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Development and validation of an ELISA for quantitation of Bovine Viral Diarrhea Virus antigen in the critical stages of vaccine production

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Bovine Viral Diarrhea Virus (BVDV) is the causative agent of a worldwide disease. The virus infects bovines of all ages, causing reproductive problems and contaminating biological products of high commercial value. The large-scale production of BVDV vaccines presents the challenge of processing antigenic proteins that are highly susceptible to the processing environment. Potency testing requires the immunization of cattle in order to determine the neutralizing antibodies titers induced by the vaccine. An alternative to the in vivo test is an in vitro measurement of key viral antigens.

This paper describes the development and validation of a sandwich-type indirect ELISA that is able to detect and quantify BVDV E2 glycoprotein in live and inactivated BVDV. Validation parameters such as repeatability, intermediate precision, and reproducibility indicated that the developed ELISA constitutes an advanced tool for evaluating the BVDV antigen throughout manufacturing and vaccine release testing.

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1. Introduction

Bovine Viral Diarrhea Virus (BVDV) is a small, enveloped single-stranded RNA virus that belongs to the genus *Pestivirus* within the *Flaviviridae* family. BVDV is the causal agent of a variety of clinical conditions, including congenital defects, diarrhea, reproductive failure, mucosal disease, respiratory disease and hemorrhagic syndrome resulting from thrombocytopenia (Baker, 1995; Perdrizet et al., 1987). In pregnant animals, trans-placental infection can lead to abortion, stillbirth, malformation and persistent infection for the calves. Persistently infected (PI) animals act as a viral reservoir and are a source of contamination in cattle herds. Furthermore, since BVDV is a contaminant of bovine-derived products and subproducts (i.e., commercial fetal calf serum), susceptible mammalian cell lines are often found contaminated with non-cytopathic strains (Bolin et al., 1994).

First described over 60 years ago, BVDV continues to cause significant economic losses to the cattle industry worldwide. In Argentina, the prevalence of BVDV antibodies in adult cattle is approximately 70% (Pacheco and Lager, 2003; Rweyemamu et al., 1990). Analyses of normal fetuses have shown that 20% are infected (Munoz et al., 1996) and 2% are seropositive (Pinto et al., 1993). The presence of both BVDV type 1 and BVDV type 2 has been reported in Argentina; however, 90% of isolates have been genotyped as BVDV type 1 (Jones et al., 2001). In Argentina the prevention strategy is the systematic vaccination of herds.

The large-scale production of BVDV inactivated vaccines presents the challenge of processing protein antigens that are highly susceptible to the processing environment. According to Title 9 of the Code of Federal Regulations (9.CFR), vaccine potency testing for batch approval requires the immunization of seronegative calves and subsequent detection of virus neutralizing antibodies (NAbs) induced by the vaccine. This procedure is expensive and time-consuming. Additionally, enrolling BVDV seronegative animals is becoming increasingly difficult due to the endemic nature of the infection. An alternative is the measurement of viral antigens by ELISA. Data reported for other viral systems supports the introduction of in vitro assays to measure vaccine potency (Claassen et al., 2004; Fournier-Caruana et al., 2003; Maas et al., 2000; Pombo et al., 2004; Rooijakkers et al., 1996).

The BVDV E2 glycoprotein is known to be the most immunogenic viral protein, capable of inducing NAbs that are involved in protection against the disease (Deregt et al., 1998; Fulton et al., 1997; Paton et al., 1992). A number of experimental recombinant vaccines against BVDV are based on the expression of glycoprotein E2 in heterologous systems, such as baculovirus (Kweon et al., 1997), bovine herpesvirus 1 (BoHV1) (Schmitt et al., 1999), and fowlpox vectors (Elahi et al., 1999). In addition, the E2 gene has been

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successfully used in plasmid DNA immunization protocols (Harpin et al., 1999; Liang et al., 2008). Altogether this data indicates that the glycoprotein E2 constitutes a critical antigen for evaluating vaccine potency in commercial BVDV production.

In this work, the development and validation of a sandwich-type indirect ELISA is described. The assay is able to detect and quantify BVDV E2 glycoprotein in live and inactivated virus. The ELISA presented a limit of quantitation of 7.81 ng/ml, and it was highly repetitive and reproducible. In conclusion, the developed ELISA constitutes an advanced tool for BVDV antigen evaluation that it is aligned with the '3Rs' initiative of refining, reducing and replacing animal experimentation.

2. Materials and methods

2.1. Recombinant antigen preparation

A stable CHO-K1 cell line that expressed a truncated version of glycoprotein E2 (tE2) was previously developed (unpublished results). Briefly, 1×10^5 CHO-K1 cells/well were transfected with 0.2 μg/ml of the recombinant plasmid (pCDNA3.1-tE2) and 2 μl of Lipofectamine Reagent (Invitrogen, California, USA) in D-MED medium without fetal bovine serum (FBS) (Internegocios S.A., Buenos Aires, Argentina). At 24 h after transfection and recovery, a selection of stable clones were carried over using Zeocin at a final concentration of 700 μg/ml in D-MEM medium and were supplemented with 10% FBS (Internegocios S.A.). Once resistant cells were obtained, they were cloned by final dilution in 96 well plates. This procedure was repeated twice. The expression of tE2 was screened by western blot. Finally, the stable cell lines were frozen in liquid nitrogen with E-MEM, 20% FBS (Internegocios S.A.) and 10% DMSO (Sigma–Aldrich, St. Louis, MO, USA).

