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Single dilution Avidity-Blocking ELISA as an alternative to the Bovine Viral Diarrhea Virus neutralization test

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A B S T R A C T

This study describes the development and validation of a blocking ELISA that measures avidity of BVDV-specific immunoglobulins (Igs) as an alternative to the classic virus neutralization test. The assay comprises a recombinant soluble E2 glycoprotein as target antigen, a neutralizing serum as detector antibody and a washing-step with a chaotropic agent to determine BVDV-specific Igs avidity. Avidity-Blocking ELISA was validated with 100 negative and 87 positive BVDV-neutralization serum samples from either infected or vaccinated bovines (inactivated commercial vaccines). Specificity and sensitivity of the Avidity-Blocking ELISA were 100% and 98.8%, respectively. The assay was standardized to use a single dilution, so that 90 samples can be tested per plate. Results expressed as Avidity Index (AI) correlated with BVDV neutralizing titers ($r = 0.94$). Unlike the virus neutralization test, the Avidity-Blocking ELISA could discriminate between infected and vaccinated animals (DIVA), suggesting that avidity measurement can be a valuable tool to achieve DIVA compliances. The data show that the avidity of anti BVDV antibodies is related to their capacity to block viral infection *in vitro*.

1. Introduction

The virus neutralization test is a sensitive and specific assay that quantifies the amount of antibody in a serum sample by determining the highest dilution required to neutralize a standard amount of virus infectivity in cell culture. This test is recognized widely as a reliable technique for the serological diagnosis of bovine viral diarrhea (BVD). Virus neutralization is applied as the reference potency test for commercial vaccines, according to title 9 of the Code of Federal Regulations (C.F.R, Title 9 – Animals and Animal Products).

However, virus neutralization assessment has several limitations. It is a time-consuming test, as data collection and processing can take about a week. The requirement of cell culture, live virus and a dedicated facility with constant monitoring of serum and cells to prevent viral contamination makes virus neutralization test expensive and difficult to deploy. Standardization is also hampered due to subjective plaque-reading. In addition, virus neutralization assay does not differentiate between BVDV natural immunity and vaccine-induced antibody responses.

These constraints support the development of alternative *in vitro* methods to neutralization. A surrogate test is paramount for developing countries, where technical and economical resources are scarce. The most eligible method for large throughput has been enzyme-linked immunosorbent assay (ELISA) (Mao et al., 2009; Martinez-Torrecuadrada et al., 2000; Nates et al., 1995). ELISA does not rely on cell cultures or live virus, it can provide a result within a few hours and it is relatively inexpensive and suitable for automation.

Attempts to attain correlation between ELISA and the virus neutralization test started two decades ago. Justewicz et al. (1987) developed an ELISA using BVDV-infected Madin-Darby Bovine Kidney (MDBK) cell monolayers as test antigen that gave a coefficient of correlation (r) of 0.63 (63%) between BVDV serum neutralization titers and ELISA (Justewicz et al., 1987). Correlation between these two tests was also assayed in the 1990s using a commercial ELISA (Graham et al., 1997; Graham et al., 1998) and more recently, in indirect ELISAs with recombinant Baculovirus-expressed antigens (Chimeno Zoth and Taboga, 2006) and a soluble-truncated BVDV's membrane-anchored E2 glycoprotein expressed in the Drosophila system (DtE2). This latter recombinant-indirect ELISA yielded titers that correlated with virus neutralizing titers with a low rate (82%) (Marzocca et al., 2007). The assessment of commercial IgG-ELISAs produced better results than the DtE2-based ELISA; however early induction of BVDV antibodies was only detected by

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neutralization assessment (Graham et al., 1997), a difference that is probably explained by this ELISA's inability to titer specific IgM in the test sera.

The quality rather than the quantity of specific antibodies determines their functional capacities (i.e.: neutralization). Bachmann et al. (1997), for instance, demonstrated that neutralizing activity against Vesicular Stomatitis Virus (VSV) correlated with avidity of specific antibodies *in vitro*. This correlation was also found for measles (Saika et al., 2008) and tick-borne encephalitis (Leonova and Pavlenko, 2009). To our knowledge, there are no studies that correlate virus neutralization, avidity of the specific Igs and responses to BVDV vaccines or natural infection.

This study describes the development and validation of isotype-independent Blocking and Avidity-Blocking ELISAs; in which serum samples prevent the binding of a custom-prepared neutralizing antibody to a recombinant E2 protein. Application of these assays demonstrates the existence of a highly significant correlation between the avidity of BVDV-bovine serum antibodies and their capacity to block viral infectivity in cell culture.

2. Materials and methods

2.1. Cells, virus and monoclonal antibodies

BVDV Singer and NADL strains, MDBK and *Spodoptera frugiperda* (SF21) cells were obtained from ATCC. BVDV stocks were produced by infecting MDBK cells, following standard procedures (Marzocca et al., 2007; Noe et al., 1994). Monoclonal antibody (MAb) 2.9.H is produced by ICT Milstein-CONICET (Buenos Aires, Argentina) and has been applied previously to a BVDV indirect ELISA (Marzocca et al., 2007) and to an antigen ELISA (Pecora et al., 2009).

2.2. Bovine serum samples

Animal procedures were performed according to standard guidelines of humane care and treatment of animals from the Laboratory Animal Welfare Act, supervised by the local animal welfare committee.

Bovine sera used to evaluate reactivity against Baculovirus-expressed truncated E2 (BtE2) were a pool of serum samples raised to recombinant full length E2 expressed in Baculovirus (Marzocca et al., 2007) and a set of sera obtained from experimentally-infected bovines, kindly provided by MV Darío Malacari from Institute of Virology, INTA (Argentina): (1) serum #134 from a BVDV Type 1b infected animal, (2) serum SB3 from a BVDV Type 1a (Singer) infected bull (Marzocca et al., 2007), (3) sera from a BVDV NADL infected cow and (4) serum CH 17/12 2008 against BVDV Type 2 from a experimentally infected calf, bled at 40 days post infection.

