Rational design of the first commercially available subunit vaccine against BVDV targeted to immune system cells

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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 reduce productive performance, growth retardation, reduced milk production, and increased susceptibility to other diseases leading to early culling of animals. There are two main measures 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 a 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. The development of this subunit targeted vaccine provides cattle and dairy producers with an inexpensive, easily administered, safe, and efficacious BVDV vaccine.

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 the 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 cattle with BVDV in the first trimester of gestation can result in the production of a 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 born from 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 immunogenicity. 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 of 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 increases 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 different experimental vaccines, improving both humoral and cellular immune responses in immunized animals as it targets the antigen to the APCs (Gil et al. 2011). The *APCH-E2* fusion gene was engineered into 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 (Dus Santos et al. 2009). Guinea pigs intramuscularly immunized with leaf extracts developed high NAb titers. In bovine vaccinated with 3 µg of alfalfa produced APCH-E2, BVDV-specific NAbs 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). 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. 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. 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 et al. 2015, 2016).

Materials and Methods

Virus strains and cells: Cytopathic BVDV-1a (Singer strain) was used to develop the subunit vaccine and to perform Virus Neutralization (VN) assays. MDBK cells were used to propagate the virus. Cells were grown in Earle's minimal essential medium (EMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2% heat inactivated FBS (Internegocios S.A.). Recombinant baculovirus expressing the APCH-E2 antigen was generated as described by Pecora et al (Pecora et al. 2015).*Spodoptera frugiperda* (SF9) cells were used to produce the recombinant vaccine. SF9 cells were cultivated in a customized SF900 II Serum Free Medium. SF9 cells were infected at a multiplicity of infection (MOI) of 3 with the recombinant baculovirus and incubated at 27 ºC for 120 h to produce the antigen.

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 ($1x10^6$, $1x10^7$,or $1x10^8 TCID_{50}$/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 ($Log_{10} < 1.37$), of Satisfactory immunogenicity if 1:24 [?] NAbs titer [?] 1:100 (Log_{10} 1.37 [?] NAbs titer [?] Log_{10} 2), or of Highly Satisfactory immunogenicity if the mean NAbs titer [?] 1:100 ($Log_{10} < 2$). (Res. SENASA 2012).

Virus Neutralization (VN) assay: according to (Aguirreburualde et al. 2013)

Competition ELISA : 96-well Nunc Maxisorp ELISA plates were coated with a bivalent llama-derived nanobody in carbonate-bicarbonate buffer (Perez Aguirreburualde 2014; Zamit 2010) directed to E2 protein from BVDV overnight at 4 oC, followed by a blocking step with 1% skim milk the next day for 1 hour at 37 oC. The subsequent steps were also incubated with these conditions. Plates were washed three time with PBS-T between steps. Then, 6 ng/well of E2 protein produced in the BEVS was added to the appropriate wells. Bovine serum samples were added in a unique 1/4 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) 25 ng/well. The ELISA Ab titer was expressed as a percentage of displacement (PD%) of the positive 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 <12%, satisfactory vaccines [?]35%ND highly satisfactory vaccines < 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 (3 ml/dose) and 54 with a conventional reproductive vaccine (5 ml/dose), which contains inactivated BVDV. All animals were vaccinated twice, beginning on study day 0 and again study day 30. Blood was drawn from all animals on study days 0, 30, 60, 120, 180 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.

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 heterogenicity of variances at different time points (varIdent). Statistical significance was assessed at p ¡0.05 for all comparisons. The analysis was conducted with Infostat software with a connection to R (Di Rienzo et al. 2013).

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 x 10^6 cells/ml were achieved and yielded approximately 5 mg/L of APCH-E2 antigen. The flowchart of antigen production is presented in **Figure 1** . The antigen is then formulated with an oil adjuvant (Marcol/Arlacel). The entire process is documented and validated following GMP guidelines.

The Argentinean National Regulatory Authority (SENASA) approved potency assay described above (See Methods). There was found to be a correlation between the guinea pig VN titer and the bovine VN titer: titers of 1:24 (Log_{10} 1.37) and 1:107 (Log_{10} 2.03) in guinea pig correlates with 1:32 (Log_{10} 1.54) and 1:134 (Log_{10} 2.13) in cattle, respectively. These VN titers also correlate with the competition ELISA PD values of 12% and 35% respectively (**Table 1**).