The developed CHO-tE2 cell line was cultured with D-MEM medium supplemented with 10% FBS (Internegocios S.A.) and 2% DMSO (Sigma–Aldrich). The supernatants were collected after 7–8 days of culture; they were then centrifuged for 30 min at 2000 × *g* and used for protein purification by immobilized metal affinity chromatography (IMAC). PMSF and leupeptin were added as protease inhibitors. A 10× Binding/Washing (BW) buffer (NaCl 3 M, NaHPO4 0.5 M) and 13 mM imidazole were added to the cell cultures in order to increase the ionic strength and reduce the non-specific protein binding. Ni-NTA resin (2 ml/l supernatant) (Qiagen, Hilden, Germany) was also used. The supernatants were incubated overnight with shaking at 4 °C. The resin was washed with 1× BW buffer (containing 13 mM imidazole) until the absorbance at 280 nm was negligible. The protein was eluted from the resin with 1× BW buffer containing 200 mM imidazole. The eluates were washed with phosphate-buffered saline (PBS) and concentrated using Amicon-Ultra 4 centrifugal filter devices (Millipore, Billerica, USA). The purity and protein concentrations were determined by SDS-PAGE and Coomassie blue staining using a bovine serum albumin (BSA) standard curve followed by densitometry analysis using Image J software. The purified proteins were stored in aliquots of 10 μl at −70 °C. The purified tE2 was used as a quality control (QC).

As a negative control, the supernatant from a stable cell line expressing β-galactosidase (CHO-lacZ) was also processed and stored at −70 °C.

The identity of the tE2 glycoprotein was evaluated by western blot. The purified tE2 was mixed with loading buffer, heated for 5 min at 100 °C and visualized on an 8% SDS-PAGE gel. The gel was electroblotted onto PDVB filters using Biorad TRANS BLOT SD semidry (Biorad, Hercules, USA) at 20 V for 1 h in transfer buffer (0.05 M Tris, 2.5 M Glycine, 20% Methanol, 0.04% SDS). The membrane was blocked in PBS 0.05% Tween-20 (PBS-T) and 3% skim milk for 90 min at 37 °C. The membrane was then incubated overnight at 4 °C with a monoclonal antibody against the E2 glycoprotein (VMRD, Pullman, USA), an anti-tE2 rabbit serum, an anti-BVDV NADL strain rabbit serum, an anti-tE2 bovine serum and an anti-BVDV Singer strain bovine serum. The membrane was washed three times with PBS-T, and it was then incubated with a peroxidase labeled goat anti-mouse IgG (H + L) at 1:1000 dilution (KPL, Maryland, USA). The signal was screened using western Lighting Chemiluminescence Reagent Plus (PerkinElmer, Boston) and Hyperfilm ECL (GE Healthcare, Pittsburgh, USA).

The stability of tE2 in crude supernatant and after purification was assayed by incubating the preparations at 37, 4, −20 and −70 °C for 1, 8 and 21 days. ELISAs were used in addition to determine the stability after three freeze–thaw procedures.

2.2. Antibodies

The monoclonal antibody (MAb) 2.9H against the E2 glycoprotein (Marzocca et al., 2007) was used as the capture antibody. This MAb is able to neutralize Singer and NADL BVDV strains.

A polyclonal antiserum to tE2 was developed in rabbit at Central Biotery, FCEyN, University of Buenos Aires. A female adult rabbit was immunized with two doses of an experimental vaccine formulated with 2.5 μg of purified tE2 in complete Freund adjuvant by subcutaneous injection. Serum samples were taken before and several times after the first immunization in order to monitor the development of the antibody response. When the antibody titer reached a plateau, serum from the rabbit was collected and stored in aliquots at −20 °C.

Rabbit management, inoculation, and sample collection were conducted by trained personnel under the supervision of a veterinarian and in accordance with the protocols approved by the FCEyN, University of Buenos Aires ethics committee on animal welfare.

2.3. Virus

The NADL, Singer, and New York1 strains of BVDV (ATCC) and an Argentinean non-cytopathic isolate (98/124) were cultured in Madin–Darby bovine kidney (MDBK) cells. The culture medium consisted of E-MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2% heat inactivated FBS (Internegocios S.A.). The virus titer was determined by the cytopathic effect in the MDBK cells for the NADL, Singer and New York1 strains and by immunofluorescence for the non-cytopathic strains. The virus stocks were clarified by centrifugation at 600 × *g* and stored at −80 °C.

2.4. ELISA procedure

U-bottom polystyrene microtiter plates (Maxisorp, NUNC, Roskilde, Denmark) were coated with 2.9H monoclonal antibody (1:400 dilution) overnight at 4 °C. The plates were then incubated with PBS-T and 1% skim milk (blocking buffer) for 1 h at 37 °C. The blocking buffer was discarded, and the samples were added at the corresponding dilution and incubated for 1 h at 37 °C.

The plates were washed three times with PBS-T and were incubated with rabbit anti-E2t serum (1:2000) for 1 h at 37 °C. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (KPL) (1:4000) was used as the secondary antibody. The plates were incubated for 1 h at 37 °C. After washing three times, the reaction was developed with H_2O_2 and 0.05% 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) for 30 min. Color development was stopped by the addition of 5% SDS. The absorbance was measured at 405 nm (OD405) in a Multiskan Ex plate reader (Thermo Scientific, Waltham, USA).

The standard curve was derived from the CHO-tE2 cultured cell supernatants. The supernatant was serially diluted at a ratio of 1:2 to the blocking buffer in order to obtain the following E2 concen-

trations: 100, 50, 25, 12.5 and 6.25 ng/ml. Calibration samples were used to estimate the concentration of the unknown samples. The assay was only accepted if the coefficients of variation (CVs) of the triplicates were less than 15% and when the slope and intercept of the calibrating curve diverged less than 15% from the expected value.

2.5. Optimization of ELISA working conditions

On the basis of the procedure described above, the optimal antigen and antibody concentrations were determined through a standard checkerboard titration procedure.