The ELISAs were validated with 187 bovine serum samples: 100 negative and 87 positive in BVDV neutralization test. Sera from BVDV vaccinated bovines ($n = 30$) comprised animals that had received at least one dose of whole virus-inactivated commercial vaccine formulated either with oil (water-in-oil emulsion) or aqueous adjuvant (alum hydroxide). Bleedings were performed at different times after vaccination (maximum 60 days). Sera from field-infected animals were obtained from farms with virus circulation. Twenty-two animals were infected acutely and were positive by RT-PCR following standard OIE procedures (OIE, 2010). All these samples and the corresponding clinical data were provided by field veterinarians. Other serum samples had been collected by our laboratory from 1997 to 2008; or kindly provided by Dr. Alejandra Romera (Institute of Virology, INTA).

2.3. ELISA control sera and Blocking Detector Antibody

Candidate serum samples to be assessed as Blocking Detector Antibody were obtained following a procedure described previously (He et al., 2007). Briefly, adult guinea pigs and rabbits were immunized with four doses of 10 μg of Drosophila-expressed truncated E2 glycoprotein (Marzocca et al., 2007) by intramuscular injection (500 μl/dose) given 2 weeks apart. Specific antibody levels were followed by the virus neutralization test and final bleeding was performed at 63 days post vaccination. Two different batches of the Blocking Detector Antibody were prepared following a Standard Operational Procedure and both could be applied consistently to the ELISAs described in this paper, with a coefficient of variation (CV) below 15% in the OD values of the control samples.

Positive control samples were selected from a pool of sera from vaccinated mice and infected cattle. Reactivity of positive and negative control samples and the Blocking Detector Antibody with BVDV and BtE2 were evaluated by PAGE-Western blot (WB) under non-reducing conditions (see Section 2.5). All selected BVDV positive animal sera reacted with BVDV-E2 and BtE2 dimers as expected.

2.4. Baculovirus-expressed truncated E2 glycoprotein (BtE2)

The ORF for a truncated form of E2 lacking the transmembrane and intracellular domains (tE2) was obtained from plasmid pMT/ss-tE2 (Marzocca et al., 2007) by restriction with BamH1/Xba1 and inserted into pVL1393 Baculovirus Transfer Vector (BD Biosciences, Franklin Lakes, NJ, USA) using the same restriction sites (pVL1393-tE2). Sf21 cells were co-transfected with pVL1393-tE2 and a linearized Baculovirus DNA (BD Biosciences) to obtain recombinant Baculovirus, according to the manufacturer's instructions. For BtE2 production, Sf21 cells grown in spinner flasks were infected in a serum-free medium at a high MOI (5 PFU/cell) and harvested 2–3 days later. Centrifuged supernatant was kept as the antigen source. Culture and infection of Sf21 cells were performed using TNMFH medium (BD Biosciences).

2.5. Analysis and quantitation of BtE2 by Western blot and ELISA

Culture supernatants containing BtE2 dimers were resolved in a 10% SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane. Strips were obtained in preparative mini-gels with 0.50 ml of recombinant Baculovirus infection supernatant. Reactivity to BtE2 was assessed with MAb 2.9.H (positive control) and bovine type-specific sera (described in Section 2.2) followed by the corresponding anti-species HRP-labeled conjugate (KPL Inc., Gaithersburg, MD, USA). For bovine sera a combined mix of anti IgG and IgM conjugates was used (diluted 1:500). Staining was carried out using 3,3′-diaminobenzidine tetrahydrochloride-hydrate (DAB, Sigma–Aldrich, St. Louis, MO, USA). To perform relative quantitation, different volumes of BtE2 (culture supernatant) were run along with aliquots of quantified DtE2 (Marzocca et al., 2007), used as standard. The bands were then analyzed and quantified with Gel-Pro Analyzer 4 (MediaCybernetics, Inc., Bethesda, MD USA) as described previously (Capozzo et al., 2006).

Quantitative antigen ELISA was adapted from Pecora et al. (2009) with the following modifications: the presence of MAb 2.9.H-captured tE2 was revealed using a commercial goat anti-BVDV antiserum (VMRD, Inc., Pullman, WA, USA) followed by anti-goat conjugate (1:3000). A standard curve was constructed from known concentrations of DtE2.

2.6. Virus neutralization test

BVDV-serum neutralizing antibodies were detected by standard virus neutralization assay as stated in the OIE Manual (OIE, 2010).

Titers were expressed as the $\log_{10}$ of the highest dilution factor able to inhibit 100 TCID (Marzocca et al., 2007). BVDV bovine antiserum (VMRD, Inc.) was used as positive control and BVDV negative fetal bovine serum (Sigma, USA) was used as negative control.

2.7. Blocking ELISA procedures

Two different tests were developed: a Blocking ELISA and an Avidity-Blocking ELISA.

Ninety-six flat bottom well plates (MICROLON®, Greiner Bio-One, USA) were coated with 50 μl of capture MAb 2.9.H diluted 1:500 in 50 mM carbonate/bicarbonate buffer pH 9.6 (Marzocca et al., 2007), and incubated overnight at 4 °C. After five washes with phosphate buffered saline (PBS), plates were blocked with non-fat dry milk (Svelty, Nestlé®. Argentina) 10% in PBS (200 μl/well) and subsequently incubated for 1 h at 37 °C, washed five times with PBS (washing step) and coated with 50 μl per well of BtE2 supernatant 0.3 μg/ml in dilution buffer (PBS 0.5%; NaCl 100 g/l; Thimerosal 0.4 g/l; Tween 20 25 μl/l; phenol red 20 μl/l; Polivinilpirrolydone-PVD 360–2.5 g/l). The plates were incubated for 1 h at 37 °C. Following a washing step, control sera were diluted 1:40 and serum samples were added in eight twofold serial dilutions starting at 1:20. Procedure was further optimized to perform a single dilution of the sample (1:40 dilution for bovine sera), which allowed to process 90 samples per plate (45 samples when assayed in duplicate). Samples were incubated for 1 h at 37 °C.

