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Development of an APC-targeted multivalent E2-based vaccine against Bovine Viral Diarrhea Virus types 1 and 2

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ABSTRACT

The aim of this study was to develop and test a multivalent subunit vaccine against Bovine Viral Diarrhea Virus (BVDV) based on the E2 virus glycoprotein belonging to genotypes 1a, 1b and 2a, immunopotentiated by targeting these antigens to antigen-presenting cells. The E2 antigens were expressed in insect cells by a baculovirus vector as fusion proteins with a single chain antibody, named APCH I, which recognizes the β-chain of the MHC Class II antigen. The three chimeric proteins were evaluated for their immunogenicity in a guinea pig model as well as in colostrum-deprived calves. Once the immune response in experimentally vaccinated calves was evaluated, immunized animals were challenged with type 1b or type 2b BVDV in order to study the protection conferred by the experimental vaccine.

The recombinant APCH I-tE21a-1b-2a vaccine was immunogenic both in guinea pigs and calves, inducing neutralizing antibodies. After BVDV type 1b and type 2 challenge of vaccinated calves in a proof of concept, the type 1b virus could not be isolated in any animal; meanwhile it was detected in all challenged non-vaccinated control animals. However, the type 2 BVDV was isolated to a lesser extent compared to unvaccinated animals challenged with type 2 BVDV. Clinical signs associated to BVDV, hyperthermia and leukopenia were reduced with respect to controls in all vaccinated calves. Given these results, this multivalent vaccine holds promise for a safe and effective tool to control BVDV in herds.

1. Introduction

The Bovine Viral Diarrhea Virus (BVDV) belongs to the pestivirus genus of the family *Flaviviridae* and it is an economically devastating disease of cattle worldwide. BVDV infections cause a broad spectrum of clinical signs ranging from mild respiratory disease to fetal death, depending on the virulence of the virus and the reproductive and immune status of the host [1]. Cattle with persistent infection are a long-term threat to herdmates because they shed BVDV for life. Persistently infected cattle may also develop mucosal disease, leading to death.

Vaccination against BVDV is an important component of prevention and control programs. One of the biggest concerns with BVDV vaccines that contain only a single BVDV genotype is their inability to cross-protect against heterologous genotypes (i.e., types 1a, 1b and 2). Then, effective protection against circulating BVDV strains requires the use of more than one genotype in the vaccine formulations to induce a wide range of protective immunity status in the cattle populations.

In Argentina, the prevalence of exposure in non-vaccinated animals is around 70% [2]. The commercial vaccines used in the country are inactivated and generally based on some of the type 1 reference strains, occasionally with the addition of BVDV-2 isolates [3]. However, a recent phylogenetic study indicated that BVDV-1b strains may predominate over the other genotypes in Argentina, and the same occurs in several countries, such as USA, Italy, Spain, Japan, India, etc. In Argentina, a marked antigenic diversity among local BVDV isolates was also noticed [4].

A single-chain antibody directed to a MHC Class II antigen epitope and denominated APCH I has been described as a good immunopotentiating molecule in different experimental vaccines, improving both humoral and cellular immune responses in immunized animals [5,6,7]. In a recent study from our group [8], we have shown that a recombinant subunit vaccine, which contains E2 protein from BVDV-1a fused to the APCH I molecule (APCH I-tE2),

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induced a high humoral immune response in guinea pigs and calves. Here, in order to obtain a broader antigenic coverage, we expressed E2 protein from three antigenically different BVDV strains fused to APCH I (belonging to BVDV-1a, -1b and -2a); the multivalent subunit vaccine was firstly subjected to an immunogenicity test in guinea pigs. This model is currently used to test commercial inactivated viral vaccines for cattle, since the neutralizing antibody titers (Nabs) of immunized guinea pigs constitute a useful predictive tool of vaccine efficacy for bovines [9]. Then, we further tested the experimental vaccine in the natural host of BVDV and for this purpose, 4 colostrum-deprived calves (CDCs) were immunized with the multivalent subunit vaccine. This animal model provides important advantages: CDC is completely naive against BVDV, and vaccination and challenge studies performed in it have no interference with previous exposure to BVDV or maternal antibodies. Moreover, it is completely suitable for studying BVDV infection and pathogenesis. In the current work, the immune response evoked by the CDCs after 2 doses of the subunit vaccine was evaluated. Thereafter, CDCs were challenged with two antigenically different field strains, belonging to BVDV-1b or BVDV-2b, and several infection parameters were assessed.

2. Materials and methods

2.1. Virus strains and cells

Cytophatic BVDV-1a (Singer strain), BVDV-1b (isolate 25366, kindly provided by Laboratorio Azul, Buenos Aires province) and BVDV-2a (VS253 reference strain) were used to develop the multivalent subunit vaccine. The noncytopathic BVDV strains 98/204 (BVDV-1b) and 98/124 (BVDV-2b) were used to perform the experimental infections. Both challenge strains were kindly provided by Dr. Odeón (INTA Balcarce, Buenos Aires). In our laboratory, MDBK cells were used to propagate all the strains. Cells were grown in Earle's minimal essential medium (EMEM) supplemented with 2% fetal bovine serum (FBS) and antibiotic cocktail.

2.2. Virus Neutralization assay

Virus Neutralization assay (VN) was used to analyze the antigenic relationships among all strains of this work, as well as to study the humoral response generated by the vaccine in guinea pigs and CDC. Briefly, 100 $TCID_{50}$ of BVDV were co-incubated for 1 h at 37 °C with 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. Reciprocals of the end point neutralization titers were calculated using the Reed and Muench method [10]. Differences in NAb titers among groups were evaluated by ANOVA test. For vaccine trials, statistical significance was assessed at $p < 0.05$ for all comparisons between groups, using the Statistix 8.0 (Analytical Software, USA).