More specifically, 2.9H MAb was immobilized onto 96-well microtiter plates in twofold serial dilutions from 1/50 to 1/1600. Correspondingly, the rabbit anti-tE2 and negative control serum were also twofold diluted from 1/125 to 1/2000. To determine the optimal conjugate dilution, the HRP-conjugated goat anti-rabbit IgG (KPL) was added to the plate at dilutions of 1/1000, 1/2000, 1/3000, 1/4000, 1/5000. The conditions that gave the highest OD405 ratio between positive and negative samples were chosen as the optimal working conditions.

The optimal blocking buffer composition was also investigated; in short, it was found that PBS-T plus 1% skim milk gave a lower background than 1% polyvinyl pyrrolidone or 5% normal horse serum. The optimal exposure time was determined by stopping the reaction after exposure for 10, 15, 20, 30, 40 and 60 min.

2.6. Limit of detection, correlation and limit of quantitation (LOQ)

Negative controls (CHO-K1 cells expressing β-galactosidase) were assayed 21 times in duplicate for the neat and 1/4 diluted samples. The limit of detection (LOD) was estimated by interpolating the mean of 42 values plus 3 standard deviations in a standard titration curve.

Linear regression curves were determined by plotting different concentrations of purified tE2 (500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98 and 0.49 ng/ml) and tE2 in crude supernatant (200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 ng/ml) versus the OD405. Eight replicates were tested in 14 (purified tE2) and 9 (tE2 in crude supernatant) independent assays in order to determine the working range, lower limit of quantitation and absolute correlation.

The linearity of the system was verified by ANOVA using Statistix8 software. The %CV associated with each concentration was calculated and used to determine the limit of quantitation (LOQ).

The LOD for BVDV E2 was determined by testing tenfold dilutions of BVDV before and after inactivation with BEI. A total of seven different BVDV batches were evaluated: four Singer, one NADL, one NY1, and one BVDV field isolate 98/124. The LOD was calculated as the viral titer ($TCID_{50}$/ml) containing an amount of E2 glycoprotein equal to or higher than 3.8 ng/ml.

2.7. Precision

Nine dilutions of purified tE2 (250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 ng/ml) were prepared for use as quality control (QC) samples. Nine twofold serial dilutions of the CHO-tE2 supernatant (200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml) used for the standard curve were also assayed according to the ELISA conditions described above. The dilutions were chosen in order to cover the entire working range. Each sample was assayed in eight replicates within the plate.

The variation between replicates and the intra-assay and inter-assay variations were calculated by ANOVA for a nested model of variance using Statistix8 software.

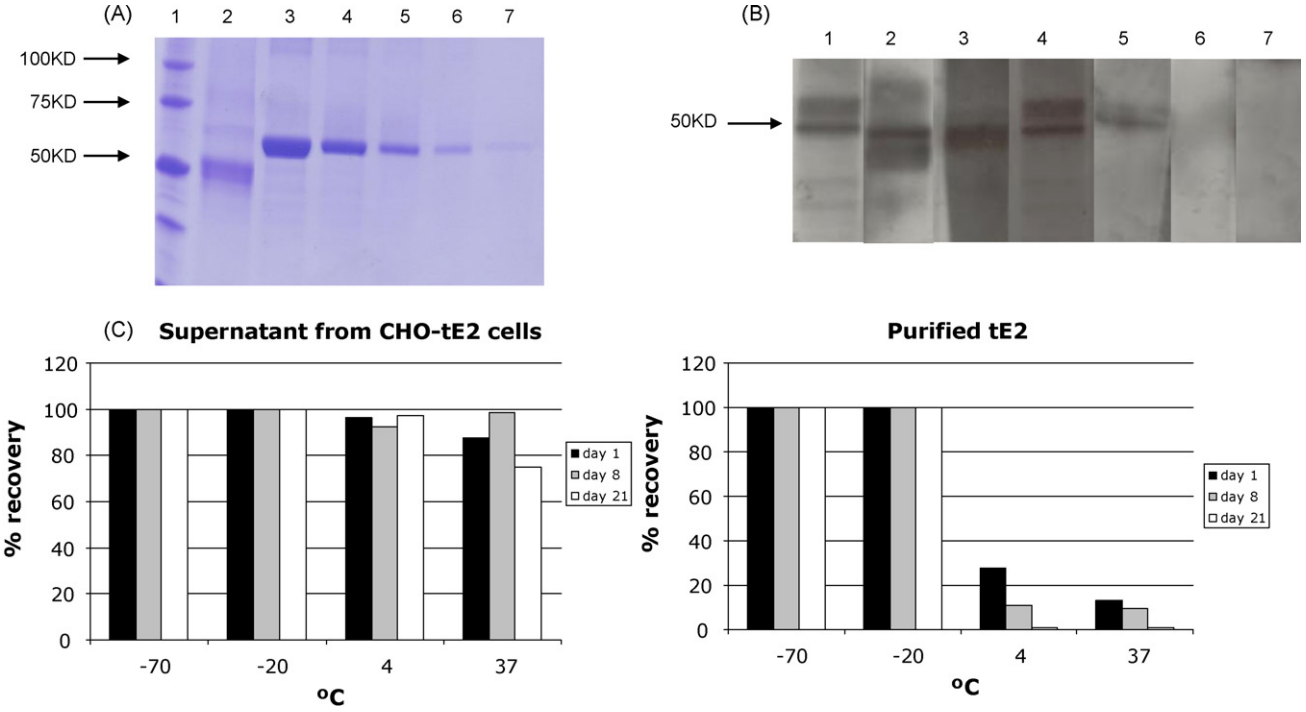

Fig. 1. (A) SDS-PAGE analysis of purified tE2 in 12.5% (w/v) polyacrylamide. Lane 1: Molecular Weight Marker (MWM), Lane 2: purified tE2, Lane 3: BSA 400 μg/ml, Lane 4: BSA 200 μg/ml, Lane 5: BSA 100 μg/ml, Lane 6: BSA 50 μg/ml, and Lane 7: BSA 25 μg/ml. (B) Antigenic characterization of tE2. Western blots were probed with a monoclonal antibody against E2 glycoprotein (VMRD) (Lane 1), an anti-tE2 rabbit serum (Lane 2), an anti-BVDV NADL strain rabbit serum (Lane 3), an anti-tE2 bovine serum (Lane 4), an anti-BVDV Singer strain bovine serum (Lane 5), a BVDV seronegative bovine serum (Lane 6) and a normal rabbit serum (Lane 7). (C) Stability of tE2 in crude supernatant and purified tE2, as measured by the percent recovery of tE2 after incubation at different temperatures for 21 days.