For the Avidity-Blocking ELISA, plates were washed twice with PBS (300 μl/well), subsequently washed with PBS-6M Urea (Promega, USA) for 15 min at room temperature and followed by two regular-PBS washing steps. Blocking Detector Antibody (50 μl/well, diluted 1:2000) was then added to the plates and incubated for 1 h at 37 °C. Non-specific adsorption was controlled by pre-treating (for 30 min at room temperature) all samples, Blocking Detector Antibody and conjugate with a custom-prepared pre-adsorption solution containing bovine and mouse BVDV-negative (naïve) sera (1 μl of undiluted serum for each sample), culture medium supernatant (TNMFH medium, 5 μl per sample) from an heterologous Baculovirus infection (titer 1×10^7 PFU/ml) (Grigera et al., 1996) and Baculovirus-mock infected SF 21 cells. Following five washing steps with PBS, BtE2-specific antibodies were detected with HRP-labeled anti-guinea pig conjugate diluted 1:1500 and incubated for 1 h at 37 °C. The colorimetric reaction was revealed with chromogen/substrate mixture ABTS/H_2O_2 [ABTS: 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] at room temperature, protected from light exposure. The reaction was stopped after 20 min by the addition of 50 μl of 2% Sodium Floride. Absorbances were read at 405 nm (Multiskan® EX, Thermo Scientific, USA). Mean OD values of samples and controls were corrected subtracting mean blank OD values (cOD). The Avidity Index (AI) was calculated as the percentage of remaining activity of the sera relative to the OD of the Blocking Detector Antibody (considered as the maximum reactivity): AI = 100 − (cOD sample × 100/cOD Blocking Detector Antibody).

During the set up, sera were run in parallel without performing the urea washing step. Data analysis showed that this step was unnecessary as Avidity Indexes can be calculated from the Blocking Detector Antibody OD with no difference in the results.

Blocking ELISA procedure was equal to the Avidity-Blocking ELISA, but without the urea washing step. The same set of serum samples were titrated by Blocking ELISA (samples were run in twofold serial dilutions). The residual activity of the tested sera (RA) relative to Blocking Detector Antibody was calculated for each dilution: RA (%) = 100 × cOD sample/cOD Blocking Detector Antibody value. Samples with RA > 52 at the first dilution (1:20) were considered negative. For positive samples, titer was expressed as the $\log_{10}$ of the sample dilution factor capable of blocking 52% of the binding of the Blocking Detector Antibody for bovine samples (cut off value).

2.8. Data analysis

Statistical analyses were carried out using biostatistics, curve fitting and scientific graphing softwares (Statistix v8, Analytical Software, USA; GraphPad Prism v4.02, GraphPad Software, USA; MedCalc v11 Software, MedCalc, Belgium). Significance was established within the 95% confidence interval for all determinations.

Positive and negative control samples were assayed along with the Blocking Detector Antibody in 20 independent assays run in quadruplicate by Blocking and Avidity-Blocking ELISA. The ODs of the standards in Avidity-Blocking ELISA performed by the same and different operator/s were compared in order to assess repeatability and reproducibility, respectively. Intra-assay, inter-assay and replicates' variations were calculated by ANOVA for a nested model of variance, applied to quantify the interaction between repeatability and reproducibility. The variation coefficient was computed as the "standard deviation/mean" ratio (in percentage). Intra plate variations were calculated for each plate and averaged. Inter-operator variations were also studied by inter-rate agreement Kappa "κ" (Edmondson et al., 2007).

Receiver operating characteristic (ROC) analysis, following Delong's method (DeLong et al., 1988) was applied, for both techniques to calculate the cut-off value for blocking ELISAs considering virus neutralization test as gold standard.

Blocking and Avidity-Blocking ELISA performances were compared to virus neutralization test by Mann–Whitney non-parametric test for independent samples. Virus neutralization titers and Avidity-Blocking ELISA results were studied by linear regression and Pearson's correlation analysis.

3. Results

3.1. Recombinant E2 as blocking ELISA antigen

This method is based on a blocking ELISA which allows testing samples from bovine and other species such as mouse sera from vaccine-development studies (data not shown). The selected antigen was a truncated form of the E2 viral protein (tE2), target of the neutralizing responses against BVDV (Bolin and Ridpath, 1996; Harpin et al., 1997). As mentioned in Section 2.4, the tE2 lacking the transmembrane and intracellular domains was expressed in the Baculovirus system (BtE2), using a consistent and cost effective production process.

BtE2 was harvested from culture supernatants. The dimeric nature of BtE2, which is necessary to preserve the conformational epitopes for binding of neutralizing antibodies (Paton et al., 1992), was analyzed by PAGE-Western blot under non-reducing conditions (Fig. 1A). Both the BtE2, as well as the secreted E2 produced in the Drosophila system (DtE2) (Marzocca et al., 2007), retained their quaternary structure, being able to form homo-dimers that reacted with MAb 2.9.H (Fig. 1A). BtE2 was also recognized by BVDV specific sera. Fig. 1B shows that sera from type-specific BVDV experimentally infected cattle (BVDV type 1a, 1b and type 2) reacted with BtE2 dimers. Visualization of reactivity with BVDV type 2-specific serum required a lower dilution factor than the other sera. These data suggest that BtE2 can be an adequate target antigen to be used in ELISA to detect antibodies against the different BVDV types.

BtE2 was quantified by density analysis and also by capture-ELISA. Production yield of two different batches was 295.66 ± 6.55 μg/ml and 199.67 ± 11.05 μg/ml. BtE2 was used without any further purification step and captured with MAb 2.9.H in the ELISA plates. The optimal capture MAb dilution and

