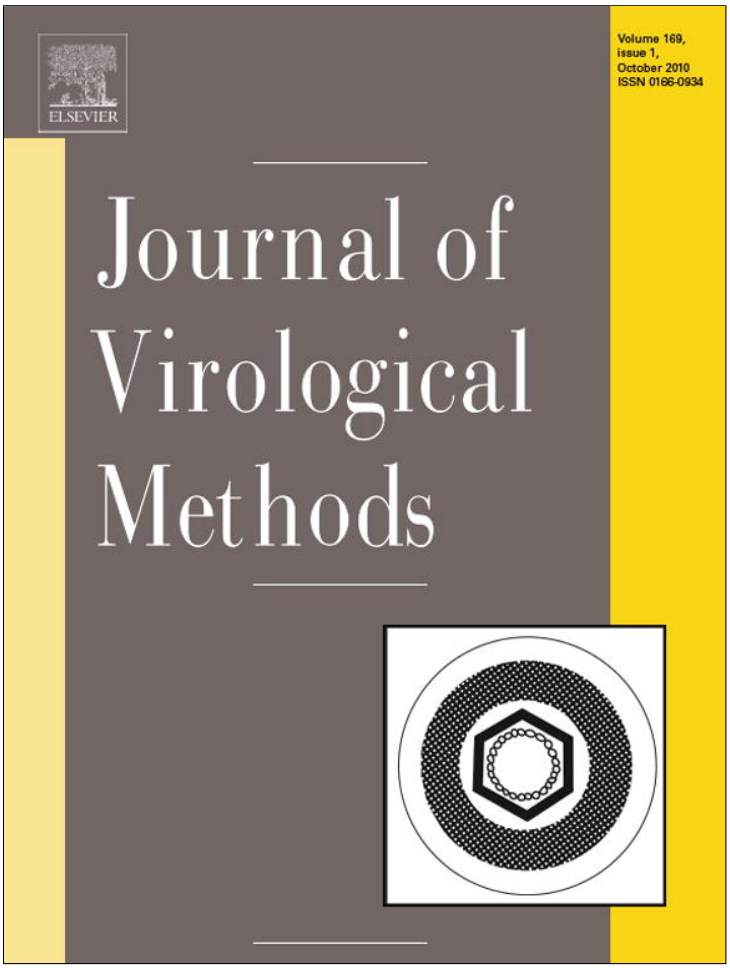

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Protocols

Validation of an indirect ELISA to detect antibodies against BoHV-1 in bovine and guinea-pig serum samples using ISO/IEC 17025 standards

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A B S T R A C T

Two ELISAs to quantify antibodies to BoHV-1 in the sera of cattle and immunized guinea pigs were developed and validated using ISO/IEC 17025 standards. The cut-off value of the assay was established at 20% positivity of a high positive control for screening of cattle. Using this threshold, the assay properly classified the OIE bovine reference sera EU1, EU2 and EU3. For vaccine potency testing, a cut-off of 40% was selected for both species. The reliability of the assays, given by their diagnostic sensitivity and specificity, using the threshold of 40% was 89.7% and 100%, respectively, for bovines and 94.9% and 100% for guinea pigs, respectively. There was almost perfect agreement between the ELISA and virus neutralization results. In addition, after vaccination, there was a good correlation between the neutralizing and ELISA antibody titers of the serum from the same bovine or guinea pig, sampled at 60 and 30 days post-vaccination, respectively ($R_{bovine} = 0.88$, $R_{guinea\ pig} = 0.92$; $p < 0.0001$). A similar correlation was observed when analyzing the mean antibody titers of groups of vaccinated animals ($R_{bovine} = 0.95$ and $R_{guinea\ pig} = 0.97$; $p < 0.0001$), indicating the relevance of the ELISAs for batch to batch vaccine potency testing in the target species and in the laboratory animal model. The intermediate precision of the assays expressed as the relative coefficient of variation (CV) of the positive control assayed over a 3-year period in the same laboratory was 22.2% for bovines and 23.1% for guinea pigs. The reproducibility of both techniques obtained in inter-laboratory assays was CV = 12.4% for bovines and CV ≈ 0 for guinea pigs, which met the requirements of the OIE (CV < 30%). The validated ELISAs represent important methods for vaccine potency testing and for controlling BoHV-1 infections.