2.3. Calculation of coefficient of antigenic similarity

In order to find the serological relationships among all the BVDV strains used in the work, hyperimmune sera were produced in 5 guinea pigs, by vaccinating them with inactivated BVDV (2.4×10^6 TCID), as described previously [4]. The neutralization end points for each virus-serum combination were combined to obtain a percent relatedness value for each pair of viruses. The coefficient of antigenic similarity (R) was calculated according to Archetti and Horsfall [11] using the following formula:

$$R = \frac{\sqrt{\text{titer strain A with antiseru B} \times \text{titer strain B with antiserum A}}}{\sqrt{\text{titer strain A with antiseru A} \times \text{titer strain B with antiserum B}}}$$

Significant antigenic differences are indicated by R values below 25 [12].

2.4. APCH I-tE2 expression

Recombinant baculoviruses expressing the E2 genes from the BVDV strains Singer (BVDV-1a), 25366 (BVDV-1b) and VS253 (BVDV-2a), were separately cloned in Dual pFastBac vector (Invitrogen) without the transmembrane domain sequence (the aminoacid sequences are detailed in supplementary Fig. 1). The signal peptide from honeybee melittin and the single chain antibody APCH I were added to the construction as described elsewhere [8]. Recombinant baculoviruses were obtained by the Bac-To-Bac baculovirus expression system (Invitrogen). Bacmids were transfected into *Spodoptera frugiperda* (SF9) cells using Cellfectin Reagent (Invitrogen) and following the manufacturer's instructions. The *S. frugiperda* (SF9) cell line was used for propagation of the recombinant baculoviruses. The amount of the APCH I-truncated E2 fusion protein (APCH I-tE2) in each preparation was determined from the intensity of immunostaining after western blotting using serial dilutions of a purified recombinant E2 protein standard which contains an histidine tag and a monoclonal antibody against Histidine (Pentahis, Qiagen). The E2 standard was previously expressed in mammalian cells and its concentration was determined by spectrophotometry and by a sandwich ELISA described elsewere [13].

2.5. Vaccination of guinea pigs

Immunogenicity of the developed subunit vaccines was first studied in a guinea pig model [9]. For the first trial, animals were immunized with 0.25 or 0.5 μg of APCH I-tE2 belonging to BVDV-1a, -1b or -2a. In the second experiment, a multivalent vaccine was used, which contained 0.25 μg of BVDV-1a APCH I-tE2; 0.5 μg of BVDV-1b APCH I-tE2; and 0.5 μg of BVDV-2a APCH I-tE2; consequently, the total amount of the combined antigens was 1.25 μg. The negative control group was immunized with mock-infected SF9 cells supernatants. Vaccines were formulated with oily adjuvant (ISA50, Seppic) containing the appropriate antigen in a proportion adjuvant:antigen of 60:40.

Animals ($n = 5$ in each group) were immunized subcutaneously with 2 doses of 0.6 ml containing the vaccine antigens, on days 0 and 21. Sera were sampled on days 0, 30 and 60 days post vaccination (dpv) to perform VN as described before against Singer, 25366 and VS253 BVDV strains.

2.6. Vaccination of Colostrum-deprived calves

Twelve colostrum-deprived, male, Holstein-Jersey calves (CDCs) were obtained from a dairy field located in Buenos Aires province. They were caught at birth to prevent them from sucking their dams and housed in biosecurity isolation units belonging to the CICVyA, INTA Castelar. The animals were bled upon arrival to perform virus isolation and PCR to discard BVDV and BVDV Nabs and they were fed milk replacer that was free of antibody against BVDV, as tested by VN.

CDCs were randomly allotted to a vaccine group ($n = 4$) and a negative control group ($n = 8$). The vaccine contained 1.5 μg of APCH I-tE2 1a, 3 μg of ACPH I-tE2 1b and 3 μg of APCH I-tE2 2a (6× of the dose used in guinea pigs). Vaccines were administered intramuscularly at 20 and 40 days of life. Details of the feeding and the

preventive antibiotics applied were described elsewhere. Vaccines were formulated with the same oil adjuvant previously described for guinea pigs.

2.7. Immune response after vaccination

CDC sera collected before vaccination and the day of the challenge were tested in VN test against the BVDV strains Singer, 25366 and VS253, as described before. A commercial ELISA which detects antibodies against p80 protein of BVDV (Ingenzim, Ingenasa), was used to check the absence of BVDV circulation and the marker characteristic of the vaccine.

In order to study and compare IgG1/IgG2 antibody responses against BVDV-1a, a sandwich ELISA was designed. Serum samples belonged to the vaccinated ($n = 4$) CDCs collected at 0 and 40 dpv. Also, sera from the same days, but belonging to 4 unvaccinated calves were used as negative controls. Briefly, U-bottom polystyrene microplates (Maxisorp, NUNC) were coated with an anti-E2 (from BVDV-1a) monoclonal antibody [14] and incubated at 4 °C ON. After blocking the plates in PBS-T, 1% skimmed milk for 1 h at 37 °C, recombinant tE2 protein was added and incubated for 1 h at 37 °C. As primary detection antibody, sera from the calves corresponding to the day of the first vaccination and to 40 dpv were used at 1:20 dilution and incubated for 1 h at 37 °C. Thereafter, peroxidase-conjugated goat anti-bovine IgG1 or IgG2 (1:1000, Serotec) were 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 in a Multiskan Fc plate reader (Thermo Scientific).

In order to detect specific cellular proliferation, peripheral blood mononucleated cells (PBMCs) were isolated by single step density separation (Histopaque 1083, Sigma). They were suspended at a concentration of 200,000 cells/well in RPMI-1640 medium (Gibco) and added to a flat-bottomed 96-well plates, each well 100 μl. Cells were stimulated in 96-well plates with the nonspecific mitogen Concavalin A (ConA, 1 μg/well, Sigma Aldrich) or UV-inactivated BVDV Singer, 25366 or VS253 (MOI: 0.1) for 3 days at 37 °C, 5% CO_2. Thereafter, XTT reagent (TACS®, Trevigen) was used to measure the cellular proliferation, following the manufacturer instructions. Finally, the stimulation index (SI) was calculated by measuring the absorbance with a microplate reader at 405 nm in a Multiskan Fc plate reader. Results are expressed as a SI according to the following formula: SI = OD of pymphocytes exposed to ConA or BVDV/OD of mock-exposed cells).