SENASA uses the guinea pig model of BVDV infection to test every batch of cattle vaccine to ensure the potency of the vaccine before it is released to the market. For BVDV vaccines, the NAb titers induced in guinea pigs were statistically validated as a reliable indicator to predict vaccine immunogenicity in bovines (See Methods). Depending on the performance in this model, vaccines can be classified into three categories: NAbs titers less than Log_{10} 1.37 are not satisfactory vaccines (not approved for sale), NAbs titers between Log_{10} 1.37 and Log_{10} 2.03 are satisfactory vaccines (approved for sale) and NAbs titers greater than Log_{10} 2.03 represent highly satisfactory vaccines (approved for sale). 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. In the farm selected to run the trial there was circulation of BVDV and therefore neutralizing antibody titers against BVDV were observed at the beginning of the study. Taking this into consideration, to present the data we subdivided each treatment into two groups: 1) animals with competition ELISA PD values [?]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 [?]35%, Nab titer increased from Log_{10}:1,43 in T0 to Log_{10}: 2,43 at T60. On the other hand, in the conventional vaccine group at T60 no significantly modification 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 has 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 non-homogeneous antibody response; some animals increased their antibody titers while others remained in their basal competition-ELISA antibody titers.

To have a better understanding of the performance of the vaccine, animals with a lower level of antibody titers (PD [?]35%) at the beginning of the experience were analyzed independently (**Figure 4B & 4C**). There were no significant differences in the mean antibody titers of these animals at day 0. 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 [?]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.

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 et al. 2015). This is a clear indication the production platform used for this new single-strain vaccine has potential as a universal and adaptable platform to develop a cost-effective and efficacious vaccine against all BVDV strains. With this strategy, it is possible to make a single vaccine to use worldwide; or, if there are significant regional strain variation, it is feasible to modify the E2 antigen to develop a region-specific BVDV vaccine. Furthermore, the single use baculovirus expression platform and the APCH targeting molecule could be used to develop new targeted vaccines against other viruses, bacteria, or parasites.

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 on 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). All commercial batches of this new targeted vaccine were able to pass this test, with several labelled with the highest potency qualification (highly satisfactory), indicating this production process is reproducible and robust.

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 presented high antibody titer levels at day 30 that remained high 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 at day 120 and continued decreasing until the end of the trial. Animals within the subunit targeted vaccine presented higher antibodies level after vaccination than the conventional vaccine group in every time-point analyzed.

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 the viral infection (S. R. Bolin and Ridpath 1996). The main of goal of a BVDV vaccination program is to protect these animals since 90% of PI animals are born from non-PI cows (Wittum et al. 2001). In the field trial it was shown that the new targeted vaccine is able to significantly increase NAbs 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 antibodies levels after vaccination.

It is also interesting to note that the standard deviation (SD) of the mean Ab titers in both groups it

is very different. The SD in the targeted vaccine is, at least, half the one observed in the conventional vaccine group at most of the analyzed timepoints (Figure 4A). This is another indication of how different, but consistently, the immune response is induced by a targeted vaccine compared to a conventional vaccine that uses inactivated BVDV in the formulation. It is also clear in Figures 4A and 4B that at day 0 there is a high variation of antibody levels going 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 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 seronegative and low-titer animals. Importantly, high BVDV-specific antibodies in the cattle did not inhibit a robust vaccine-specific immune response to the new targeted antigen.

In conclusion, the targeted vaccine represents a new and improved vaccine against BVDV with the advantages of attenuated vaccines in terms of immunogenicity but with the safeness 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 that an inactive vaccine was associated with an emerging disease named bovine neonatal pancytopenia (Deutskens et al. 2011). Therefore, veterinarians and farmers demand the introduction of safe and efficacious vaccine. This new subunit targeted vaccine satisfies these requirements and 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.

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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, participate 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. The data are not publicly available due to privacy or ethical restrictions.

