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**A novel MHC-II targeted BVDV subunit vaccine induces a neutralizing immunological response in guinea pigs and cattle.**

**Running Head:** Results of the first targeted vaccine against BVDV

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## Abstract

Bovine viral diarrhea virus (BVDV) is a major cause of economic loss in the cattle industry, worldwide. Infection results in reduced productive performance, growth retardation, reduced milk production, and increased susceptibility to other diseases leading to early culling of animals. There are two primary methods used to control the spread of BVDV: the elimination of persistently infected (PI) animals and vaccination. Currently, modified live or inactivated vaccines are used in BVDV vaccination programs, but there are safety risks or insufficient protection, respectively, with these vaccines. Here we report the development and efficacy of the first targeted subunit vaccine against BVDV. The core of the vaccine is the fusion of the BVDV structural protein, E2, to a single-chain antibody, APCH, together termed, APCH-E2. The APCH antibody targets the E2 antigen to the major histocompatibility type II molecule (MHC-II) present on antigen-presenting cells. Industrial production of the vaccine is carried out using the baculovirus expression vector system (BEVS) using single-use manufacturing technologies. This new subunit vaccine induces strong BVDV-specific neutralizing antibodies in guinea pigs and cattle. Importantly, in cattle with low levels of natural BVDV-specific neutralizing antibodies, the vaccine induced strong neutralizing antibody levels to above the protective threshold, as determined by a competition ELISA. The APCH-E2 vaccine induced a rapid and sustained neutralizing antibody response compared to a conventional vaccine in cattle.

## Introduction

Bovine viral diarrhea virus (BVDV) belongs to the genus Pestivirus, family Flaviviridae. This virus has a worldwide distribution and infects ruminants. 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 (Ridpath 2010). It is also one of the etiologic agents of Bovine Respiratory Disease (BRD), which is a major health problem and the main cause of economic losses in raising cattle (Griffin 1997). Infection of pregnant cows with BVDV in the first trimester of gestation can result in the production of a persistently infected (PI) animal (Grooms 2004). Cattle with a persistent infection are a long-term threat to herd-mates because they shed BVDV for life and represent the main reservoir of the virus within the herd. More than 90% of new PI calves are born to healthy cows that became infected during gestation, the other 7% to 10% of PI calves are offspring of PI cows (Wittum et al. 2001). Vaccination against BVDV is an important component of prevention and control programs since it can prevent clinical signs, reduce viral spread and the birth of new PI animals. Currently, only modified live vaccines (MLV) and

inactivated vaccines are used in vaccination programs. Both have historical disadvantages; MLV in terms of safety and inactivated vaccines in terms of immunoprotection. Subunit vaccines provide the opportunity of developing safe and effective vaccines as has been shown with the new human recombinant vaccines against shingles (Herpes zoster) and meningitis B (*Neisseria meningitidis* group B) that have received US-FDA approval in recent years. In the field of veterinary medicine, the challenge is to produce a recombinant vaccine that induces a protective immune response at a cost affordable price.

The BVDV genome consists of a single-stranded, positive sense RNA molecule of approximately 12.3 kb in length. E2 is the major structural glycoprotein of the BVDV envelope and the most immunoprotective protein of the virus (Deregt et al. 1998; Fulton et al. 1997; Paton, Lowings, and Barrett 1992). Neutralizing antibodies (NAbs) induced in infected animals are mainly directed against E2 (Donis 1995). The first attempt by our group to produce a protective subunit vaccine against BVDV was based on a secreted version of the BVDV E2 glycoprotein. Sera from animals vaccinated with E2 neutralized several BVDV strains within a genogroup (Pecora et al. 2014; S. Bolin et al. 1988). Moreover, it was demonstrated that NAbs raised against E2 prevented infection from BVDV (Bolin 1995; Toth et al. 1999; Pecora et al. 2015). The E2 subunit was initially expressed in stably transfected CHO-K1 cells, reaching a yield of 0.3 mg/L. The immunogenicity of this first generation E2 antigen vaccine was studied using guinea pigs, as a laboratory animal model, and field trials were conducted in cattle. Animals vaccinated with this E2 subunit vaccine developed high NAb titers and were protected against viral infection (Pecora et al. 2016). Results obtained in this initial trial were promising, but the low quantity of antigen produced in the CHO-K1 cell-line made large scale commercial production cost inhibitory for veterinary medicine purposes. To address this issue, two important modifications were introduced: 1) the protein production system was changed to transgenic alfalfa plants (*Medicago sativa*, L.) and the viral E2 glycoprotein was targeted to the antigen-presenting cells (APC) in order to increase its immunogenicity.

The coding sequence of the BVDV E2 glycoprotein was fused to the coding sequence of APCH, a single chain antibody, creating a fusion gene termed *APCH-E2*. APCH is a single-chain antibody directed to the major histocompatibility complex type II (MHC -II) antigen epitope and has been designated as a potent immunomodulating molecule in at least two different vaccine models, improving both humoral and cellular immune responses in immunized animals as it targets the antigen to the APCs (Gil et al. 2011; Pecora, Malacari, Perez Aguirreburualde, et al. 2015). The *APCH-E2* fusion gene was engineered into the alfalfa

genome and the antigen was produced in alfalfa leaves, yielding up to 1 µg/g (antigen/ wet alfalfa) and production of the fusion antigen remained stable after vegetative propagation. A methodology based on an aqueous two-phase system was standardized for concentration and partial purification of APCH-E2 from alfalfa. Guinea pigs intramuscularly immunized with leaf extracts developed high BVDV-specific NAb titers. In bovine vaccinated with 3 µg of alfalfa produced APCH-E2, BVDV-specific NAb titers were induced and vaccinated animals did not shed BVDV after a viral challenge (isolate 98/124, type IB) (Aguirreburualde et al. 2013). Results with transgenic alfalfa plants were promising, but there were two major issues that should be resolved in order to transform the plant-derived APCH-E2 antigen in an industrial product: 1) inhibitory scaling-up issues with the extraction and purification process, and 2) the unknown regulatory aspects for parenteral administration of a viral antigen derived from transgenic-plants.