Relative expression levels of bovine cytokines IL-4, IL-10, IL-12 and IFNγ mRNA were studied by quantitative Real Time PCR (q-RT PCR). Briefly, total RNA was isolated from the CDC PBMCs with RNeasy Mini Kit spin columns (Qiagen), according to the manufacturer's instructions. After applying the RQ1 DNAse treatment to remove any residual genomic DNA, the concentration and purity of RNA recovered was assessed using a NanoDrop nd1000 spectrophotometer (Thermoscientific). The RNA samples were reverse transcribed into cDNA by using MMLV retrotranscriptase (Promega) and OligoDT primers (B071-40, Biodynamics). To quantify the cytokines mRNA expression in the samples, a real time Sybr Green PCR relative method was used. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was simultaneously processed in q PCR for each sample as an endogenous control.

All the components for the reactions were added and mixed using strips, and each sample was prepared in triplicate. q-RT PCR reactions were performed using an Applied Biosystem RT PCR 7500 equipment in a total volume of 25 μl containing 12.5 μl of MasterMix (B124-100, Biodynamics), ROX fluorophore (54881, Invitrogen), 10 μM of each pair of primers and 5 μl of DNA. The reaction cycle was 95 °C for 10 min, 45 cycles for 95 °C for 15 s and 60 °C for 1 min. At the end, a dissociation curve was performed.

The data was collected and analyzed with Applied Biosystems sequence detection software 1.3.1. The threshold was set manually for each gene. The Ct value of each gene was normalized for differences in amount of total RNA in the reaction using the GAPDH gene as an endogenous control. Relative quantification of gene expression was determined by the standard curve method (ABI PRISM 7700 sequence detection system, user bulletin #2). Gene expression was measured by relative quantification, which compared the threshold cycle (Ct) of the sample corresponding day 6 post booster with the Ct generated by a reference sample referred to as the calibrator (days of the first vaccination).

2.8. Proof of concept challenge

Two challenge groups were randomly formed, each one containing 2 vaccinated and 4 unvaccinated CDCs. All animals were challenged at 60 days of life with 5×10^6 $TCID_{50}$ of BVDV-1b 98/204 (group 1) or BVDV-2b 98/124 (group 2). Inoculum was administered intranasally, in a total volume of 4 ml.

After challenge, a clinical examination was performed daily to monitor depression, nasal and ocular discharges, diarrhea and anorexia (supplementary Table 1). Blood was collected daily from each calf using EDTA-coated tubes from day 1 pre-challenge through day 15 post-challenge, and white blood cell (WBC) counts were conducted by a clinical laboratory (Rapella, Buenos Aires City). The greatest declines in circulating white blood cells were calculated by dividing the lowest recorded circulating lymphocyte reading for each animal by the baseline lymphocyte reading for that animal and multiplying by 100 to generate a % decline. Rectal temperatures were recorded twice a day [15].

2.9. Virus isolation and Real Time PCR

Nasal and ocular swabs and blood samples for virus isolation and q-RT PCR were collected daily during 16 days, starting 1 day before challenge. Samples were diluted (1:5) in maintenance medium (Minimum Essential Medium containing 2% FBS and antibiotic/antimycotic solution). 30 μl of diluted samples were seeded in duplicate on monolayers of MDBK cells in a 96-well tissue culture plates. After 1 h of incubation, plates were washed and fresh cell culture medium was added. After 3 days, plates were frozen and thawed and the samples were passaged twice on MDBK. After the third passage, presence of BVDV was detected via immunofluorescence staining using a commercial anti-BVDV polyclonal antibody conjugated with FITC (CJ-F-BVD, VMRD).

A q-RT PCR was performed to search for genetic material of BVDV to confirm virus isolation results. A similar scheme was followed to that described above for the cytokine q-RT PCR, but the cDNA was obtained with High Pure Viral RNA Kit (Roche) and the quantification was performed by an absolute method, through the use of a plasmid containing the 5'UTR of BVDV. In this protocol, the stage of removal of genomic DNA with DNAse was skipped.

2.10. Animal welfare

Guinea pigs and CDCs handling, inoculation and sample collection was 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, (Protocols 10/2011 and 40/2013).

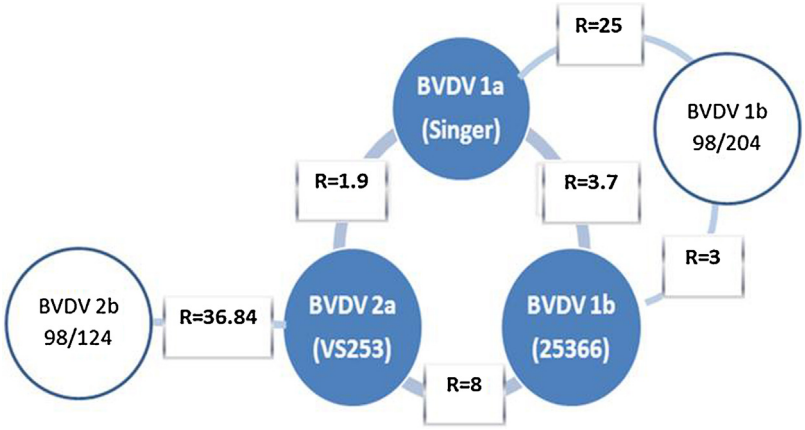

Fig. 1. Coefficients of antigenic similarity among BVDV strains used in the whole study. Strains written in white were used for the vaccine development. Strains written in black were used for the challenge. *R* values <25 indicate significant antigenic differences.