Figure Captions

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

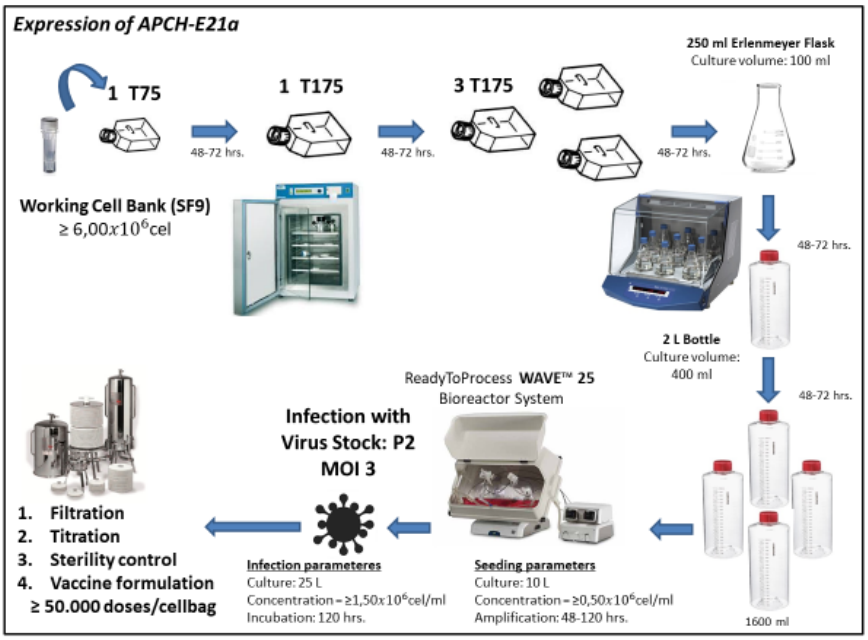

Figure 2: Guinea Pig Model

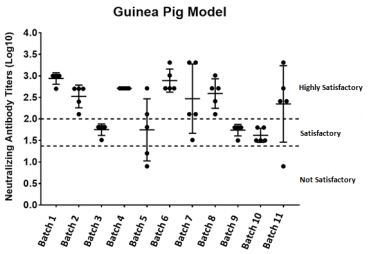

Figure 2: Results obtained with all the batches of the targeted vaccine that were approved to be released into the market using the guinea pig model of vaccine potency. The dots within each batch (1 to 11) represents a guinea pig and the lines and bars represents the mean NAb titers ± standard deviation,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

Table 1. Vaccine classification points for BVDV

SPECIES VIRUS NEUTRALIZATION	**BVDV VACCINE POTENCY** Not Satisfactory	**BVDV VACCINE POTENCY** Satisfactory	**BVDV VACCINE POTENCY** Highly satisfactory
GUINEA PIG	$\bar{Y} < 1.37$	1.37 [?] $\bar{Y}$ [?] 2.0	$2.0 < \bar{Y}$
BOVINE	$\bar{Y} < 1.54$	1.54 [?] $\bar{Y}$ [?] 2.13	$2.13 < \bar{Y}$
ELISA			