Table 1
Limit of detection.

Assay	Negative control	
	Neat	1/4 diluted
N[a]	21	21
Overall mean	0.14	0.08
Standard deviation	0.02	0.01
%CV	11.82	11.59
LOD[b]	3.80 ng/ml	4.38 ng/ml

[a] Each assay was performed in duplicate.
[b] The LOD was calculated by interpolating the mean value in the titration curve.

2.8. Inter-laboratory assay

Fifteen randomly coded samples including dilutions of purified tE2, tE2 in crude supernatant, BVDV stocks and negative controls (supernatant from CHO-lacZ) together with capture, primary and developing antibodies were sent to four laboratories (Adventicious Virus Laboratory, Gastroenteric Virus Laboratory and Herpesvirus Laboratory from Institute of Virology, INTA-Castelar and Biogenesis-Bago S.A.).

The laboratories were requested to follow the ELISA protocol supplied with the samples. Each laboratory performed eight assays every 7–10 days, and the results were reported to the authors for further analysis. The %CV for intra-laboratory assays was then calculated. In addition, the reproducibility was calculated by ANOVA for a nested model of variance using Statistix8 software.

2.9. Detection of BVDV during antigen batch production

Inactivated BVDV preparations were obtained by treatment with 1% (v/v) 0.1 M binary bromoethylenimine (BEI) for 25 h at 37 °C as described by Bahnemann (1976).

Prior to the ELISA testing, live and inactivated virus preparations were mixed with 0.25% Triton X114 (Sigma–Aldrich) and were incubated for 30 min at 4 °C. The mixture was then incubated in a water bath for 15 min at 28–37 °C. After a brief centrifugation, the aqueous phase of the sample was used in the ELISA.

Neat and 1/4 diluted samples of live BVDV and inactivated BVDV were assayed in duplicate. The quantitation of BVDV E2 was performed in eight replicates of a Singer strain production, and the %CV for intra-assay precision was calculated.

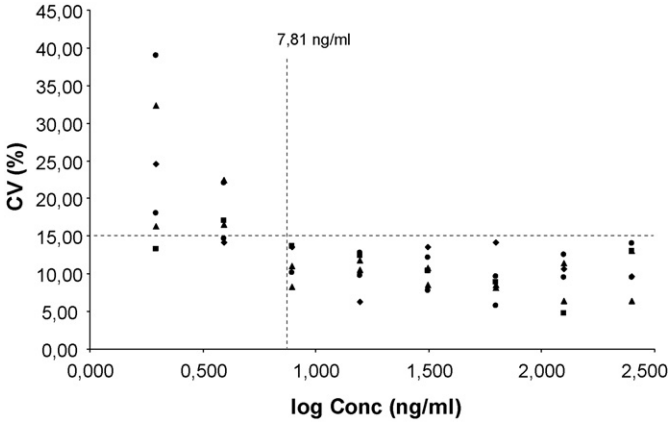

Fig. 3. Different dilutions of purified tE2 were assayed, and the %CVs were plotted as a function of tE2 concentration.

2.10. Specificity

Four BVDV strains (NADL, Singer, NY1), the 98/124 BVDV field isolate and six bovine viruses (bovine herpesvirus 1, bovine herpesvirus 5, bovine rotavirus, bluetongue virus, parainfluenza type 3 virus and bovine respiratory syncytial virus) were assayed during the ELISA development to evaluate specificity.

2.11. Evaluation of BVDV vaccines in cattle

The vaccines were formulated with an oil adjuvant containing the appropriate antigen in an adjuvant to antigen ratio of 60:40. Six different specifications of BEI-inactivated virus containing 7.81, 7.52, 0.78, 0.15, 0.08 and 0.015 μg of E2 glycoprotein were used as the antigen. As a negative control, the same formulation was made using a mock antigen.

Groups of eight BVDV seronegative bovines were immunized through intramuscular injections using 3 ml of each vaccine and were boosted 30 days after the initial immunization. An additional non-vaccinated group was included. Sera were obtained at 0, 30 and 60 days post immunization.

Serum neutralizing antibodies were detected by a virus neutralization assay. Briefly, 100 $TCID_{50}$ of BVDV (Singer strain) were co-incubated with 75 μl of 1/4 serial dilutions of the inactivated