With the aim of overcoming these difficulties, the APCH-E2 antigen construct was engineered into the baculovirus expression vector system (BEVS)(Pecora, Malacari, Pérez Aguirreburualde, et al. 2015). BEVS was chosen because of the system's advantages: it is safe, easy to use, and readily amenable to manufacturing scale-up (Kost, Condreay, and Jarvis 2005). In 2017, after more than ten years of research and development, this baculovirus produced APCH-E2 antigen was the basis of the first subunit and targeted vaccine licensed to be used in cattle for the control of BVDV. To get the governmental approval a controlled field study with seronegative calves and a placebo control group was performed; the targeted vaccine was able to induce a strong immune response after inoculation with two doses, 30 days apart. At the peak of the immune response, 60-120 days post vaccination (dpv), the mean NAb titer was  $> \text{Log}_{10} 4.2$  and the mean NAb titer was  $> \text{Log}_{10} 2$  at 360 dpv. No animals on the placebo group presented antibody against BVDV over the course of the trial (Pecora, Malacari, Pérez Aguirreburualde, et al. 2015). Here, we report the immunogenicity and efficacy of this new APCH-E2 commercial vaccine as tested in guinea pigs and a field trial in cattle under normal production conditions. This BEVS derived APCH-E2 vaccine induced a strong antibody response in all vaccinated animals and correlated with protection in experimentally challenged calves (Aguirreburualde et al. 2013; Pecora, Malacari, Perez Aguirreburualde, et al. 2015; Pecora et al. 2016).

## Materials and Methods

**Antigen production:** Recombinant baculovirus expressing the APCH-E2 (BEVS-APCHE2) antigen was generated as described by Pecora et al (Pecora, Malacari, Perez Aguirreburualde, et al. 2015). *Spodoptera*

*frugiperda* (Sf9) cells were used to produce the recombinant fusion antigen. Sf9 cells were cultivated in a customized SF900 II Serum Free Medium (ThermoFisher) and cultured as shown in Figure 1. Briefly, Sf9 cells were multiplied using a series of static and shaking cultures with increasing volumes to cultivate enough cells to seed 10L of media in a single-use bioreactor (GE, WAVE™ 25). Sf9 Cells were cultured at 27 °C to a cell density of  $1.5 \times 10^6$  cells/ml in the bioreactor culture, which was then infected with the BEVS-APCHE2 virus at a multiplicity of infection (MOI) of 3 and incubated at 27 °C for 120 h to produce the antigen. To purify the antigen, the culture is processed through a series of filtration steps. The concentration of the APCH-E2 antigen is determined via an in-house ELISA (Pecora et al. 2009). Harvested antigen is stored at  $4 (\pm 2)$  °C prior to formulation.

**Vaccine:** Vedevax BLOCK® (Bioinnovo SA, Buenos, Aires, Argentina) is a pre-mixed, ready-to-use, BVDV vaccine formulated with a water-in-oil adjuvant. The vaccine is a two-dose regimen (3 ml/dose, \$0.9 USD/dose) administered 21-30 days apart to cattle 6 months or older. The vaccine is licensed in Argentina (File N°: 0057519/2014) and Peru (File N°: B.14.5.02.I.0016) and is the only one-of-its-kind in the world.

Conventional vaccine: is a commercially available reproductive  $Al(OH)_3$ -adjuvant vaccine which contains inactivated BVDV in its formulation. The vaccine is a two-dose regimen (5 ml/dose, \$0.5 USD/dose) administered 21-30 days apart to cattle 6 months or older.

**Vaccination of guinea pigs:** 5 guinea pigs per group were vaccinated twice, at day 0 and 21, with 0.6 ml (1/5 of the bovine dose) and bled 30 days post-second vaccination. Sera were analyzed by Virus Neutralization (VN) assay. According to a dose response assay conducted in guinea pigs and bovines using vaccines formulated with increasing titers of BVDV per dose ( $1 \times 10^6$ ,  $1 \times 10^7$ , or  $1 \times 10^8$  TCID<sub>50</sub>/dose), a vaccine can be classified as of Not Satisfactory immunogenicity if the induced mean NAb titer in guinea pig was lower than 1:24 ( $\log_{10}$  titer < 1.37), of Satisfactory immunogenicity if  $1:24 \leq$  NAbs titer  $\leq$  1:100 ( $\log_{10}$  1.37  $\leq$  NAbs titer  $\leq$   $\log_{10}$  2), or of Highly Satisfactory immunogenicity if the mean NAbs titer  $\geq$  1:100 ( $\log_{10}$  titer > 2). (Res. SENASA 2012).

**Virus Neutralization (VN) assay:** The cytopathic BVDV-1a (Singer strain) was used in the VN assays. The MDBK cell culture conditions, virus propagation conditions, and the virus neutralization assay were performed as previously described (Aguirreburualde et al. 2013).

**Competition ELISA:** 96-well Nunc Maxisorp ELISA plates were coated with a bivalent llama-derived nanobody in carbonate-bicarbonate buffer directed to E2 protein from BVDV overnight at 4 °C, followed by a blocking step with 1% skim milk the next day for 1 hour at 37 °C. The subsequent steps were also incubated with these conditions. Plates were washed three times with PBS-Tween20 (0.1%) between steps. Then, 6 ng/well of BEVS produced APCH-E2 was added to the appropriate wells. Bovine serum samples were added in a unique ¼ dilution in PBS-0.05% Tween 20. After a 1 h incubation, a rabbit polyclonal antiserum to E2 was added in a dilution 1/40 followed by a peroxidase-labeled anti-rabbit IgG (KPL) at 25 ng/well. The ELISA Ab titer was expressed as a percentage of displacement (PD%) of the positive rabbit hyperimmune serum against the E2 protein that was considered the 100% signal. The cutoff point of the ELISA was established in PD% = 10%. Using this assay, the concordance between PD% and vaccine quality was established as follows: low-quality vaccines <PD 12%, satisfactory vaccines 12% ≤ PD ≤ 35%PD and highly satisfactory vaccines PD > 35% (Manuscript under preparation).