3. Results

3.1. Antigenic similarity among strains

In order to obtain a subunit vaccine covering a wide range of circulating BVDV, E2 glycoproteins from three different BVDV strains (from genotypes 1a, 1b and 2a) were cloned and fused to a Single Chain Antibody. It was determined that the three BVDV strains selected were antigenically different, since *R* values were <25 for each combination. Challenge strains, BVDV-1b 98/204 and BVDV-2b 98/124 demonstrated to be antigenically different between them as well. Results of the antigenic similarities among strains are shown in Fig. 1.

3.2. APCH I-tE2 fusion proteins expression in BEVS

Plasmids encoding APCH I-tE2 from BVDV-1a, -1b and -2 were constructed and used to infect insect cells. The expression of the fusion proteins was analyzed by SDS-PAGE followed by western blotting. All chimeric proteins were detected with the expected electrophoretic mobility corresponding to 75 KDa (Fig. 2). Amidasa treatment with *PNGase* F reduced the size of the proteins band to about 65 KDa, indicating that the expression products were glycosylated. These proteins expressed by the BEVS showed the same electrophoretic mobility to that found with this fusion protein expressed in mammalian cells, confirming their correct folding and glycosylation (data not shown), although the nature of carbohydrates moieties added were not determined.

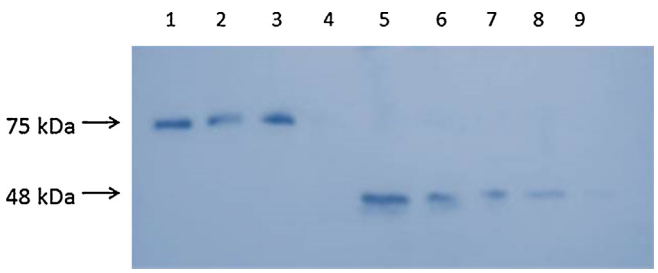

Fig. 2. A western blot image of APCH-tE2 proteins, using a monoclonal antibody against Histidine. Lines 1, 2 and 3: APCH-tE2 from BVDV-1a, -1b and -2a expressed in baculovirus expression system (75 KDa). Line 4: uninfected SF9 cells. Lines 5, 6, 7, 8 and 9: serial 2-fold dilutions of tE2 standard from BVDV-1a (20 μg/ml) (48 KDa).

3.3. Humoral immune response in guinea pigs

In a first vaccination trial, in which the APCH I-tE2 proteins from BVDV-1a, -1b and -2a were tested separately, all vaccinated animals developed specific antibody responses against the homologous BVDV strain, meanwhile the negative control animals remained seronegative. However, animals vaccinated with APCH I-tE2 1a showed higher humoral responses than the other groups (Fig. 3a). In a second vaccination trial, guinea pigs were immunized with a multivalent vaccine containing APCH I-tE2 from BVDV-1a, -1b and -2a and all animals mounted antibody responses against the 3 different viral strains. Once again, geometric mean antibody titers (GMT) against BVDV Singer were slightly higher than those obtained with the other two viral variants (antibody titers reached 2.64, 2.1 and 1.7 against BVDV-1a, -1b and -2 at 60 dpv, respectively). No synergistic immunogenicity effect was detected by using the three APCH I-tE2 proteins (Fig. 3b).

3.4. Vaccination of Colostrum deprived calves (CDCs)

3.4.1. Humoral response

Based on the results obtained in the experimental guinea pig model, the immunogenicity of the APCH I-tE2 multivalent subunit vaccine was evaluated in the natural host of BVDV. At the day of the first vaccination, CDCs were seronegative for BVDV-1a, -1b and -2. GMT at the day of challenge induced by the multivalent vaccine were 2.15, 1.13 and 1.43 against BVDV-1a, -1b and BVDV-2, respectively (Fig. 4a). Until that day, none of the calves had developed antibodies against p80 protein confirming the absence of BVDV circulation (Fig. 4b).

Besides, levels of IgG1 and IgG2 antibodies anti BVDV-1a were compared between the day of the first vaccination and the day of challenge. In vaccinated CDCs, IgG2 levels against BVDV-1a were not changed in that time period, while IgG1 values were doubled (Fig. 4c). Unvaccinated CDC did not develop IgG1 or Nabs against BVDV.

3.5. Cell-mediated immune response induced by APCH I-tE2 fusion proteins

PBMCs from vaccinated CDCs exhibited a significant increase in proliferation following stimulation with Concavalin A or with inactivated BVDV-1b at 6 days post booster compared to the day of first vaccination or control animals (Fig. 5). No significant