GUINEA PIG & BOVINE	$\bar{Y} < 12\%$	12% [?] $\bar{Y}$ [?] 35%	$35\% < \bar{Y}$

Figure 3: Antibody Response

Fig 3.A: Virus Neutralization Assay

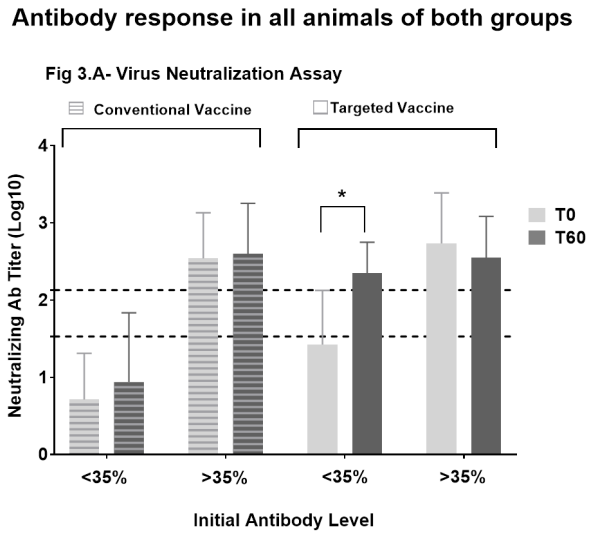

Fig 3.B: Competition ELISA

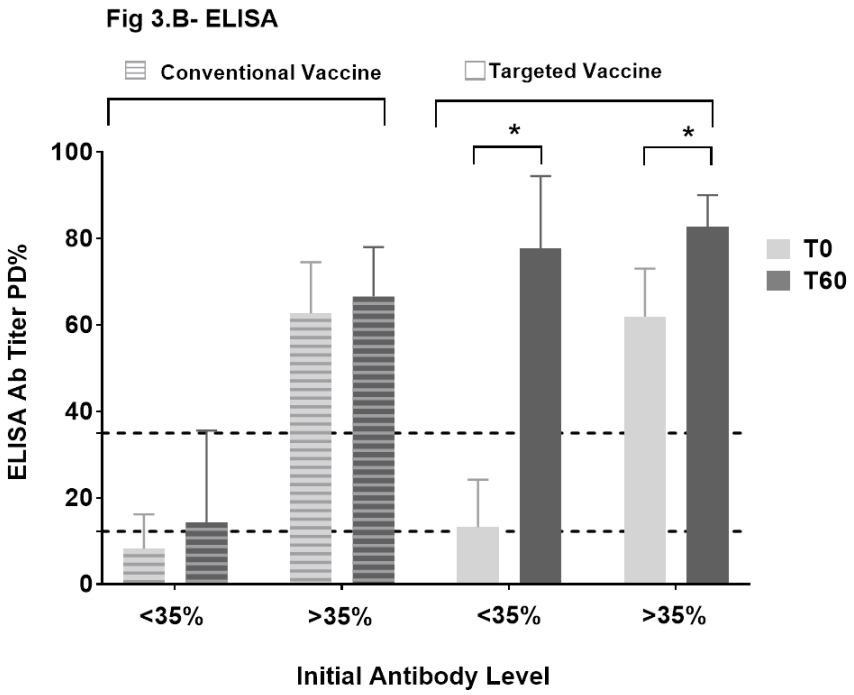

Figure 3 A & B: Antibody responses in all animals of both groups were evaluated by VN assays (Fig 3.A) at 0 and 60 days and by a specially designed competitive ELISA to BVDV (Fig 3.B). Light Grey Bars: T0 Dark Grey Bars: T60; Striped Bars: Conventional Vaccine; Solid Bars: Targeted Vaccine. For bovine samples a 35% displacement ELISA value correlates with a 1/32 NAb titer.