**Field trial in cattle:** The trial was performed in Estancia Lavalle, Mercedes, Corrientes, Argentina. A total of 107 Brangus cows were divided randomly into two groups, 53 vaccinated with the targeted vaccine and 54 with a conventional reproductive vaccine, which contains inactivated BVDV. All animals were vaccinated twice, beginning on study day 0 and again on study day 30. Blood was drawn from all animals on study days 0, 30, 60, 120, 180, 300 and 360. Sera were analyzed individually by competition ELISA. Results are expressed as a percentage of displacement of a positive hyperimmune serum against the E2 protein. Serum samples of all animals at day 0 and 60 (T0 & T60, respectively) were also evaluated by virus neutralization assay. The field trial was carried out in a commercial herd under normal management conditions. There were no changes in animal feeding, health, movement or any other parameter or condition during the trial. None of the animals had a negative reaction to the targeted vaccine and no mortality was observed in both groups.

**Statistical analysis:** Differences in mean antibodies values among groups were evaluated by a general mixed model of repeated measures throughout time considering vaccine group and time as fixed factors and the animal as a random factor followed by Tukey multiple comparisons test. The matrix of variance-covariance was modeled assuming an AR1 autocorrelation effect due to the sampling of the same animals through time (AR1) and heterogeneity of variances at different time points (varIdent). Statistical significance was assessed at  $\alpha \leq 0.05$  for all comparisons. The analysis was conducted with Infostat software (Infostat® Software Estadístico) with a connection to R.

## Results

To produce the antigen at industrial scale; the cell culture conditions, the protocol of infection with the recombinant baculovirus, and the downstream processing of the antigen, needs to be standardized. In order to setup the industrial method, different spinner flasks and bioreactors, customized media with and without fetal bovine serum (FBS), culture and infection conditions, and filtration systems were tested and analyzed (data not shown). The process was validated using the single-use WAVE™ bioreactor system, SF900 II serum free media and a two-step filtration system. As a result, cell concentrations of up to  $6 \times 10^6$  cells/ml were achieved and yielded approximately 5 mg/L of APCH-E2 antigen. This yield allows the production of 3.3 dose per ml of culture with an antigen production cost that is similar to transgenic plants and lower of mammalian cells (**Table S1**). The flowchart of antigen production is presented in **Figure 1**. The antigen is then formulated with an oil adjuvant (Marcol/Arlacel) to get a water-in-oil stable emulsion. The entire process is documented and validated following GMP guidelines.

The Argentinean National Regulatory Authority (SENASA) approved the potency assay described above (See Methods). There was found to be a correlation between the guinea pig VN titer and the bovine VN titer:  $\text{Log}_{10}$  titer: 1.37 and  $\text{Log}_{10}$  titer 2.03 in guinea pig correlates with  $\text{Log}_{10}$  titer 1.54 and  $\text{Log}_{10}$  titer 2.13 in cattle, respectively. These VN titers also correlate with the competition ELISA PD values of 12% and 35% respectively (**Table S2**).

To date, eleven commercial batches of the targeted vaccine has been submitted to SENASA. All of them have been approved, with seven being classified as highly satisfactory and four as satisfactory vaccines in this guinea pig model. A dot blot graph of NAb titer results for batches 1 to 11 is presented in the **Figure 2**.

A field trial in cattle was performed to evaluate the immune response of this new vaccine and to compare the performance of this targeted vaccine with a conventional vaccine formulated with killed BVDV. The field trial was carried out in a commercial herd under normal management conditions with approval from SENASA. There were no changes in animal feeding, health, movement or any other parameter or condition during the trial. On the farm selected to run the trial, there was circulation of BVDV; therefore, neutralizing antibody titers against BVDV were observed at the beginning of the study (**Figure 3**). Taking this into consideration, to present the data we subdivided each treatment into two groups: 1) animals with competition ELISA PD values  $\leq 35\%$ , and 2) animals with competition ELISA PD values  $> 35\%$  at the start of the trial. The PD 35% threshold was selected because it correlates with the competition ELISA measurements with



the cutoff limit used to evaluate the satisfactory efficacy of vaccines in the SENASA-approved guinea pig model (**Figure 3 A & B**). A significant increase in the NAb titer was observed in the animals within the targeted vaccine group that started the trial with a competition ELISA result  $\leq 35\%$ , NAb titers increased from  $\text{Log}_{10}(1.43)$  at T0 to  $\text{Log}_{10}(2.43)$  at T60. On the other hand, in the conventional vaccine group at T60 no significant change was observed in the NAb titers of the animals regardless of their T0 titers. All the animals in both groups were temporally sampled and the immune response was evaluated by ELISA (**Figure 4 A, B & C**). The targeted vaccine group had a greater immunological response than the conventional vaccine based on inactivated BVDV virus, in terms of the induction of antibodies to BVDV and the duration of the immune response. The targeted vaccine group developed a strong antibody response to BVDV at 30 days after the first dose of vaccine (**Figure 4A**). The antibody titers to BVDV remained high during the trial up to 360 days post-vaccination. Contrastingly, animals in the conventional vaccine group presented a non-homogeneous antibody response; some animals increased their antibody titers while others remained at their basal competition-ELISA antibody titers. Significant differences were found between groups at all the timepoints analyzed from 30 dpv to the end of the trial.