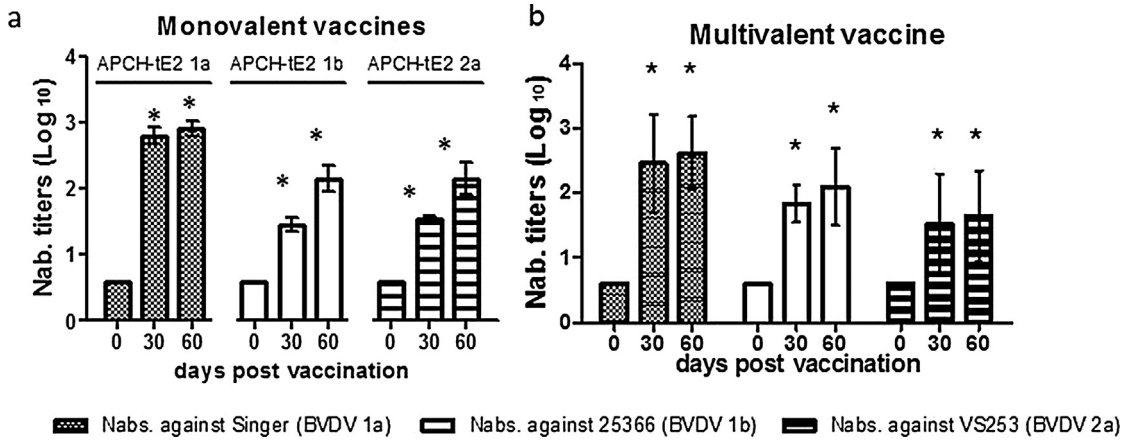

Fig. 3. Humoral immune response in guinea pigs. (a) Animals were immunized at day 0 and 21 with oily vaccines containing 0.2 μg of APCH-tE2 1a ($n = 5$), 0.5 μg of APCH-tE2 ($n = 5$) or 0.2 μg of APCH-tE2 2a ($n = 5$). At 30 and 60 days post vaccination (dpv) animals were bled and sera were evaluated by virus neutralization (VN) assay against Singer, 25366 or VS253 strains. (b) Animals ($n = 5$) were immunized with an oily vaccine containing APCH-tE2 from BVDV-1a, -1b and -2. At 30 and 60 dpv animals were bled and sera were evaluated by VN assay against Singer, 25366 and VS253 strains. Each bar represents the average titer of the group. Asterisks (*) indicate significant differences from the animals immunized with the multivalent vaccine with respect to the unvaccinated control group ($n = 5$), for each of the strains used in the VN ($p < 0.05$).

proliferative response was observed in response to the BVDV type 1a or 2a in the vaccinated calves.

To address the question of whether vaccination with the multivalent subunit formulation could modify the levels of cytokines, mRNA levels of specific of cytokines were analyzed at day 6 post booster and they were compared to their relative levels at the day of the first vaccination dose. It was observed that IFNγ and IL-12 levels increased 2-fold times at that time point. This up-regulation

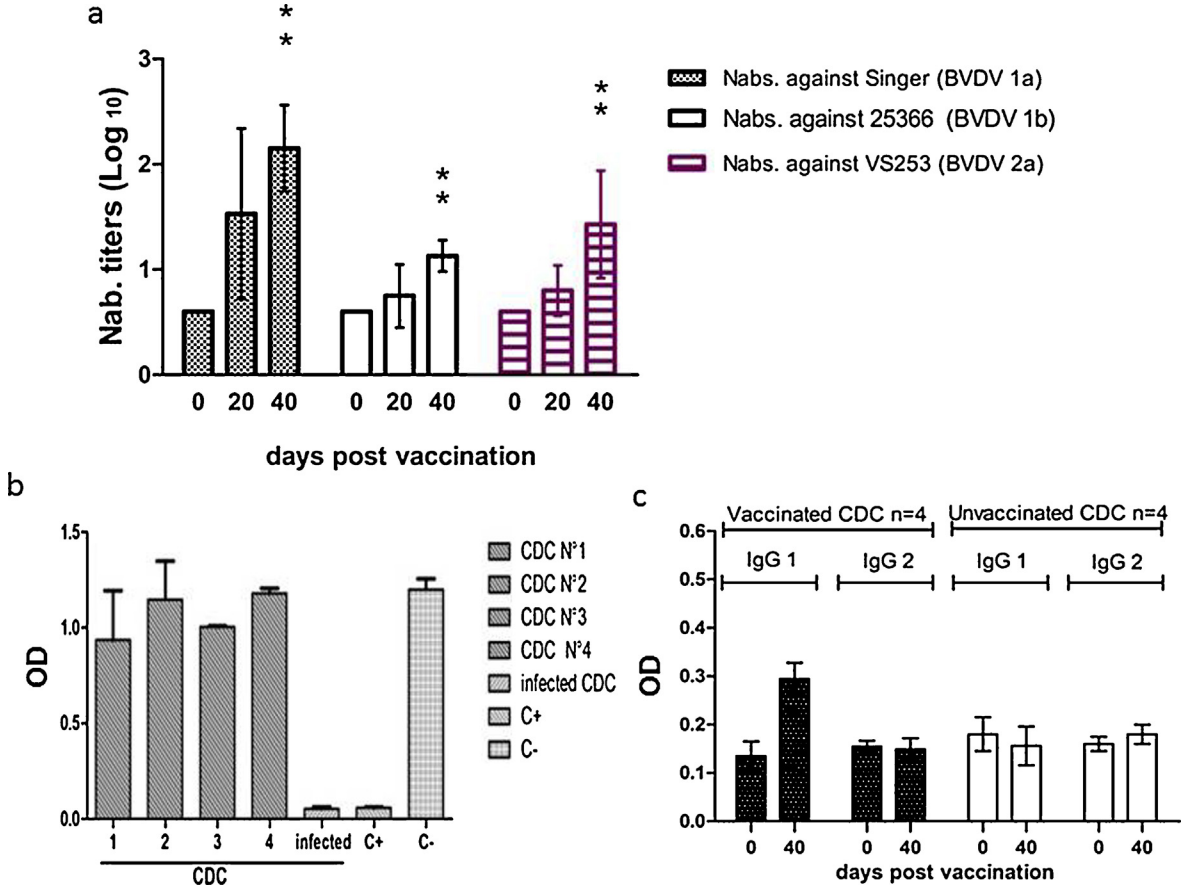

Fig. 4. Humoral immune response in the 4 colostrum-deprived calves (CDCs) immunized with an oil vaccine containing APCH-tE21a, 1b and 2. (a) Virus neutralization (VN) assay against Singer, 25366 and VS253. Each bar represents the average titer of the group. Asterisks (*) indicate significant differences from the animals immunized with the multivalent vaccine with respect to the negative control group, for each strain used in the VN test ($p < 0.05$). (b) Commercial competition ELISA which detects antibodies against p80 protein from BVDV in CDC sera. (c) Increase in IgG1 antibodies against BVDV-1a in CDCs after immunization. n = number of calves in each group. Error bars: SD.