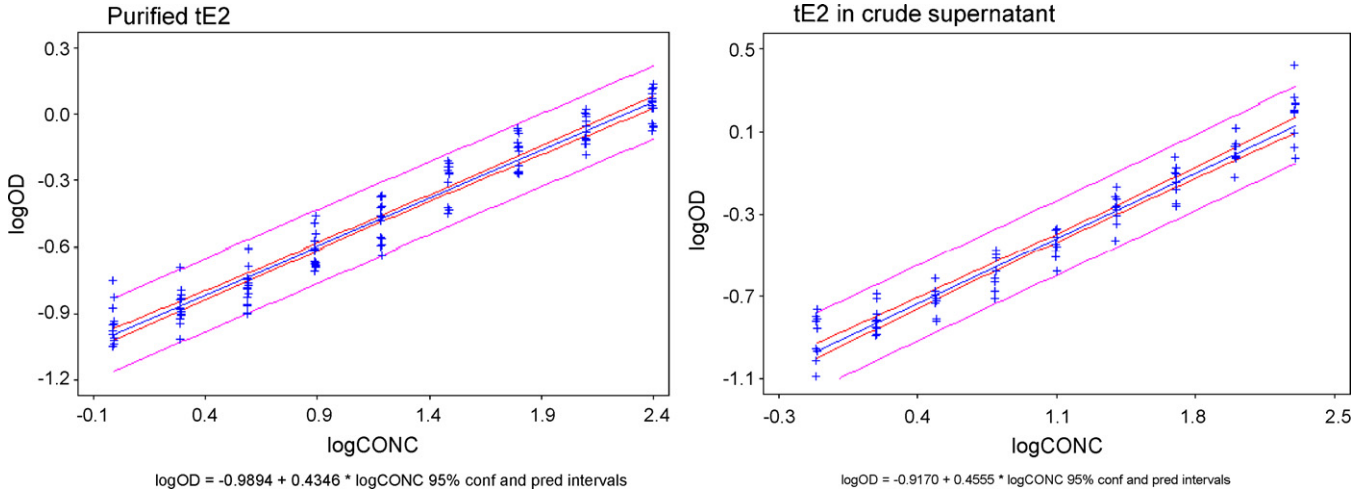

Fig. 2. Eleven concentrations of purified tE2 and tE2 in crude supernatant were tested in eight replicates and in 14 and 9 independent assays, respectively. Linear regression curves were determined according to the following equation: $y = ax + b$, where y is the log of the absorbance at 405 nm, x is the log of the concentration in ng/ml, and a and b are the calibration slope and intercept, respectively. The central line represents the curve obtained using this equation. Middle and external lines represent confidence and prediction intervals, respectively.

Table 2
Repeatability and intermediate precision for calibration curve parameters.

Variation source	σ^2	% Variation	σ	%CV
Slope				
Replicate, $\sigma_0{}^2$	0.0027	31.28	0.052	11.70
Plate, $\sigma_2{}^2$	0.0057	65.98	0.076	17.10
Assay, $\sigma_3{}^2$	0.00023	2.73	0.015	3.47
	Sum = 0.0087	100		
Intercept				
Replicate, $\sigma_0{}^2$	0.036	83.20	0.19	21.20
Plate, $\sigma_2{}^2$	0.0013	3.12	0.037	4.11
Assay, $\sigma_3{}^2$	0.00059	13.68	0.077	8.60
	Sum = 0.043	100		

serum samples for 1 h at 37 °C. The mixture was then transferred to microtiter plates with 3×10^4 MDBK cells/well. The plates were incubated for 72 h at 37 °C with 5% CO_2. Control wells without virus were used for each serum sample in order to minimize toxicity.

The serum neutralization titer was considered as the highest serum dilution that inhibited 50% of the viral cytopathic effect. Titers were expressed as the $\log_{10}$ of the highest dilution able to inhibit 100 $TCID_{50}$.

BVDV positive serum obtained from an experimentally infected cattle and BVDV negative serum from a reference animal were used as the positive and negative controls, respectively.

Cattle management, inoculation, and sample collection were conducted by trained personnel under the supervision of a veterinarian and in accordance with protocols approved by the INTA's ethics committee on animal welfare.

3. Results

3.1. Antigen preparation

Recombinant tE2 protein, expressed in a soluble form in the supernatant of the CHO-tE2 cells, was purified by IMAC. After purification and concentration, a tE2 preparation with a protein concentration of 200 μg/ml and 80.69% purity was obtained (Fig. 1A). The purified tE2 protein reacted with several specific antibodies: a monoclonal antibody against the E2 glycoprotein (VMRD), an anti-tE2 rabbit serum, an anti-BVDV NADL strain rabbit serum, an anti-tE2 bovine serum and an anti-BVDV Singer strain bovine serum (Fig. 1B). These results indicated that this protein could be a suitable antigen for ELISA development.

The stability of both tE2 in crude supernatant and purified tE2 were assayed at 1, 8 and 21 days post-production by incubating the preparations at 37, 4, −20 and −70 °C. The tE2 in crude supernatant was shown to be stable under the evaluated conditions (Fig. 1C). Furthermore, the tE2 stability was not affected after three freeze–thaw cycles. On the other hand, the purified tE2 in PBS was not stable at 37 or 4 °C and could only be conserved at −20 or −70 °C (Fig. 1C). For this reason, the tE2 in crude supernatant was chosen as the reagent for the generation of the calibration curves.

3.2. Optimization of the ELISA procedure

Assay optimization involved the study of several parameters, including the concentration of antibodies, sample dilution, incubation time, and choice of primary and secondary antibodies and blocking buffer. The selection of optimal conditions was based on the degree of non-specific binding and the signal obtained in the calibration curves. The optimal dilutions for capture, primary antibody binding and conjugate addition were set at 1:400, 1:2000 and 1:4000, respectively. After these conditions were checked, the blocking buffer was optimized. It was established that when compared to the other blocking buffers, the PBS-T plus 1% skim milk gave the highest OD405 ratio between positive and negative samples. Finally, it was demonstrated that the optimal exposure time was 30 min.

3.3. Limit of detection, working range and calibration

The limit of detection (LOD) of the ELISA was defined as the mean value plus three standard deviations of the OD405 negative control value. The overall mean OD405 obtained from 21 independent assays was calculated and used to estimate the LOD by interpolating the mean value in the titration curve (Table 1).

Eleven twofold dilutions of purified tE2 (from 500 to 0.49 ng/ml) were prepared, and an aliquot of each sample was tested in 14 independent assays, each replicated eight times. Linear regression curves were determined by plotting the concentrations of the tE2 versus the absorbance on a log scale. A linear dose–response curve was obtained (from 250 to 0.98 ng/ml of purified E2t) with a coefficient of correlation $R^2 = 0.944$, $p < 0.001$ (Fig. 2A).

