3.

4. As the reviewer suggest, all the subtitles were renamed in order to make clear the description of the contents

4. Figure 6 has been removed since the same data can be obtained from Table II. I have also reworked the font size of the figures and the figure legends.

5- The reviewer has also suggested making a total revision of the Cattle studies.
A- Trial 2 was deleted.
B- Cattle´s trial I and the guinea pig study were put together as serological evaluation of the tE2 protein.
C- I agree with the reviewer that the most important result for the evaluation of the E2 protein;

D The reviewer asked about the genotype of the challenge virus and the day when the challenge was conducted. Both responses were included in MyM section. We use type 1 virus and the challenge was made on day 55 post vaccination.

6- The anti BVDV antibody used in the IFA was policlonal serum.

7-   The reviewer has also suggested to use titer units as 1:XX instead of log10. I have changed "titres log 10" for "1: xx " in all these cases were the text referrers specifically to SN antibody titers .

8 Figures have been improved to make them clearer as suggested by the reviewer

9 Finally the complete structure and design of the manuscript was modified according the reviewer propose