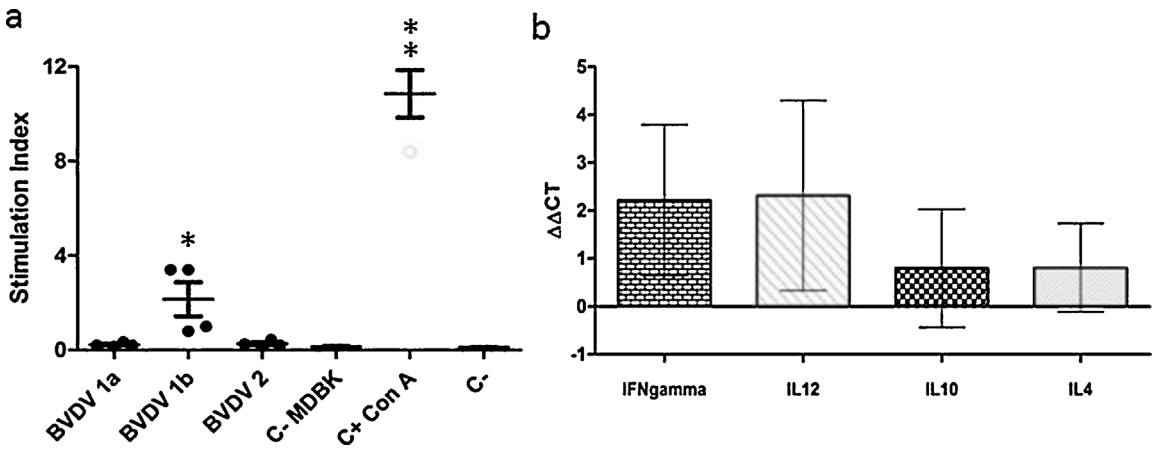

Fig. 5. Cellular immune response after vaccination in 4 colostrum-deprived calves (CDCs) measured at 6 days post booster. (a) Specific lymphocyte proliferation following UV-inactivated BVDV-1a, -1b or -2 stimulation of isolated PBMCs. Proliferation is expressed as group means of corrected OD (COD = OD virus − OD negative control) values after 3 days of stimulation and addition of XTT® reagent. Standard deviations and statistical significance are indicated by upward deflection lines and asterisks ($p \leq 0.05$ (*) or $p \leq 0.01$ (**)), respectively. Stimulation with the superantigen Concavalin A was used as a positive proliferative control. (b) Changes in expression of IFNγ, IL-12, IL-10 and IL-4 mRNA in PBMC of vaccinated CDCs. Data are expressed as fold difference of expression at 6 days post booster compared with values obtained at the day of the first vaccination.

trend was lower in the case of the other two cytokines evaluated (IL-10 and IL-4) (Fig. 5b). Despite this data, these assays did not render statistically significant results.

3.6. BVDV-1b challenge

After BVDV-1b challenge, vaccinated CDCs showed good levels of protection since BVDV was not detected in buffy coats or nasal swabs samples (assessed by VI and q-RT PCR). In contrast, control unvaccinated calves showed 6 days of viremia and 4 days of nasal discharge (Table 2a). Regarding to WBC counts, unvaccinated calves showed a decrease average at 5 dpi of 60% ± 10, while vaccinated animals presented a transient decrease of 41% and 52% at the same day post-infection (Table 1a). Clinical scores were around 10 in the 4 unvaccinated CDCs, due to respiratory signs including cough and nasal secretions. Clinical signs were almost undetectable in the 2 vaccinated animals. One of them presented a score of 3, because of nasal discharge at day 7 and slight depressed sensorium at day 8. The other CDC did not show any clinical sign associated to BVDV (Fig. 6a). All the CDCs showed 2 peaks of slight pyrexia (39.5 °C) at 3 and 8 dpi (Table 3).

Table 1
Comparison in greatest recorded decline in circulating white blood cell (WBC) count in colostrum-deprived calves (CDCs).

(a) Animal	Max. % decline in WBC	dpi
Unvaccinated CDCs ($n = 4$)	60 ± 10	7
Vaccinated CDC no. 2	52	6
Vaccinated CDC no. 4	41	6

(b) Animal	Max. % decline in WBC	dpi
Unvaccinated CDCs ($n = 4$)	60 ± 10	7
Vaccinated CDC no. 1	37	6
Vaccinated CDC no. 3	33.3	6

The average greatest daily decline in circulating lymphocyte count recorded per animal is shown following infection with each of the 2 viruses: 98/204 (BVDV-1b) (a) or 98/124 (BVDV-2b) (b).
dpi: days post infection.

3.7. BVDV-2b challenge

Similarly to what we found in calves challenged with BVDV-1b, vaccinated animals presented a good level of protection

Table 2
Proportion of vaccinated and nonvaccinated calves challenged with type 1b (a) or type 2 (b) BVDV in which virus was isolated from buffy coats (BC) and nasal swabs (NS) after challenge.

(a)

	Days post infection																	Duration
Sample	Group	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	(n of days)
BC	Unvaccinated	0/4	0/4	0/4	0/4	3/4	4/4	4/4	4/4	4/4	3/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	5–6
BC	Vaccinated	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0
NS	Unvaccinated	0/4	0/4	0/4	0/4	0/4	0/4	2/4	1/4	1/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1–2
NS	Vaccinated	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0

(b)

	Days post infection																	Duration
Sample	Group	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	(n of days)
BC	Unvaccinated	0/4	0/4	2/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	2/4	0/4	0/4	0/4	0/4	0/4	8–10
BC	Vaccinated	0/2	0/2	0/2	0/2	0/2	0/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1–2
NS	Unvaccinated	0/4	0/4	0/4	0/4	3/4	4/4	4/4	3/4	4/4	3/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	5–6
NS	Vaccinated	0/2	0/2	0/2	0/2	0/2	0/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1–2

Virus isolation results were positive (+) or negative (−) for the day indicated. Duration of viremia and virus shedding is shown in the last column.