Serial dilutions of tE2 in crude supernatant (from 200 to 0.39 ng/ml) were also tested in nine assays, each replicated eight times. A linear response was obtained in the range of tE2 between 200 and 0.78 ng/ml ($R^2 = 0.94$, $p < 0.001$). Values for the slope and intercept were similar to those obtained in the regression curve performed with purified tE2 (Fig. 2B). Based on these results, the purified tE2 was used for the QC, and the tE2 in crude supernatant was used for the calibration curves.

The lower limit of quantitation (LOQ) was 7.81 ng/ml. This value corresponded to an acceptance CV of less than 15% (Fig. 3).

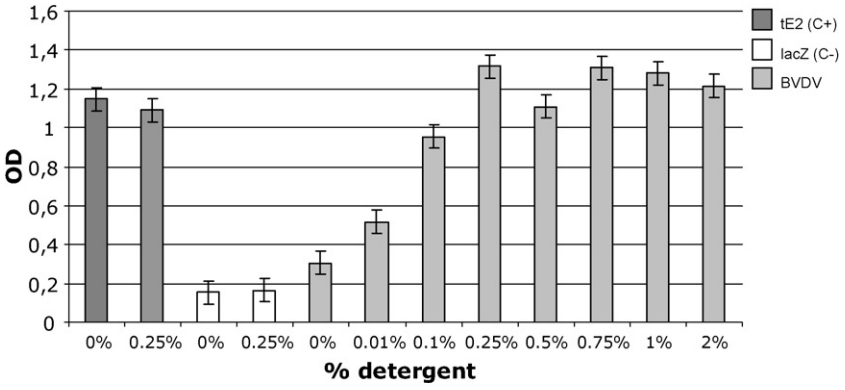

Fig. 4. BVDV was treated with different concentrations of Triton X114 and incubated 30 min at 4 °C, and the mixture was then incubated in a water bath 15 min at 37 °C. After a brief centrifugation, the upper aqueous phase of the sample was used in the ELISA. Positive (tE2) and negative (lacZ) controls were incorporated in the assay.

Table 3
Limits of detection of different batches of live and inactivated BVDV strains.

BVDV strain	Batch	Inactivation	LOD ($TCID_{50}$/ml)
BVDV Singer	1	Yes	$6 \times 10^{3.5}$
		No	$6 \times 10^{3.5}$
	2	Yes	$6 \times 10^{3.75}$
		No	$6 \times 10^{3.75}$
	3	Yes	6×10^{3}
		No	$6 \times 10^{3.5}$
	4	Yes	$6 \times 10^{4.5}$
		No	$6 \times 10^{4.5}$
BVDV NADL	1	No	6×10^{3}
BVDV NY1	1	No	$6 \times 10^{4.5}$
BVDV 98/124	1	No	$6 \times 10^{4.5}$

Table 4
Intra-assay precision of BVDV E2 (Singer strain) quantitation.

Replicates	E2 (ng/ml)
1	36.85
2	32.36
3	28.84
4	28.84
5	26.34
6	28.70
7	28.60
8	36.72
%CV	12.87%

3.4. Repeatability and intermediate precision

The precision of the tE2-ELISA was assessed using the variation in the parameters of the calibration curve within the plate and both intra- and inter-assay variation data (Table 2).

The intra-plate variation presented a CV of 11.7% for the slope and 21.2% for the intercept. The estimated intra- and inter-assay variations for the slope were 17.1% and 3.47%, respectively. The intra- and inter-assay variations for the intercept were 4.11% and 8.6%, respectively.

3.5. Optimization of BVDV detection by tE2-ELISA

The BVDV Singer strain (10^6 $TCID_{50}$), produced by infecting MDBK cells, generated a very low reaction signal in the developed ELISA. In order to enhance the BVDV detection, the virus was exposed to physical (sonication, freeze–thaw treatment) and chemical (detergent) processing. The use of physical methods did not increase the reaction signal. On the other hand, when Triton X114 was used, the sensitivity was greatly increased. In order to standardize the optimal concentration of the detergent for BVDV detection, the BVDV was mixed with different concentrations of Triton X114 (0%, 0.01%, 0.1%, 0.25%, 0.75%, 1%, and 2%) and assayed in the ELISA (Fig. 4). A concentration of 0.25% of Triton X114 was chosen for virus detection in the subsequent experiments. The treatment of the purified tE2 and the negative control with 0.25% Triton X114 did not interfere with the reaction signal.

The LOD for BVDV E2 was also determined by testing serial dilutions of different virus stocks before and after inactivation with BEI ($n = 4$). The results showed that the detection of both live BVDV and inactivated BVDV was in the range of $10^{3.5}$ and $10^{4.5}$ $TCID_{50}$/ml (Table 3). The quantitation of E2 in BVDV production (Singer strain), assessed in eight replicates, showed a CV of 12.87% (Table 4).

The specificity was evaluated using other bovine viruses as the antigen; some of these other viruses are commonly included in commercial vaccines together with BVDV (bovine herpes virus 1 and 5, bluetongue virus, bovine rotavirus, parainfluenza type 3 virus and bovine respiratory syncytial virus). No reactivity was observed with these viruses (data not shown). In addition, NADL and New York BVDV type 1 strains and an Argentinean non-cytopathic isolate were tested, all of which were successfully detected in the tE2-ELISA (Table 3).

3.6. Inter-laboratory assay

A panel of 15 randomly coded samples was sent to four laboratories. The collaborators received the samples to be tested together with the capture and primary antibodies, conjugates and reagents for developing the reaction. The coating, washing and blocking buffers were prepared in each laboratory. The assays were performed every 7–10 days. The samples included crude supernatant to create calibration curves, purified tE2 at a concentration of 30 μg/ml, BVDV with Triton X114 addition, mock antigen and the supernatant from CHO-lacZ cells. The results showed that when each replicate was run on different days in the same laboratory, individual CVs varied from 8.66% to 22.37% (Table 5). Individual CVs generated by negative controls ranged between 7.32% and 13.77% (data not shown).