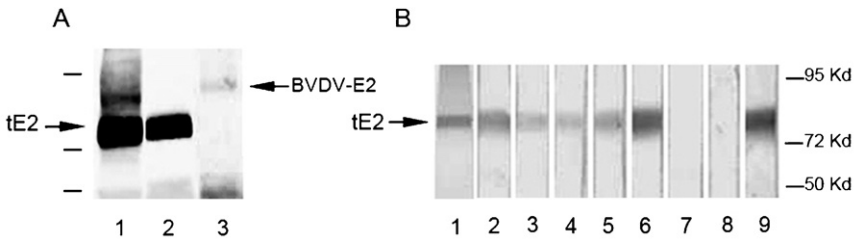

Fig. 1. *Drosophila melanogaster*–expressed truncated E2 (DtE2, 100 ng – lane 2), 1 μl aliquot of BtE2 supernatant (lane 1) and BVDV (Singer strain, lane 3) were analyzed by 10% SDS–PAGE under non-reducing conditions, followed by Western blot. Reactivity was assessed with MAb 2.9.H (anti E2). Arrows indicate the position of tE2 and BVDV-E2 dimers. Horizontal lines on the left correspond to molecular weight markers (values expressed in kilodaltons, panel B). Western blot analysis showing the reactivity of BtE2 with different sera from bovines infected experimentally with BVDV. Strips containing BtE2 were incubated with a 1:1000 dilution of the Blocking Detector Antibody (lane 1); 1:40 dilution of serum #134 from a cow infected with BVDV Type 1b (lane 2); serum SB3 from a BVDV Type 1a (Singer strain) infected cow (lane 3); serum from a bull infected with BVDV NADL (lane 4); bovine serum against recombinant E2 (lane 5); and a 1:20 dilution of serum from a BVDV Type 2 infected calf bled at 40 days post infection (lane 6). Negative bovine and mouse sera were diluted 1:20 (lanes 7 and 8, respectively). MAb 2.9.H. (1:100) was used as positive control (lane 9). Reaction was revealed with DAB.

antigen concentration were established by standard checkerboard titration. Culture yielded 150 plates per ml.

3.2. Avidity-Blocking ELISA: set-up

Blocking ELISAs were optimized to follow a standard procedure. Two protocols were assayed: a regular blocking ELISA using a guinea-pig neutralizing sera as Blocking Detector Antibody and an Avidity-Blocking ELISA that incorporates an additional urea-washing step.

The selected Blocking Detector Antibody had virus neutralization titer over 3.60, strong binding signal to BtE2 in Western blot and low reactivity to other components of the antigen preparation (Fig. 1B, lane 1). A 1:2000 dilution of the Blocking Detector Antibody producing an OD = 1.00 by direct ELISA was fixed as the maximum reactivity for the blocking ELISAs.

Three different concentrations of urea-PBS, ranging from 6 to 8 M, were assessed to set up Avidity-Blocking ELISA, based on previous studies on avidity ELISAs performed with bovine sera (Bjorkman et al., 1999; Ozkul et al., 2008; Pajuaba et al., 2010). Positive and negative samples could be differentiated by the three conditions (data not shown) therefore, concentrations of urea in the washing step were selected based on the variability of OD values produced by the Blocking Detector Antibody. Table 1 shows results from 25 independent determinations of the OD values, assessed with or without the 15 minutes-urea washing step. No differences were found between the OD values of the Blocking Detector Antibody treated or not with 6, 7 or 8 M urea ($p > 0.05$, ANOVA). However, the 6 M urea solution yielded the least variability (CV = 6.29%) between the OD values of the Blocking Detector Antibody, either treated or untreated with the chaotropic solution (Table 1). This latter concentration was selected to set up the Avidity-Blocking ELISA.

Positive and negative control samples were then assayed by the Blocking and Avidity-Blocking ELISA, along with the Blocking Detector Antibody in 20 replicates (different plates) run in quadruplicates. The results showed that both ELISAs could discriminate between positive and negative control samples showing significant differences between mean values ($p < 0.05$). Intra-plate Mean Coefficient of Variation (CV) was CV = 9% for the Blocking ELISA values and even less variable (CV = 6%) for the Avidity-Blocking ELISA values, indicating very high assay repeatability (Jacobson, 1998).

Avidity-Blocking ELISA measurements obtained for control samples and the Blocking Detector Antibody by two different operators in 20 independent assays were compared. Coefficients of variation for all control samples were below 10.16%, indicating the high reproducibility of the assay (Table 2). The kappa value (κ) used to determine the level of agreement between the two operators for each diagnostic test, showed that there was substantial agreement for the negative control serum and the Blocking Detector Antibody ($\kappa = 0.723$ and 0.704, respectively), and good agreement ($\kappa = 0.884$) for the positive control serum (Edmondson et al., 2007).

3.3. Evaluation of virus neutralization positive and negative bovine serum samples

One hundred virus neutralization negative and 87 virus neutralization positive bovine serum samples were evaluated by Blocking and Avidity-Blocking ELISA. As mentioned in Section 2.7, Avidity-Blocking ELISA assay was performed using a single serum dilution of the sample. Standardization experiments indicated that the linear relationship between OD and dilution (ELISA titer and positive/negative ratio) in Avidity-Blocking ELISA, was strongest at a serum dilution of 1:40 and this single dilution was used for avidity testing, following the criteria stated by Graham et al. (1998).

The percentage of Residual Activity (RA) at the first dilution in Blocking ELISA and Avidity Index (AI) in the Avidity-Blocking ELISA, were calculated as indicated in Section 2.7. Optimal cut-off values were established by ROC analysis by comparing false negative (FN) and false positive (FP) rates for every possible cut-off (Table 3). ROC curves were created by plotting the sensitivity against 1-specificity for different cut off values of Blocking and Avidity-Blocking ELISA (Fig. 2). The best cut-off for the Avidity-Blocking ELISA was AI = 21, which yielded the highest sensitivity: 98.1% and 100% specificity (Fig. 2B). Specificity and sensitivity by Blocking ELISA were 93.3% and a 98.8% respectively, for a cut off value of RA = 52% (Fig. 2A).

3.4. Correlation between the Blocking ELISA titers and avidity indexes with BVDV neutralization titers

Results obtained by both blocking ELISAs were compared to the virus neutralization titers (Fig. 3). Blocking ELISA titers and avidity indexes were calculated as indicated in Section 2.7. Virus neutralization titers of the tested serum samples ranged from negative (titer < 0.6) to 3.9. Both the blocking ELISA titers and avidity indexes correlated with the BVDV neutralization titers. Correlation coefficients (Pearson's "*r*") were of 0.82 for Blocking ELISA (Fig. 3A) and 0.94 for Avidity-Blocking ELISA, following a linear regression model for the avidity ELISA values, with high coefficient of regression, $r^2 = 0.90$ (Fig. 3B).