To have a better understanding of the performance of the vaccine, animals with a lower level of antibody titers (PD  $\leq 35\%$ ) at the beginning of the trial were analyzed independently (**Figure 4B & 4C**). There were no significant differences in the mean antibody titers of these animals at day 0 (T0). Results in Figure 4B and 4C show that animals that started the trial with PD antibody titers below 35% and were vaccinated with the targeted vaccine ultimately reached similar levels of antibody titers to those animals that started with PD% titers  $>35\%$  by the end of the study period. In contrast, the animals in the PD  $\leq 35\%$  antibody titer subgroup of the conventional vaccine group had a small increase in antibody titers at day 120, peaking at 27% of displacement in the competition ELISA, which then declined by the end of trial (day 360) and finished with a mean titer of 9% of displacement in the competition ELISA. Furthermore, these animals within the conventional vaccine group never attained similar antibody titers to the conventionally vaccinated animals of the PD  $>35\%$  subgroup. Within the PD  $\leq 35\%$  subgroup there were eleven seronegative animals (PD  $\leq 10\%$ ), six in the targeted vaccine group and five in the conventional vaccine group. The six seronegative animals in the targeted vaccine group had a similar antibody increase as those animals in the PD  $\leq 35\%$  subgroup at all the timepoints analyzed (T 30, 60, 120, 180, 300 & 360), meaning they responded positively to the vaccine. Conversely, seronegative animals in the conventional vaccine group had a small increase in their antibody

levels, but the mean titer was lower than the PD  $\leq$ 35% subgroup at all the timepoints analyzed (data not shown).

## Discussion

The commercial production and downstream processing of this novel targeted vaccine, was established and standardized. The antigen is produced in Sf9 cells using a baculovirus expression vector system with customized media in single-use wave bioreactors. It is a flexible technology, with potential incorporation of multiple antigens into a single formulation. Studies from our group have previously shown that a recombinant subunit vaccine containing BEVS derived E2 proteins from three different strains of BVDV (BVDV-1a, -1b and -2a) each, fused to the APCH molecule, was able to induce protection in colostrum-deprived calves challenged with BVDV (Pecora, Malacari, Perez Aguirreburualde, et al. 2015)..

The guinea pig model is used by the Argentine government authorities since it is a reliable tool that consistently predicts the performance of the vaccine in the field. This model for BVDV vaccine potency testing was presented at the XXII Seminar on Harmonization of Registration and Control of Veterinary Medicines Americas Committee for Veterinary Medicines (CAMEVET) (<https://rr-americas.oie.int/en/events/xxii-seminar/> Mexico, 2016) and a group of experts from different countries is revising the guideline in order to implement this guinea pig model in different American countries (validation study in progress).

In the cattle field trial, this new targeted vaccine induced a more potent and longer lasting immune response when compared to the conventional inactivated vaccine. All animals within the subunit vaccine group developed high antibody titer levels at day 30 that remained elevated until the end of the trial at day 360 post vaccination. In contrast, the conventional vaccine group animals did not have a significant increase in antibody titers at days 30 or 60, and subsequently, the antibody levels decreased to basal levels by day 120 and continued decreasing until the end of the trial. Animals within the subunit targeted vaccine presented higher antibody levels after vaccination than the conventional vaccine group at every time-point analyzed. In addition, the level of antibodies reached by the targeted vaccine in this field trial are similar to the results published by Downey-Slinker et al using a live attenuated vaccine for BVDV (Downey-Slinker et al. 2016) suggesting the targeted vaccine may produce similar levels of protection as a live attenuated vaccine, but with the safety of an inactivated virus vaccine.

The northern region of Argentina is characterized as having a wet, hot, and subtropical weather. Within cattle herds in this region, such as the one chosen for this field trial, it is common to find animals with varying levels of BVDV-specific antibody titers. In these instances, it has previously been shown that animals with lower BVDV-specific antibody titers are more susceptible to the viral infection (S. R. Bolin and Ridpath 1996). The main goal of a BVDV vaccination program is to protect these animals since 90% of PI calves are born from non-PI cows (Wittum et al. 2001). In the field trial it was shown (Figure 4C) that the new targeted vaccine is able to significantly increase NAb titers to levels that correlate with protection in these seronegative and low-titer animals suggesting an increase in protection from day 30 post-vaccination. This potential protection lasted throughout the course of the trial (360 days post-vaccination) (S. R. Bolin and Ridpath 1996). On the other hand, the susceptible bovine population within the conventional vaccine group had no significant changes in antibody levels after vaccination.

It is also interesting to note that the standard deviation (SD) of the mean Ab titers in both groups is very different. The SD in the targeted vaccine is, at least, half the SD observed in the conventional vaccine group at most of the analyzed timepoints (Figure 4A). This may suggest a more consistent immunological response is induced by the targeted vaccine than conventional vaccine or a consequence of circulating BVDV in the herd that affected the antibody responses of the two groups differently. It is also clear in Figure 4A that at day 0 there is a high degree of variation of antibody levels across the entire herd, ranging from zero to 87% PD. At day 180, all animals of the targeted vaccine group are concentrated in a range from 55% to 90% PD, but animals in the conventional vaccine group exhibit a greater variation, ranging from 16% to 88% PD. In the conventional vaccine group, animals with high antibody titers to BVDV at the beginning of the trial maintained high antibody titers at the end of the trial. In contrast, animals with no or low antibody titers did not increase their antibody level over the course of the trial and, therefore, remained susceptible to virus infection. On the other hand, in the targeted vaccine group, all animals reached high antibody titers to BVDV independent of their initial antibody titers, indicating the targeted vaccine is able to induce a potent immune response in low-titer and, more importantly, in seronegative animals.

The targeted vaccine represents a new and improved vaccine against BVDV with the advantages of attenuated vaccines in terms of immunogenicity but with the safety profile of inactivated vaccines. Safety is a key issue in BVDV control programs since the vaccination of pregnant cattle with an attenuated vaccine can lead to the development of a persistently infected animal (Palomares et al. 2013) and an emerging disease

named, bovine neonatal pancytopenia, has been associated with an inactivated vaccine (Deutskens et al. 2011). Therefore, veterinarians and farmers demand the introduction of safe and efficacious vaccine. This novel subunit targeted vaccine satisfies safety requirements since it can be applied to pregnant cattle without the risk of generating PI animals and efficacious requirements since it is potent enough to confer a long-lasting immune response even in seronegative animals. It implies a reduction in the doses applied over the lifespan of a cow, reducing the cost of vaccination and animal movements. It is also a flexible platform that can be used to produce a new generation of targeted vaccines against a variety of viral, bacterial, or parasite antigens.