The inter-laboratory tests produced a CV of 4.39% and 11.82% for tE2 and BVDV quantitations, respectively (Table 6).

3.7. Evaluation of neutralizing antibodies induced by experimental BVDV vaccines containing different doses of E2

Six experimental vaccines were formulated to different specifications of inactivated BVDV containing 0.015–7.81 μg of E2 as

Table 5
Quantitation of tE2 and BVDV in the inter-laboratory assay.

Assay no.	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
	tE2[a] (μg/ml)	BVDV[b] (μg/ml)	tE2 (μg/ml)	BVDV (μg/ml)	tE2 (μg/ml)	BVDV (μg/ml)	tE2 (μg/ml)	BVDV (μg/ml)
1	35.72	29.13	25.73	19.88	31.05	24.05	25.76	19.59
2	33.29	37.09	32.71	24.88	36.49	27.24	23.28	24.53
3	26.75	29.97	30.23	27.45	30.44	25.38	21.60	26.36
4	29.16	33.35	27.16	27.23	30.18	28.09	21.65	27.06
5	32.39	35.74	32.71	28.20	36.59	23.25	32.27	30.91
6	30.53	35.81	33.85	27.93	29.93	33.05	23.47	31.98
7	28.64	31.94	33.85	19.68	23.64	28.86	34.83	29.86
8	32.21	34.74	39.99	28.67	33.79	26.78	36.49	25.53
Mean	31.08	33.47	32.03	25.49	31.52	27.09	27.42	26.98
SD	2.88	2.90	4.43	3.70	4.20	3.09	6.13	4.00
CV	9.27	8.66	13.84	14.51	13.32	11.39	22.37	14.81

A calibration curve was calculated for each assay and was used to quantify E2 in the concentrated crude supernatant and BVDV samples.
[a] tE2 was purified as described in Section 2 and was diluted to a concentration of 30 μg/ml.
[b] BVDV was treated with Triton X114 as described in Section 2.

Table 6
Reproducibility of tE2 and BVDV quantitation.

Variation source	σ^2	% Variation	σ	%CV
tE2 quantitation				
Assay, σ_0^2	20.80	92	4.56	14.90
Laboratory, σ_2^2	1.80	8	1.34	**4.39**
	Sum = 22.61	100		
BVDV quantitation				
Assay, σ_0^2	11.89	51.65	3.45	12.20
Laboratory, σ_2^2	11.13	48.34	3.34	**11.82**
	Sum = 23.02	100		

The bold values signifies $p < 0.05$.

measured by the developed ELISA. The potency of these vaccines was evaluated in BVDV seronegative cattle. Groups of eight bovines were immunized with each vaccine, and the development of neutralizing antibodies was monitored. A clear dose-dependent response was observed at 30 days post vaccination with neutralizing antibodies titers higher than two in animals immunized with vaccines containing 1.52 and 7.81 μg of E2. After the second immunization, neutralizing antibodies titers increased in all groups, with the exception of the group receiving 0.015 μg E2/dose. Vaccines containing 0.78, 1.52 and 7.81 μg of E2 were able to evoke a high neutralizing antibodies response (Fig. 5).

4. Discussion

In Argentina, with a livestock population in excess of 50 million and widespread BVDV infection, the strategy for the prevention of BVD is vaccination. Although vaccination is not mandatory, there is an increasing interest to protect cattle against BVD due to the worldwide distribution of the disease, the emergence of more virulent BVDV strains and the consequent economic losses in the dairy and beef industries.

Currently, inactivated and attenuated live vaccines are commercially available worldwide. Nevertheless, an increasing number of cattle with high antibody titers to BVDV suggests that exposure to field strains has not been diminished despite the use of both herd management and vaccination programs. Only inactivated BVDV vaccines are licensed in Argentina by the National Authority of Animal Health (SENASA). However, these vaccines may induce a short and weak immune response if insufficient amounts of viral antigen are present in the formulation.

The conventional evaluation of vaccine potency is based on the immunization of seronegative animals and the evaluation of neutralizing antibodies titers induced by the vaccination (Vallat, 2008b). Hence, the evaluation and quantitation of viral antigens by ELISA have become an attractive option to monitor the critical stages of vaccine production.

Table 7
Validation parameters of tE2-ELISA.

Validation parameters	Result	Acceptance criteria[a]
Repeatability		
Intra-plate	11.70% (slope), 21.20% (intercept)	%CV < 20
Inter-plate	17.10% (slope), 4.11% (intercept	
Intermediate precision	3.47% (slope), 8.60 (intercept)	%CV < 25
Reproducibility	4.39% (tE2 quantitation) 11.82% (BVDV quantitation)	%CV < 30
Limit of detection	3.8–4.3 ng/ml	
Limit of quantitation	7.81 ng/ml	
Working range	250–0.98 ng/ml	
Specificity	Positive reaction with BVDV cytopathic (Singer, NADL) and non-cytopathic (NY1, 98/124) strains Negative reaction with BoHV1, BoHV5, BTV, BRV, PI3 virus and BRSC	

[a] Breve et al. (2008), Pombo et al. (2004), Hansen et al. (2008) and OIE (Manual of Diagnostic tests and vaccines for terrestrial animals, 2008a).

Furthermore, the ELISA overcomes many of the limitations associated with plaque forming assays (Pheasey et al., 2006), and as it can be used for both live and inactivated viruses, it is particularly suitable in an industrial context.

In the present study, a sandwich-type indirect ELISA for the detection of BVDV E2 was developed successfully. The ELISA was studied using several validation parameters (Table 7). Various commercial and experimental antibodies were tested both as capture and detection antibodies. The combination of the MAb 2.9H and the polyclonal rabbit anti-tE2 antibody as the capture and detection antibodies, respectively, resulted in the best signal-to-noise ratio.