It was observed that vaccinated animals had low avidity antibodies, though virus neutralization titers ranged between 0.90 and 2.70 (open circles, Fig. 3B). Therefore, bovine samples were further discriminated between vaccinated or field infected animals. Fig. 4

Table 1
Reactivity of the Blocking Detector Antibody in the Avidity-Blocking ELISA after treatment with different concentrations of urea.

Urea concentration[a]	Number of determinations[b]	Mean[c]	Std. deviation	Std. error	Lower 95% CI of mean	Upper 95% CI of mean	CV%[d]
0 M	25	1.067	0.061	0.012	1.042	1.093	5.72
6 M	25	0.951	0.060	0.012	0.926	0.976	6.29
7 M	25	0.930	0.195	0.039	0.849	1.010	21.01
8 M	25	0.810	0.163	0.033	0.743	0.877	20.14

[a] Urea concentration (M) applied in the washing step.
[b] Values of 25 independent assays.
[c] No significant differences were found between mean OD values obtained for the diverse treatments ($p > 0.05$).
[d] Coefficient of variation (in percentage).

Table 2
Variability between operators for the Blocking Detector Antibody, positive and negative control sera run by Avidity-Blocking ELISA to evaluate reproducibility.

Samples and conditions			Analysis of covariance			Inter-rate agreement (κ)[a]	CV%
Control sample	Operator	Number of evaluations	MEAN	SE	p-Value		
Negative	1	20	0.943	0.008	0.72	0.723[b]	10.16
	2	20	0.922	0.050			
Positive	1	19	0.318	0.021	0.75	0.884[c]	7.98
	2	19	0.315	0.020			
Blocking Detector Antibody	1	20	0.998	0.025	0.12	0.704[b]	6.80
	2	20	1.025	0.056			

[a] Criteria from Edmondson et al. (2007).
[b] Substantial agreement between operators: $0.61 \leq \kappa \leq 0.80$.
[c] Good agreement: $\kappa > 0.80$.

Table 3
Results from ROC analysis of bovine serum samples tested by the Blocking and Avidity-Blocking ELISA.

Test	Sample size (n)	Area under ROC curve	Standard error[a]	Criterion	Sensitivity	Specificity
Blocking ELISA	187	0.991	0.004	>52[b]	98.5	93.3
Avidity-Blocking ELISA	187	0.996	0.003	>21[c]	98.8	100

[a] Delong's method (DeLong et al., 1988).
[b] Residual Activity value (RA).
[c] Avidity Index value (AI).

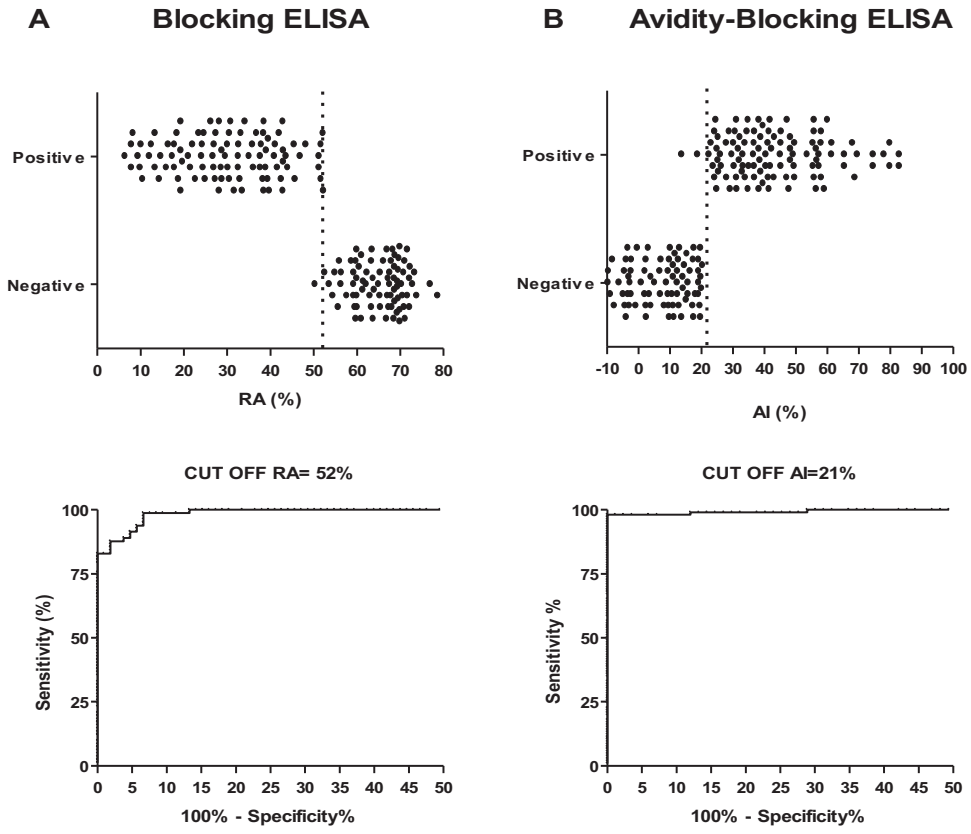

Fig. 2. Percentage of Residual Activity "RA" (Blocking ELISA; panel A) or Avidity Index "AI" (Avidity-Blocking ELISA; panel B) for individual virus neutralization positive and negative serum samples. Dotted vertical lines indicate Cut Off values as calculated by ROC analysis. Lower panels show ROC curves for the established cut off values.