In conclusion, the development of this novel subunit targeted vaccine provides cattle and dairy producers with an economical, easily administered, safe, and efficacious BVDV vaccine.

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**Animal Welfare:** Guinea pig and cattle 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).

**Authors contribution:** DB is the leader of this project, JB produced the antigen and the vaccine used in cattle, LR analyzed serum samples from guinea pig and cattle, AP made the recombinant BEVS construct, JAE discovered the APCH molecule, MA was the veterinarian in charge of the field trial, VP conducted the statistical analysis, participated in the experimental design, and revised the article, AW is the PI of the lab, he maintained program funding and helped design and supported the project and revised the manuscript. All authors attest they meet the ICMJE criteria for authorship.

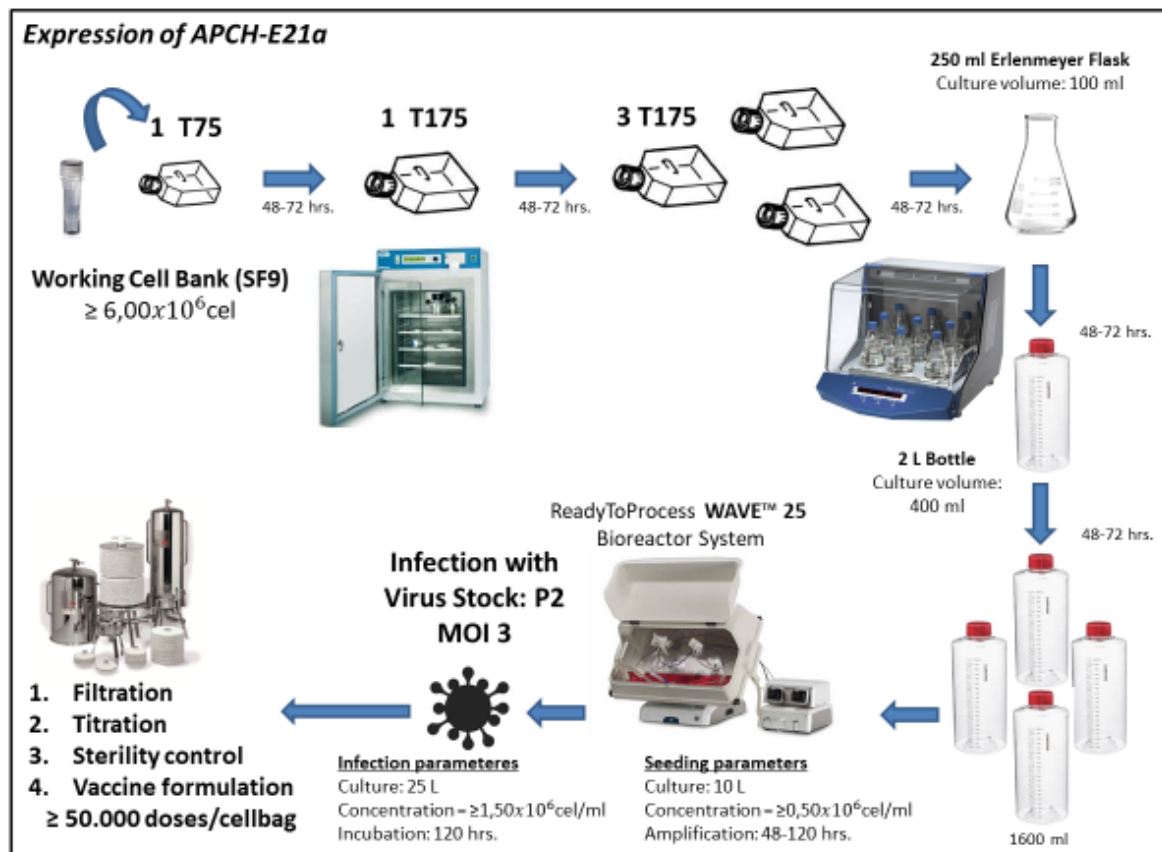
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**Competing interests:** D Bellido, J Baztarrica, A Wigdorovitz and M Acosta work for Bioinnovo - Vetanco SA; and J.M Escribano works for Algenex.

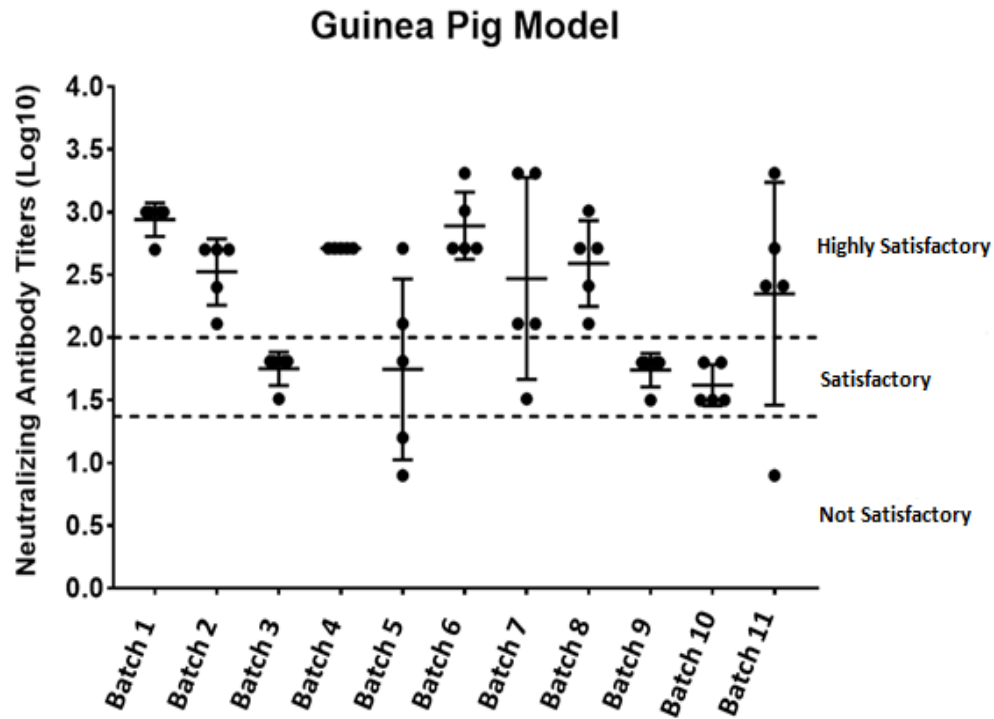
**Data availability statement:** The data that support the findings of this study are available on request from the corresponding author. Certain data, including DNA sequences, vaccine processing and formulation, or farm location is considered proprietary.