An IMAC-purified tE2 preparation was used as a QC. The tE2 in crude supernatant was chosen as the reagent for the routine calibration curves because it was stable after prolonged incubation (21 days) at 37 °C or after three freeze–thaw cycles. Moreover, the protein in crude supernatant was stable for up to 6 months at −20 °C (data not shown). Both the crude preparations and purified tE2 showed a similar response when assayed at different

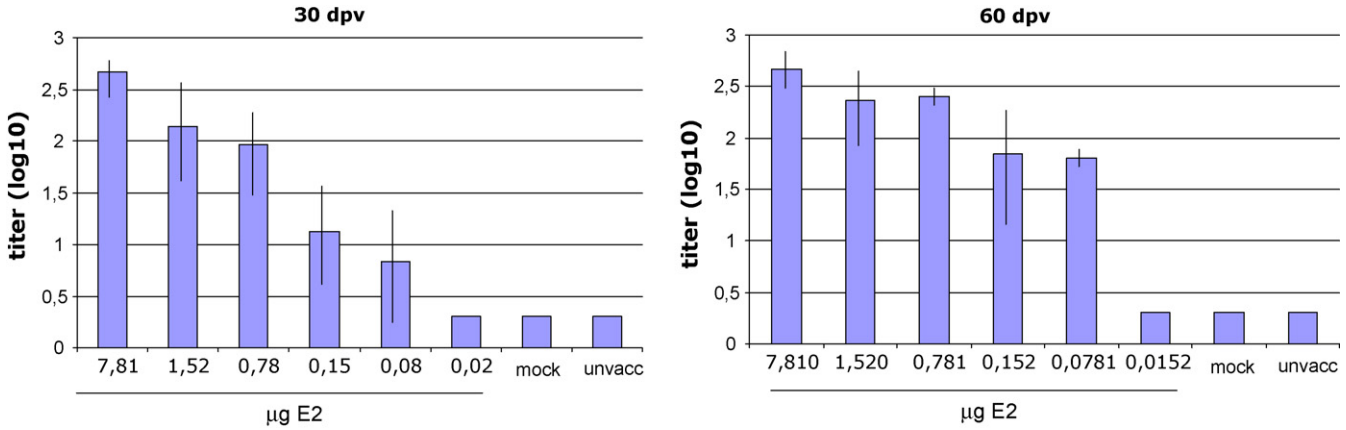

Fig. 5. BVDV conventional vaccines were prepared in an oleous formulation with six specifications of virus containing different doses of E2. Groups of bovines ($n = 8$) were immunized with the experimental vaccines. As negative controls, one group was immunized with a mock antigen, and one group remained unvaccinated. Neutralizing antibodies were analyzed as described in the Section 2.

concentrations in the ELISA, indicating no interference by culture medium proteins. The precision was assessed by monitoring the variation at three levels: intra-plate, inter-plate and inter-assay. The assay displayed suitable repeatability and intermediate precision (Table 7). To complete the validation, an inter-laboratory assay was performed involving four laboratories. The variations between laboratories were acceptable (CV 4.39% and 11.82% for quantitation of tE2 and BVDV, respectively), indicating that the ELISA is reproducible.

To evaluate the applicability of the ELISA to measure the BVDV antigen, the detection of live and inactivated virus was optimized. The results showed that the treatment of viral samples with 0.25% Triton X114 was the most effective method to enhance the detection of E2 in BVDV samples. Under the optimized conditions, it was possible to detect cytopathic (Singer and NADL) and non-cytopathic (New York and 98/124) strains of BVDV type 1 with a LOD that ranged between $10^{3.5}$ and $10^{4.5}$ $TCID_{50}$/ml. The specificity of the ELISA was demonstrated by the absence of reactivity when other virus preparations were used as the antigen. This is particularly important in the cases of bovine herpes virus 1 and 5, parainfluenza type 3 virus and bovine respiratory syncytial virus, since these viruses are usually included with BVDV in multivalent vaccine formulations.

It is important for manufacturers of inactivated vaccines to test the immunogenicity of final vaccine formulations. For both practical and ethical reasons, the replacement of in vivo potency tests by more rapid and reliable in vitro methods is highly desirable. Several strategies involving the quantitation of antigen content in viral vaccines have been reported (Claassen et al., 2004; Fournier-Caruana et al., 2003; Maas et al., 2000; Pombo et al., 2004; Rooijakkers et al., 1996). In addition, Ludemann and Katz (1994) have previously proposed the advantages of using an in vitro assay for testing inactivated BVDV vaccines. The ELISA developed by the authors allowed the detection of BVDV in inactivated vaccines used to immunize cattle; however, in that case, no association was noted between the neutralizing antibody titers in the serum samples and the OD readings in the ELISA.

In this work, BVDV seronegative cattle were immunized with inactivated BVDV vaccines containing increasing amounts of E2 antigen, which were quantified with the developed ELISA. The analysis of neutralizing antibodies titers obtained with each formulation demonstrated a clear correlation to the amount of E2 antigen present in the vaccine. This result suggests that the quantitation of E2 in inactivated BVDV vaccine constitutes an additional tool to control the amount of specific antigen to be incorporated in final formulation.

Future studies will be focused on the optimization of antigen detection in oleous and aqueous formulations that are of particular interest for batch release and vaccine stability studies. Moreover, in order to assess the applicability of this methodology for testing vaccine potency, it will be interesting to compare the in vitro determination of BVDV vaccines with the level of neutralizing antibodies induced by those vaccines in guinea pigs and cattle.

In summary, the new ELISA is easy to produce and perform, time-saving and suitable for the evaluation of key BVDV protein antigens in the critical steps of vaccine production.

The intended use of the new ELISA is to assay the antigens of BVDV used in inactivated vaccines as an alternative to the expensive and time-consuming immunization trials in cattle. In addition, in vitro testing of vaccines can reduce significantly process development costs and provide rapid and accurate data.

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