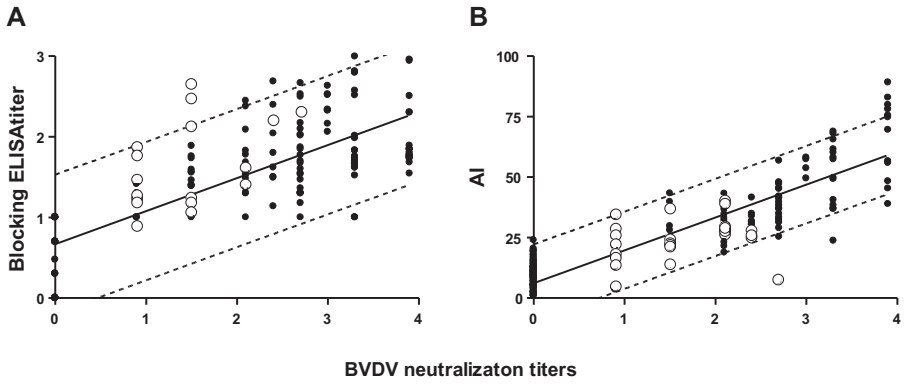

Fig. 3. Correlation between BVDV neutralization titers and the Blocking ELISA titer (A) or Avidity Index "AI" (B) of bovine serum samples. Blocking ELISA titers correspond to the $\log_{10}$ of the dilution factor giving a Residual Activity (RA) equal to 52%. For the Avidity-Blocking ELISA, an Avidity Index value greater than 21% was considered positive. Linear regression curves and intervals of 95% prediction band are depicted with full and dotted lines, respectively. Each dot indicates an individual value. Open circles in panel B correspond to avidity values of sera from BVDV vaccinated bovines (inactivated vaccines).

shows Blocking ELISA titers (Fig. 4A) and avidity indexes (Fig. 4B) from vaccinated and RT-PCR confirmed acute-infected animals. The blocking ELISA behaved as a virus neutralization test: it detected positive sera either from infected or vaccinated animals but did not discriminate between them ($p = 0.4369$; Fig. 4A). In contrast, avidity indexes measured by Avidity-Blocking ELISA were significantly different between these groups ($p < 0.05$, Fig. 4B), suggesting that avidity measurement, unlike virus neutralization test and Blocking ELISA, distinguished between acute-infected and killed-virus vaccinated animals.

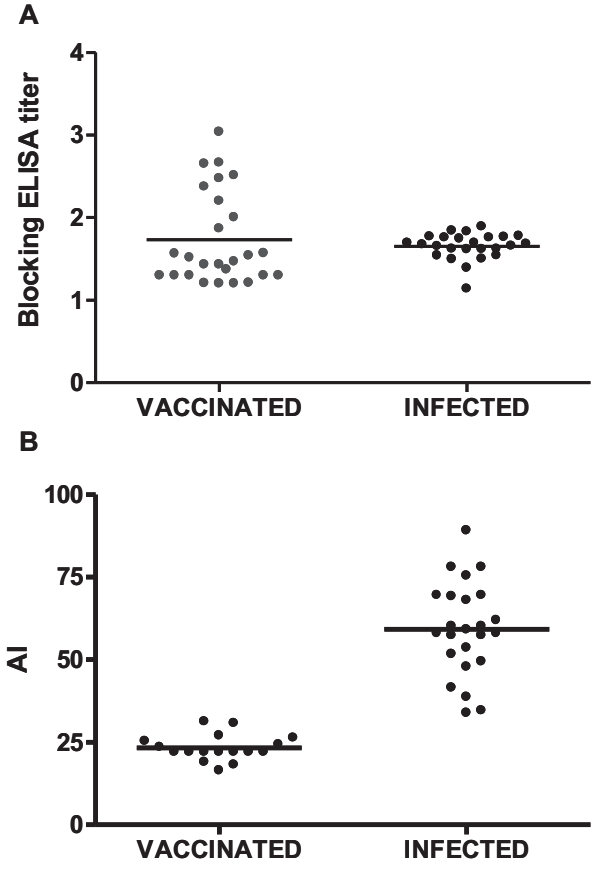

Fig. 4. Blocking ELISA titers (panel A) and Avidity Indexes "AI" (panel B) of serum Igs obtained from bovines infected with BVDV or immunized with inactivated commercial vaccines. Circles correspond to individual AI or Blocking ELISA titers. Horizontal lines indicate mean values.

4. Discussion

A single dilution BVDV Avidity-Blocking ELISA was developed. This assay performs as an indirect *in vitro* assessment of a biological assay (virus neutralization). Avidity indexes calculated by Avidity-Blocking ELISA correlated with virus neutralization titers, allowing the assessment of BVDV-specific antibodies in serum samples in only 1 day, without the need of cell culture, live virus manipulation or dedicated facilities. An alternative assay to virus neutralization test is paramount for BVDV vaccine potency testing in South America, as resources and technical capabilities to deploy the neutralization test are limited.

This assay relies on a truncated E2 glycoprotein, reactive to sera from BVDV Type 1a, 1b and BVDV-2 experimentally infected animals; and a custom designed polyclonal serum (the Blocking Detector Antibody) produced following a standardized procedure (He et al., 2007). During the set up of these assays, we evaluated the combined use of two anti E2 neutralizing MAbs as Blocking Detector Antibody. Blocking ELISAs performed with these MAbs rendered low specificity (73%) and the precision was unacceptable (Positive Predictive Value = 0.597). In this assay, Avidity-Blocking ELISA values did not correlate with BVDV-neutralizing titers (data not shown). This may be due to the fact that, unlike the hyperimmune guinea-pig serum, these MAbs detected only two neutralizing epitopes. A combination of multiple MAbs covering a wider range of neutralizing epitopes along the E2 glycoprotein could potentially be assayed in the future in order to further improve these tests.

The use of the Blocking Detector Antibody in a blocking ELISA simplified avidity testing. Unlike other Avidity ELISAs that run samples in parallel, with and without urea (Bjorkman et al., 1999; Capozzo et al., 2006; Ozkul et al., 2008; Pajuaba et al., 2010) avidity indexes in this single dilution Avidity-Blocking ELISA are calculated relative to the Blocking Detector Antibody-OD value. This allows BVDV Avidity-Blocking ELISA to test up to 90 samples per plate; which is equivalent to 5.6 complete sets of samples from inactivated-vaccine potency tests (8 prevaccination and 8 postvaccination sera), as stated by the code 9.CFR (C.F.R, Title 9 – Animals and Animal Products).