### Figure Captions

**Figure 1:** Production flowchart of the APCH-E2 antigen.



**Figure 2:** Guinea Pig Model



**Figure 2:** Results obtained with all the batches of the targeted vaccine that were approved to be released into the market using the guinea pig model of vaccine potency. The dots within each batch (1 to 11) represents an individual guinea pig and the lines and bars represent the mean NAb titers  $\pm$  standard deviation, the dotted-dashed lines represent the split point for vaccine classification according to SENASA's predetermined level of immunogenicity.

**Figure 3: Antibody Response**

**Fig 3.A: Virus Neutralization Assay**

## Antibody response in all animals of both groups

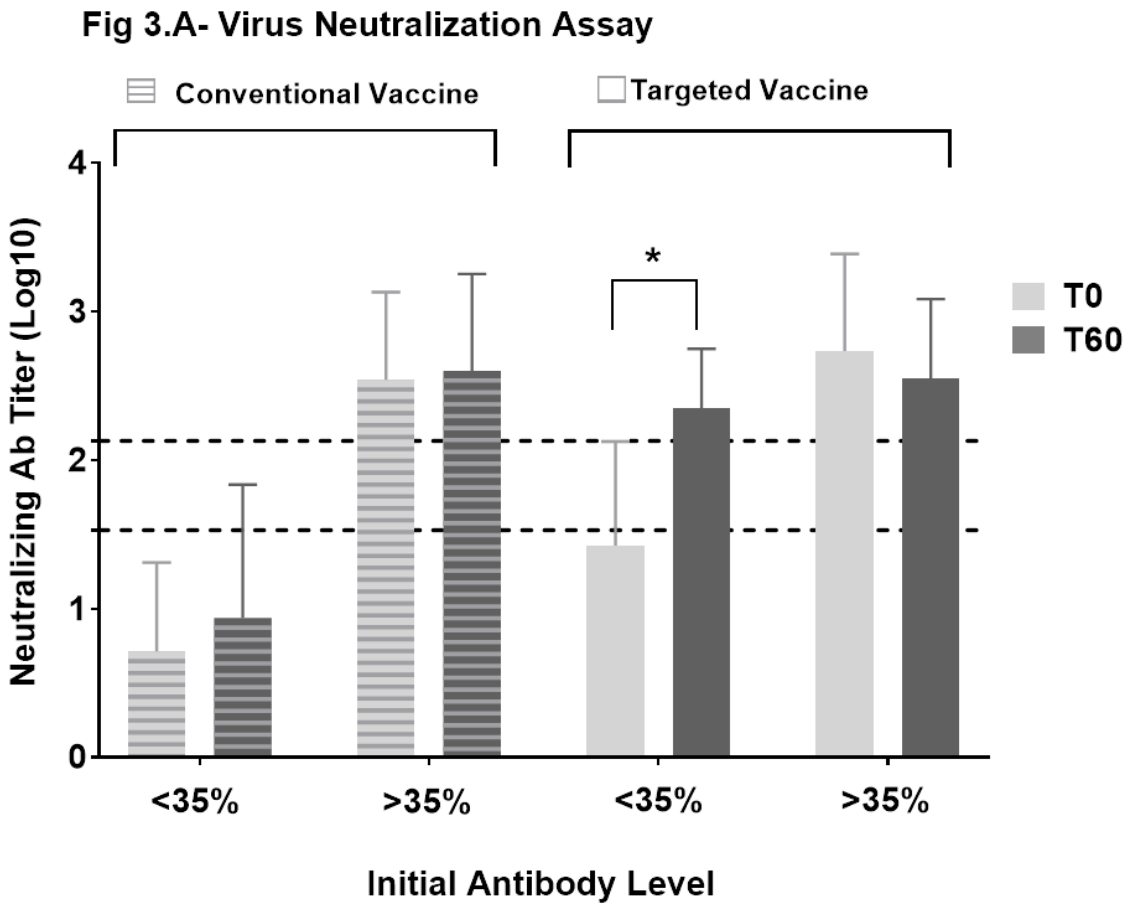
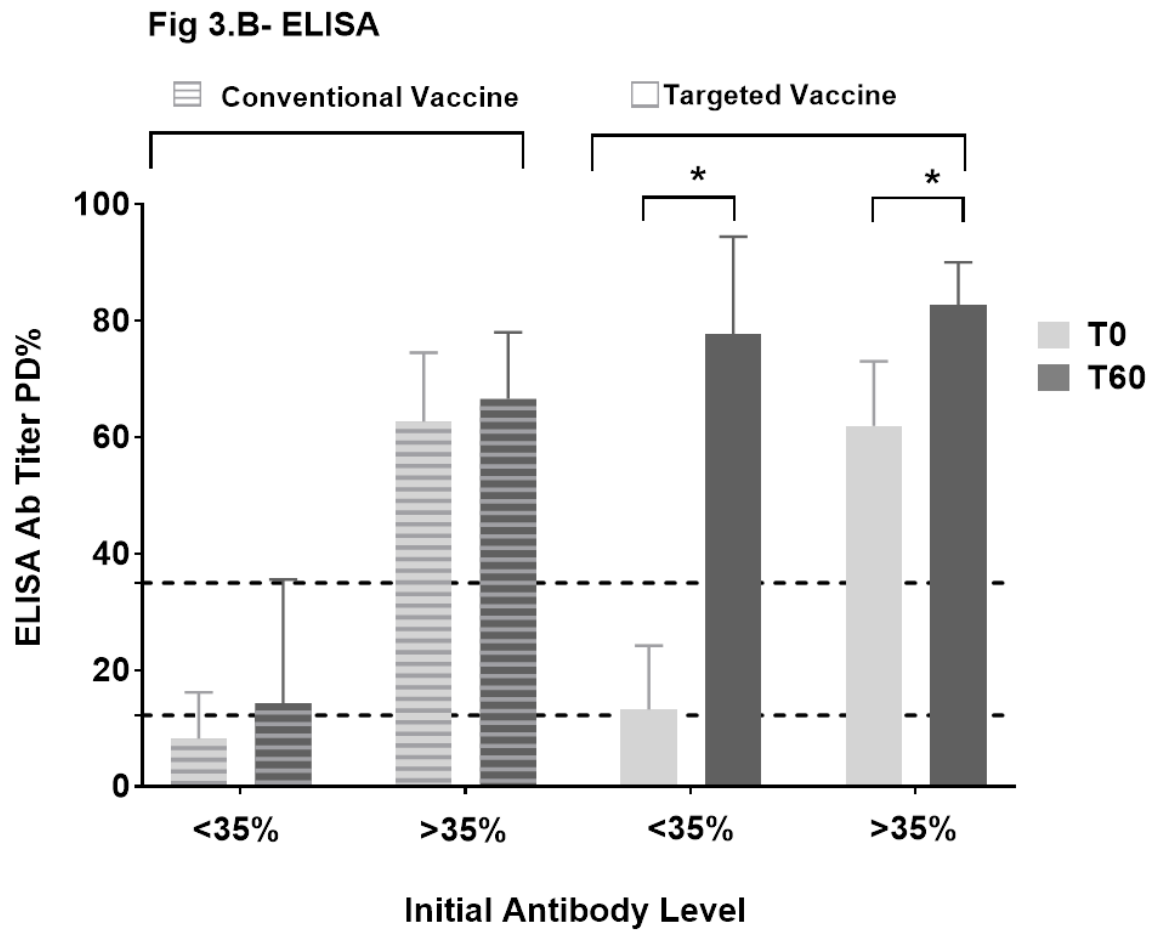