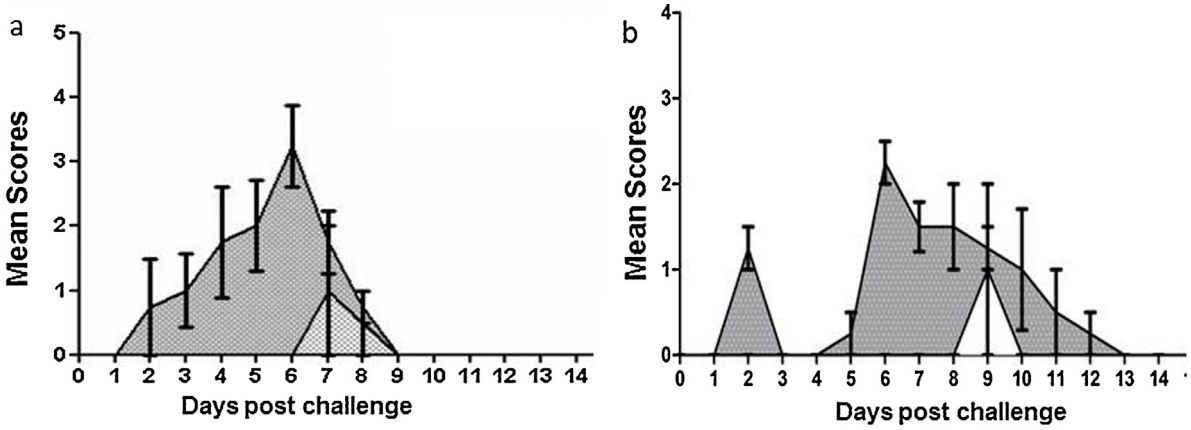

Fig. 6. Mean daily clinical scores (CS) of colostrum-deprived calves (CDCs) following challenge with 5×10^6 $TCID_{50}$ of BVDV, assigned according to Table 2. Clinical signs were observed daily from the day of the challenge to 14 days after challenge. Area under curves (AUC) in dark gray indicates the CS of unvaccinated CDCs ($n = 4$). In both challenge groups (with BVDV-1b or -2b) unvaccinated calves showed CS = 10. (a) Challenge with BVDV-1b (98/204): Vaccinated calf no. 2 did not present clinical signs associated to BVDV throughout the experiment. Vaccinated calf no. 4 (AUC in light gray) showed a CS = 3 because of a muco-serous nasal secretion at 7 days post infection (dpi) and a slight depression at 8 dpi. (b) Challenge with BVDV-2b (98/124): Vaccinated calf no. 1 did not present clinical signs associated to BVDV throughout the experiment. Vaccinated calf no. 3 (AUC in white) showed a CS = 2 because of loose feces at 9 dpi.

against BVDV-2b. While unvaccinated animals presented 10 days of viremia and 6 days of nasal discharge, vaccinated calves showed 1 or 2 days of positive virus isolation in both type of samples (at 5 and 6 dpi), as shown in Table 2b. Analysis of the WBC count demonstrated that while unvaccinated CDCs presented a decrease average in WBC of 67% ± 10 from 2 to 7 dpi, the 2 vaccinated animals showed a transient WBC decrease of 33% and 37% at 6 dpi (Table 1b).

Unvaccinated animals presented gastrointestinal symptoms including diarrhea and hematochezia after challenge with BVDV-2b, meaning a score of 10. In contrast, vaccinated CDCs showed scores of 0 and 1, due to a mild diarrhea in one of the two calves (calf no. 3, Fig. 6b). With respect to the rectal temperatures, all the unvaccinated CDCs showed 2 peaks of pyrexia, at 3 and 7 dpi, while one of the vaccinated animals did not showed pyrexia and the other (calf no. 3) presented elevated rectal temperatures at 7 dpi.

4. Discussion

Inactivated and modified-live vaccines are widely used to protect cattle from BVDV infection; however, both types of vaccines have significant shortcomings. Thus, there is a need for developing more efficacious and safer vaccines. A subunit vaccine is safe in terms of horizontal and vertical transmission, and allows discrimination between vaccinated and infected animals. However, the generation of strong immune responses to subunit vaccines sometimes requires the use of potent adjuvants, high antigen doses and repetitive vaccinations. To overcome these difficulties, one strategy is to target the antigen to antigen-presenting cells. In this regard, a single chain antibody (scFv) was developed to achieve this function [5]. This ScFv, denominated APCH I (Antigen Presenting Cells homing I), recognizes a MHC Class II DR molecule in most mammalian species and has been seen to potentiate subunit vaccines formulated as protein fusion antigens or in the context of DNA vaccination [6,7].

As expected, the recombinant proteins were immunogenic in guinea pigs, as demonstrated by production of Nabs against the three BVDV strains after two vaccination doses (Fig. 3). Interestingly, APCH I-tE2-1a seems to be more immunogenic than their counterparts tE2-1b and 2a, since in spite that all vaccine formulations contained the same quantities of recombinant protein, the highest GMT were found against BVDV-1a. When the three proteins were included in the same vaccine, once again Nabs titers were higher against BVDV-1a compared to BVDV-1b or BVDV-2a. No synergistic effect was observed in the immunogenicity of this multivalent vaccine. These two results were also evident in the vaccination experiment in CDCs, which received 6 times the dose of guinea pigs.

Multiple immunological assays, when used simultaneously, can provide a broader, more in depth evaluation of immune response of calves to vaccination. Each of our assays was chosen to evaluate different aspects of the vaccine-induced immune response in the CDCs. Besides the development of Nabs against the 3 BVDV variants, all vaccinated calves produced IgG1 antibodies against BVDV-1a. Unlike IgG2 antibodies, IgG1 are capable of binding to a receptor (FcR) of the mammary gland secretory epithelium, which selectively captures and concentrates this isotype when colostrum is produced [16]. This Ab isotype may not be important for CDCs from this work, but it could be important for pregnant cows, which are an important animal population to be vaccinated against BVDV.