Both ELISAs were validated with 187 bovine sera (Table 2) yielding high specificity and sensitivity when compared to the virus neutralization results. Sensitivity was higher (Chimeno Zoth and Taboga, 2006; Cho et al., 1991; Justewicz et al., 1987) or similar to values reported for other BVDV-ELISAs (Pacheco and Lager,

2003). Specificity for Avidity-Blocking ELISA was 100%. This same value had been achieved by two indirect ELISAs which used MDBK infected cells (Cho et al., 1991) or a Baculovirus expressed E2 protein (Chimeno Zoth and Taboga, 2006) as target antigens. However, this last test had lower sensitivity (88.3%) than the Avidity ELISA developed in this study (98.10%). The indirect ELISA that applies the truncated Drosophila-expressed E2 (Marzocca et al., 2007) was more sensitive (Sensitivity = 100%) but less specific than the Avidity-Blocking ELISA (98%). Another advantage of the Avidity-Blocking ELISA is the lower intra and inter-plate variability in OD values.

Indirect ELISAs have not yielded good correlation with virus neutralization test [i.e.: $r = 0.82$ (Marzocca et al., 2007)] probably because they detect preferentially IgG isotypes and do not discriminate antibodies according to their quality. Although IgG1 has been shown to be the most prevalent isotype associated with BVDV neutralization (Howard et al., 1985), the presence of BVDV-neutralizing IgM in the primary response may explain why the virus neutralization test detects seroconversion before indirect ELISAs (Graham et al., 1998).

The developed blocking ELISAs correlated with virus neutralization titers, supporting our hypothesis that the combination of tE2 with a high-neutralizing detector serum can measure BVDV neutralizing antibodies more efficiently than conventional indirect ELISAs. Avidity index determination by single-dilution Avidity-Blocking ELISA proved to be more suitable than Blocking ELISA to replace virus neutralization test. Correlation coefficient respect to neutralization titers rose from 82% with Blocking ELISA to 94% with Avidity-Blocking ELISA.

Determination of the avidity index of serum-antibodies has been applied mainly to assess recall immune responses and distinguish anamnestic cross-reactive antibodies from primary specific responses, which may be useful in some clinical situations (Chan et al., 2007). Avidity testing has been used for several diseases, at herd or individual levels (Hamkar et al., 2006; Pannuti et al., 2004; Polack et al., 1999). To date, there is no published information on avidity testing of humoral responses against BVDV.

Infected animals have higher serum Ig avidity indexes than those immunized with inactivated commercial vaccines (Fig. 4B). This tendency suggests that this single dilution Avidity-Blocking ELISA may be a useful tool as a test to discriminate infected from vaccinated animals (DIVA test), in areas where inactivated vaccines are applied. There is still a need for a reliable DIVA assay for this disease. The latest candidate, p80 blocking ELISA, did not give consistent results on an individual animal level (Raue et al., 2011). The differential Ig avidity indexes between vaccinated and infected animals demonstrated in this work suggest that this trend should be further explored as an indicator of the immune status of the herd.

The data presented above show that the ability of anti BVDV antibodies to block viral infection relates to their avidity to bind BVDV E2 glycoprotein *in vitro*. The use of Avidity-Blocking ELISA, performed with a single dilution of the sample could be considered as a quick, safe and inexpensive alternative to the virus neutralization test. Further studies at herd level are needed to determine and validate the final application of this test.

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References

Bachmann, M.F., Kalinke, U., Althage, A., 1997. The role of antibody concentration and avidity in antiviral protection. Science 276, 2024–2027.

Bjorkman, C., Naslund, K., Stenlund, S., Maley, S.W., Buxton, D., Uggla, A., 1999. An IgG avidity ELISA to discriminate between recent and chronic Neospora caninum infection. J. Vet. Diagn. Invest. 11, 41–44.

Bolin, S.R., Ridpath, J.F., 1996. Glycoprotein E2 of bovine viral diarrhea virus expressed in insect cells provides calves limited protection from systemic infection and disease. Arch. Virol. 141, 1463–1477.

C.F.R, C.o.F.R. Title 9 – Animals and Animal Products. Part 113, Standard Requirements: 113.31 Bovine Virus Diarrhea Vaccine. National Archives and Record administration, Washington.

Capozzo, A.V., Ramirez, K., Polo, J.M., Ulmer, J., Barry, E.M., Levine, M.M., Pasetti, M.F., 2006. Neonatal immunization with a Sindbis virus-DNA measles vaccine induces adult-like neutralizing antibodies and cell-mediated immunity in the presence of maternal antibodies. J. Immunol. 176, 5671–5681.

Chan, K.H., Sonnenberg, K., Niedrig, M., Lam, S.Y., Pang, C.M., Chan, K.M., Ma, S.K., Seto, W.H., Peiris, J.S., 2007. Use of antibody avidity assays for diagnosis of severe acute respiratory syndrome coronavirus infection. Clin. Vaccine Immunol. 14, 1433–1436.

Chimeno Zoth, S., Taboga, O., 2006. Multiple recombinant ELISA for the detection of bovine viral diarrhoea virus antibodies in cattle sera. J. Virol. Methods 138, 99–108.

Cho, H.J., Masri, S.A., Deregt, D., Yeo, S.G., Thomas, E.J., 1991. Sensitivity and specificity of an enzyme-linked immunosorbent assay for the detection of bovine viral diarrhea virus antibody in cattle. Can. J. Vet. Res. 55, 56–59.

DeLong, E.R., DeLong, D.M., Clarke-Pearson, D.L., 1988. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 44, 837–845.

Edmondson, M.A., Givens, M.D., Walz, P.H., Gard, J.A., Stringfellow, D.A., Carson, R.L., 2007. Comparison of tests for detection of bovine viral diarrhea virus in diagnostic samples. J. Vet. Diagn. Invest. 19, 376–381.

Graham, D.A., Mawhinney, K.A., McShane, J., Connor, T.J., Adair, B.M., Merza, M., 1997. Standardization of enzyme-linked immunosorbent assays (ELISAs) for quantitative estimation of antibodies specific for infectious bovine rhinotracheitis virus, respiratory syncytial virus, parainfluenza-3 virus, and bovine viral diarrhea virus. J. Vet. Diagn. Invest. 9, 24–31.

Graham, D.A., McShane, J., Mawhinney, K.A., McLaren, I.E., Adair, B.M., Merza, M., 1998. Evaluation of a single dilution ELISA system for detection of seroconversion to bovine viral diarrhea virus, bovine respiratory syncytial virus, parainfluenza-3 virus, and infectious bovine rhinotracheitis virus: comparison with testing by virus neutralization and hemagglutination inhibition. J. Vet. Diagn. Invest. 10, 43–48.