Fig 3.B: Competition ELISA



**Figure 3 A & B:** Antibody responses in all animals of both groups were evaluated by VN assays (Fig 3.A) at study day 0 and 60 and by a specially designed competitive ELISA to BVDV (Fig 3.B). Light Grey Bars: T0 Dark Grey Bars: T60; Striped Bars: Conventional Vaccine; Solid Bars: Targeted Vaccine. For bovine samples, a 35% displacement ELISA value correlates with a 1/32 NAb titer. Dotted-dashed lines represent the split point for vaccine classification according to SENASA's predetermined level of immunogenicity. \*Denotes statistically significant differences ( $p < 0.05$ ).

**Figure 4 A, B & C:** Kinetics of antibody response after vaccination

**Fig 4.A:** Targeted Vaccine vs Conventional Vaccine. All Animals



Fig 4.A- Targeted Vaccine vs Conventional Vaccine

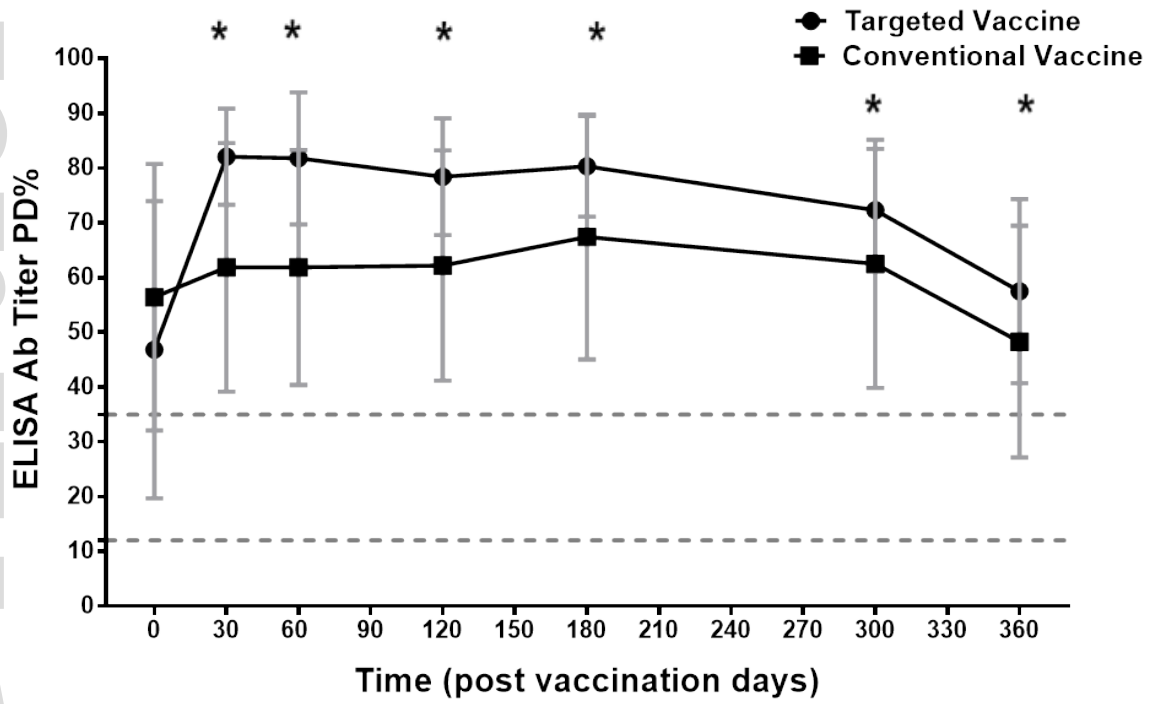