Table 3
Timeline of the study.

Day	0	20*	40*	60•	75
Clinical examination	x	x	x		x x x x x x x x x x x x x
Serum for antibody detection (VN and IgG isotype profile)	x		x	x	x
PBMC isolation (lymphoproliferation and mRNA cytokine quantitation)		x	x		
Whole blood for virus detection	x				x x x x x x x x x x x x x
Nasal swabs for virus detection					x x x x x x x x x x x x x
Days post infection			0	7	14

12 colostrum-deprived calves (CDCs) were immunized twice at a 20-day interval (*) with an experimental subunit vaccine against BVDV ($n = 4$) or maintained unvaccinated (controls, $n = 8$), then challenge with BVDV-1b or -2b was performed ($n = 6$ in each group: 2 vaccinated CDCs +4 unvaccinated CDCs) (•). Clinical examinations, collection of serum for antibody detection and whole blood for virus detection and isolation of peripheral blood mononuclear cells (PBMCs) for lymphocyte proliferation assays and q RT-PCR for bovine cytokines are indicated with crosses at the indicated post-infection day (dpi).

This characteristic of the vaccine may be valuable under field conditions where multiple variables can affect the process of colostrum, undoubtedly having a higher concentration of IgG1 antibodies against BVDV in it would be an advantage.

Although humoral memory is an important factor in preventing and controlling BVDV infection, the scientific evidences suggest that both humoral and cell-mediated immune responses are important in protection against BVDV-induced disease in cattle [17]. To achieve this assessment, some cellular immunity parameters were studied in the vaccinated CDCs. Firstly, cellular proliferation was analyzed, and results indicated that only stimulation of lymphocytes with BVDV-1b generated a significant proliferation in all vaccinated calves, although this virus genotype was not the best inducer of a humoral immune response. In contrast, the antibody response was higher against BVDV-1a but the lymphoproliferative response against this genotype was not significant. Differences between the titers of the 3 BVDV strains or in the MOI used to stimulate the PBMC can be discarded; and this different results among the BVDV strains used was noticed in independent assays when using CDC lymphocytes corresponding to different times post vaccination. In this study, no correlation between the ability of recombinant proteins to induce antibodies and to stimulate lymphocytes was found, but this could be explained because the E2 protein possesses both B and T antigenic epitopes [18]. To date, these epitopes have not been fully characterized in the E2 protein from the model virus BVDV-1a and still less the ones corresponding to E2 from BVDV-1b and -2.

The evaluation of certain cytokine expression to characterize vaccine responses has been used previously to assess immune responses to infection or vaccination [18]. Several methods allow quantitation of cytokine expression at the protein and at the mRNA level of PBMCs. In that sense, quantitative Real Time PCR is a high sensitivity technique which results useful to quantify minimal physiologic changes in genes expression and it has a limit of detection from 10 to 100 folds higher than other methods [21]. For this purpose, a q-RT PCR technique was standardized to quantify the cytokine mRNA expression using a relative method. Our studies provided a trend in the nature of the T helper (Th) response induced by the vaccine: IFNγ and IL-12 mRNA levels were up-regulated, suggesting a Th1 polarization, which is a desired characteristic for vaccines against viral pathogens. However, it is noteworthy that the immunological profile generated by this subunit vaccine may also be given by components such as the adjuvant used and residues of the baculoviral lysates. The vaccine was formulated with an oily adjuvant and the water in oil emulsion leads to a Th1 response [19]. It is also possible that the formulation had incorporated CpG residues from the baculoviral lysates which would have induced a Th1 immune response via TLR9 [20]. CpG motifs activate monocytes and macrophages and make them the secretion of IL-12.

The reason because our findings in cytokine stimulation were not statistically relevant could be explained because of insufficient assay sensitivity, or perhaps to the fact that the assays were not carried out at the optimum time post-vaccination in relation to recirculation of responsive cells in peripheral blood. Furthermore, lymphocytes from which the mRNA was extracted have not been stimulated with the specific antigens previously, and this would certainly increase the observed trend. Further studies will be needed to determine the best sampling point for each cytokine of interest to monitor the vaccine response.

The protective ability of the multivalent vaccine (APCH I-tE2 1a-1b-2a) was assessed in a proof of concept experiment, in which two field strains belonging to BVDV-1b and -2b were used. As revealed by the calculation of the antigenic indexes, challenge strains were antigenically different from the strains used to develop the vaccine, which is a desirable characteristic in the design of vaccine-challenge trials [15].

After the experimental infections, clinical signs associated with the BVDV-1b or the BVDV-2 strains were respiratory or gastrointestinal, respectively, in the unvaccinated CDCs. In both challenge groups, vaccinated animals showed much lower or no clinical signs associated to the disease. Besides a milder reduction in WBC counts, vaccinated CDCs showed a significant virus protection, compared to unvaccinated animals which showed 6 or 10 days of viremia when challenged with BVDV-1b or -2b, respectively. In spite of that a reduced number of animals were used in the protection experiment; data are enough encouraging to extend the studies with this new BVDV vaccine approach to a larger number of animals in each group that will allow us to certify its protective value. Further trials could be conducted to explore another vaccination schedule or another adjuvant in the composition of the vaccine, and final experiments should be carried out in adult cattle. Finally, meanwhile this novel formulation is being evaluated by the National Authorities for Animal Health (SENASA) to be registered as the first subunit vaccine for cattle.

As expected, vaccinated CDCs lacked antibodies against p80 protein from BVDV up to the day of challenge, whereby the presence of BVDV was discarded and it further corroborated the "DIVA" characteristic of this subunit vaccine. Discrimination between vaccinated and infected animals is highly relevant for BVDV eradication programs.

Given these promising results, we consider that the use of a multivalent subunit vaccine in combination with a comprehensive BVDV control program should result in decreased incidence of persistent infection in calves and therefore minimize the risk of BVDV infection in the herd.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.07.106

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