Grigera, P.R., Garcia-Briones, M., Periolo, O., la Torre, J.L., Wagner, R.R., 1996. Immunogenicity of an aphthovirus chimera of the glycoprotein of vesicular stomatitis virus. J. Virol. 70, 8492–8501.

Hamkar, R., Mahmoodi, M., Nategh, R., Jelyani, K.N., Eslami, M.B., Mohktari-Azad, T., 2006. Distinguishing between primary measles infection and vaccine failure reinfection by IgG avidity assay. East Mediterr. Health J. 12, 775–782.

Harpin, S., Talbot, B., Mbikay, M., Elazhary, Y., 1997. Immune response to vaccination with DNA encoding the bovine viral diarrhea virus major glycoprotein gp53 (E2). FEMS Microbiol. Lett. 146, 229–234.

He, Q., Velumani, S., Du, Q., Lim, C.W., Ng, F.K., Donis, R., Kwang, J., 2007. Detection of H5 avian influenza viruses by antigen-capture enzyme-linked immunosorbent assay using H5-specific monoclonal antibody. Clin. Vaccine Immunol. 14, 617–623.

Howard, C.J., Clarke, M.C., Brownlie, J., 1985. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to bovine viral diarrhoea virus (BVDV) in cattle sera. Vet. Microbiol. 10, 359–369.

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. Rev. Sci. Tech. 17, 469–526.

Justewicz, D.M., Magar, R., Marsolais, G., Lecomte, J., 1987. Bovine viral diarrhea virus-infected MDBK monolayer as antigen in enzyme-linked immunosorbent assay (ELISA) for the measurement of antibodies in bovine sera. Vet. Immunol. Immunopathol. 14, 377–384.

Leonova, G.N., Pavlenko, E.V., 2009. Characterization of neutralizing antibodies to Far Eastern of tick-borne encephalitis virus subtype and the antibody avidity for four tick-borne encephalitis vaccines in human. Vaccine 27, 2899–2904.

Mao, N.Y., Zhu, Z., Jiang, X.H., 2009. Comparison and evaluation of enzyme-linked immunization assay kits with plague reduction neutralization test for detection of measles IgG antibody. Zhongguo Ji Hua Mian Yi 15, 215–218.

Martinez-Torrecuadrada, J.L., Lazaro, B., Rodriguez, J.F., Casal, J.I., 2000. Antigenic properties and diagnostic potential of baculovirus-expressed infectious bursal disease virus proteins VPX and VP3. Clin. Diagn. Lab. Immunol. 7, 645–651.

Marzocca, M.P., Seki, C., Giambiagi, S.M., Robiolo, B., Schauer, R., Dus Santos, M.J., Scodeller, E.A., La Torre, J.L., Wigdorovitz, A., Grigera, P.R., 2007. Truncated E2 of bovine viral diarrhea virus (BVDV) expressed in *Drosophila melanogaster* cells: a candidate antigen for a BVDV ELISA. J. Virol. Methods 144, 49–56.

Nates, S.V., Rey, G.Y., Giordano, M.O., Depetris, A.R., Boshell, J., 1995. Neutralization enzyme-linked immunosorbent assay for evaluation of immunity to measles virus. Viral Immunol. 8, 47–52.

Noe, W., Bux, R., Berthold, W., Werz, W., 1994. Optimization of vaccine production for animal health. Cytotechnology 15, 169–176.
OIE, W.O.f.A.H., 2010. OIE Terrestrial Manual. Chapter Bovine Viral Diarrhoea. (2008) Paris.
Ozkul, A., Demir, B., Karaoglu, T., Alkan, F., Dincer, E., Oncel, T., Burgu, I., 2008. Maturation of immunoglobulin G avidity after inactive gE deleted bovine herpesvirus type 1 (BHV-1) marker vaccination. Viral Immunol. 21, 3–11.
Pacheco, J.M., Lager, I., 2003. Indirect method ELISA for the detection of antibodies against bovine diarrhea virus in bovine serum. Rev. Argent. Microbiol. 35, 19–23.
Pajuaba, A.C., Silva, D.A., Mineo, J.R., 2010. Evaluation of indirect enzyme-linked immunosorbent assays and IgG avidity assays using a protein A-peroxidase conjugate for serological distinction between Brucella abortus S19-vaccinated and -infected cows. Clin. Vaccine Immunol. 17, 588–595.
Pannuti, C.S., Morello, R.J., Moraes, J.C., Curti, S.P., Afonso, A.M., Camargo, M.C., Souza, V.A., 2004. Identification of primary and secondary measles vaccine failures by measurement of immunoglobulin G avidity in measles cases during the 1997 Sao Paulo epidemic. Clin. Diagn. Lab. Immunol. 11, 119–122.
Paton, D.J., Lowings, J.P., Barrett, A.D., 1992. Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus. Virology 190, 763–772.
Pecora, A., Perez Aguirreburualde, M.S., Rodriguez, D., Seki, C., Levy, M.S., Bochoeyer, D., Dus Santos, M.J., Wigdorovitz, A., 2009. Development and validation of an ELISA for quantitation of bovine viral diarrhea virus antigen in the critical stages of vaccine production. J. Virol. Methods 162, 170–178.
Polack, F.P., Auwaerter, P.G., Lee, S.H., Nousari, H.C., Valsamakis, A., Leiferman, K.M., Diwan, A., Adams, R.J., Griffin, D.E., 1999. Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody. Nat. Med. 5, 629–634.
Raue, R., Harmeyer, S.S., Nanjiani, I.A., 2011. Antibody responses to inactivated vaccines and natural infection in cattle using bovine viral diarrhoea virus ELISA kits: assessment of potential to differentiate infected and vaccinated animals. Vet. J. 187, 330–334.
Saika, S., Matsunaga, T., Ogawa, T., Ichinohe, S., 2008. Gelatin particle agglutination (PA) antibody: comparison to neutralizing antibody (NT) and hemagglutination inhibition (HI) antibody and relationship to IgG avidity. Kansenshogaku Zasshi. 82, 310–316.