Fig 4.B: Targeted Vaccine vs Conventional Vaccine. Initial Ab Level > 35%

Fig 4.B- Targeted Vaccine vs Conventional Vaccine  
Initial Ab Level >35%

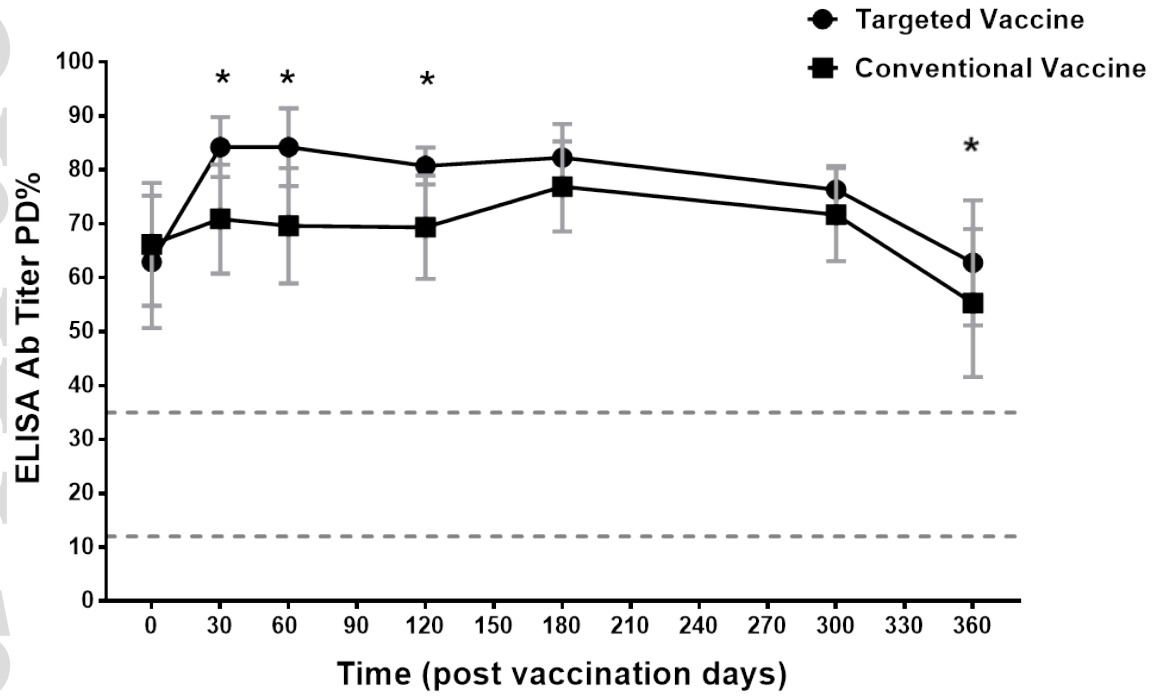
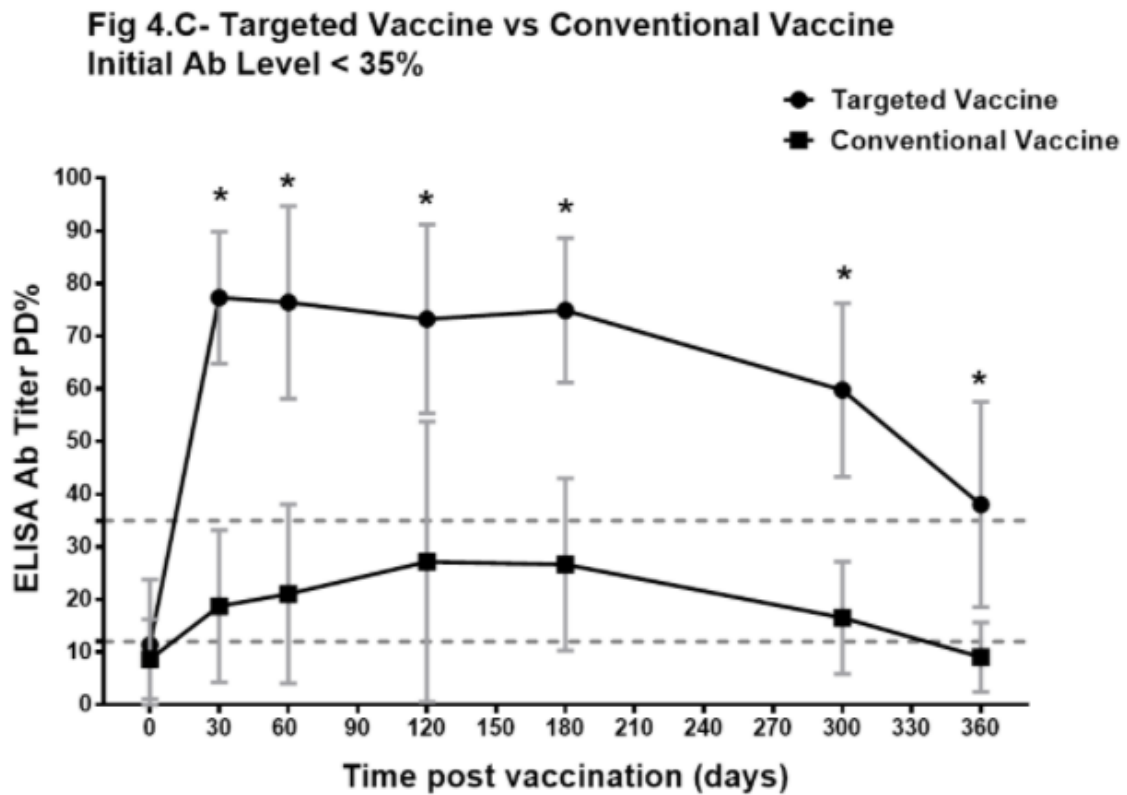


Fig 4.C: Targeted Vaccine vs Conventional Vaccine. Initial Ab Level < 35%



**Figure 4 A, B & C:** Kinetics of antibody response to BVDV after vaccination. A: All animals; B: animals with initial antibody titers >35% by PD-ELISA; C: animals with initial antibody titers <35% PD. ●: Targeted vaccine group; ■: Conventional vaccine group. Dotted-dashed lines represent the split point for vaccine classification according to SENASA's predetermined level of immunogenicity. \*Denotes statistically significant differences ( $p < 0.05$ ).

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