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

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

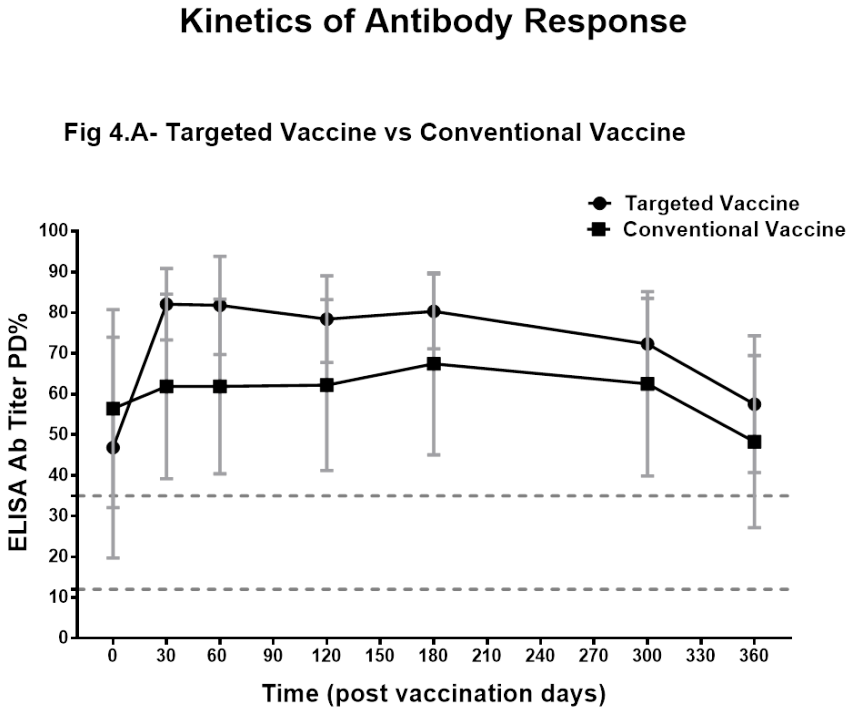

Fig 4.B: Targeted Vaccine vs Conventional Vaccine. Initial Ab Level > 35%

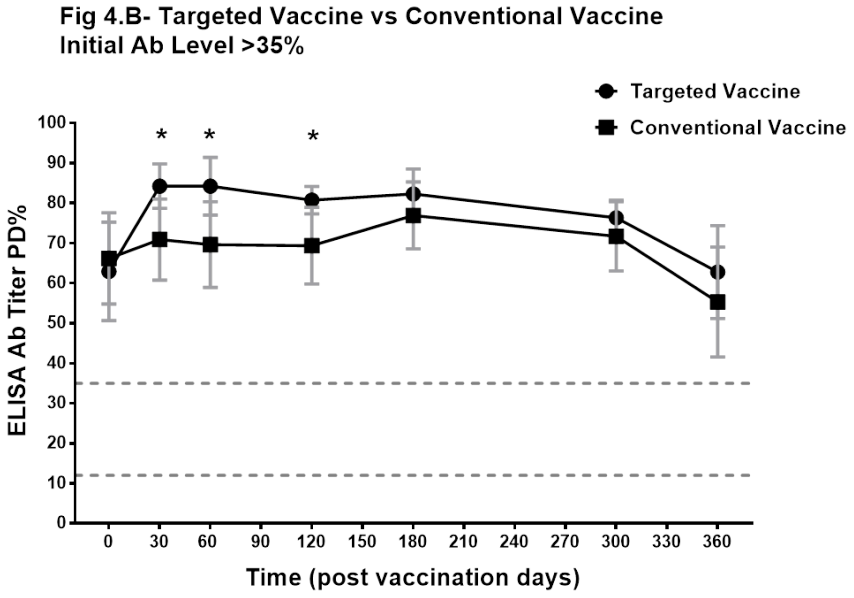

Fig 4.C: Targeted Vaccine vs Conventional Vaccine. Initial Ab Level < 35%

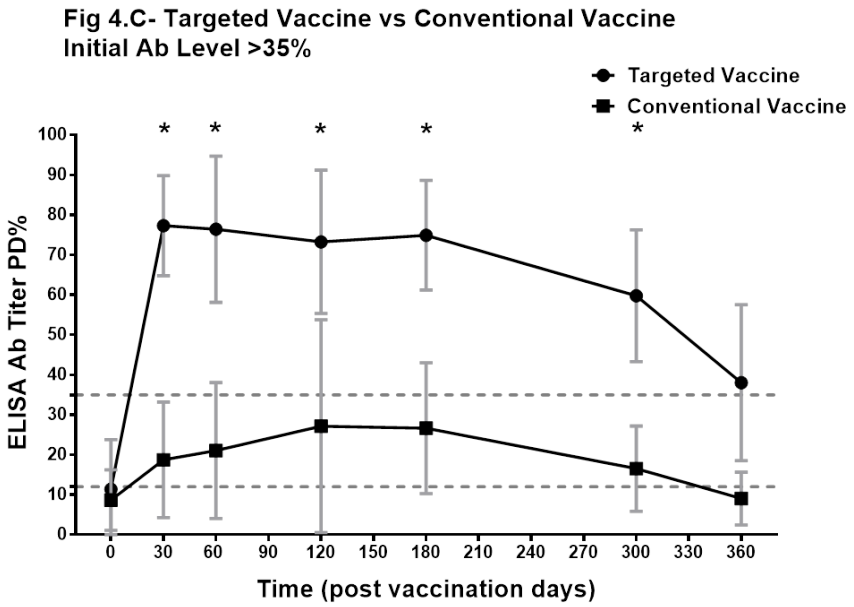

Figure 4 A, B & C: Kinetics of antibody response to BVDV after vaccination. A: All animals; B: animals with initial antibody titers >35% by PD-ELISA; C: animals with initial antibody titers <35% PD Targeted vaccine group; Conventional vaccine group. Dotted-dashed Lines at 12% and 35% indicate the PD% values that correlate with the threshold establish for qualifying vaccines: low-quality vaccines, <12% PD, satisfactory vaccines <35% PD.

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