1. Introduction

Infectious bovine rhinotracheitis (IBR) and pustular vulvovaginitis (IPV) are highly contagious respiratory and reproductive diseases of domestic cattle caused by bovine herpesvirus 1 (BoHV-1). BoHV-1 infection is distributed worldwide causing important economic losses in the livestock industry. Vaccination is a very useful method to control the disease (OIE2.4.13, 2008). The implementation of strict control programs based on management restrictions and the application of inactivated or live "marker" immunogens that are capable of differentiating between infected and vaccinated animals (DIVA vaccines) allowed for the eradication of the infection from several European countries (OIE2.4.13, 2008). These countries demand international trade of BoHV-1 free cattle. In the rest of the world, the classic attenuated and killed BoHV-1 vaccines are still available (Ackermann and Engels, 2006; OIE2.4.13, 2008).

In Argentina, BoHV-1 infection is endemic, reaching prevalences between 25 and 85% (Campero et al., 2003; Campos et al., 2009; Moore et al., 2003). The eradication programs currently in progress in the European Community have created the need to improve the Argentinean status of BoHV-1 infection in cattle. In Argentina, vaccination against BoHV-1 is not mandatory, and due to regulatory restrictions, only killed vaccines can be used to prevent viral diseases in cattle including IBR. The future implementation of IBR

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eradication programs requires DIVA gE-vaccines such as the vaccine that has been developed in our institute (Puntel et al., 2002), and an alternative method for vaccine potency testing with demonstrated concordance with the host species (Parreno et al., 2010) and well standardized and harmonized assays for BoHV-1 antibody detection. The aim of the present study was the statistical validation of two indirect enzyme linked immunosorbent assays (ELISAs) for the detection of antibodies to BoHV-1 in cattle and guinea pigs sera. The validation was designed to meet the ISO/IEC 17025 standards for test accreditation, and it included the estimation of the diagnostic sensitivity and specificity of the assay, repeatability and intermediate precision within a laboratory over time and the intermediate precision and reproducibility in an inter-laboratory assay.

To estimate the relevance of the assays, the concordance of the assay results with the virus neutralization test results and the correlation between the ELISA and neutralizing antibody titers in vaccinated animals were also investigated. Finally, the absolute analytical sensitivity of the test was estimated using the international bovine reference samples provided by the OIE reference laboratory for BoHV-1 (Kramps et al., 2004).

The ELISA for bovine sera was developed in the Virology Institute, INTA, Argentina, in 1990 (Bratanich et al., 1990). It was used extensively for research (Del Medico Zajac et al., 2006; Romera et al., 2000). Also, after its extensive validation, which included the analysis of OIE international bovine reference samples (EU1, EU2 and EU3), the ELISA represents a safe, fast, sensitive and specific diagnostic method for application in serological surveys to evaluate the BoHV-1 infection status in cattle and for international trade purposes (Greiner and Gardner, 2000a,b). In addition, the assay constitutes a critical method in a guinea pig model that can be used as an alternative method for vaccine potency testing, which is in alignment with the 3R initiative of refining, reducing and replacing animal experimentation (Hendriksen, 2009).

2. Materials and methods

2.1. International bovine reference sera

Three European Union standard bovine reference sera of IBR-EU1 (strong positive), IBR-EU2 (weakly positive) and IBR-EU3 (negative), which were kindly provided by Dr Kramps, (Central Veterinary Institute, The Netherlands), were used as external reference standards for ELISA validation (Kramps et al., 2004; Perrin et al., 1993). The lyophilized samples were reconstituted upon arrival with 1 ml of distilled water, aliquoted and stored at −20 °C as recommended. After reconstitution, the solution in the IBR-EU1 vial was a 1:16 dilution of the EU1 serum in negative EU3 serum. For the analysis in the ELISA, 0.4 ml of the sample was diluted with 0.6 ml of buffer to prepare a 1:40 dilution. The sample was analyzed in serial four-fold dilutions starting at 1:160. The weakly positive serum EU2 and the negative serum EU3 were reconstituted and tested in the ELISA at a starting dilution of 1:4. To save this precious reference material, samples were only assayed by ELISA.

2.2. Internal bovine positive control serum

The bovine positive control serum of the assay was derived from a naturally infected calf. The animal belonged to a beef farm suffering an IBR outbreak in 1993. The diagnosis was made by detection of virus shedding in nasal swabs. The circulating BoHV-1 strain was analyzed by restriction enzyme analysis and the profile was similar to the one of the reference strains, the Los Angeles subtype 1.1 (Romera, unpublished data). After the confirmation of the diagnosis, the animal was identified and serially sampled. The sera were tested for the BoHV-1 antibody by virus neutralization. The serum samples with the highest antibody titers to BoVH-1 were selected to ensure a large enough volume for a reference sample with a known high neutralizing antibody titer. The samples were pooled, diluted 1:2 in glycerol and stored at −20 °C. The set of fractions used during the first 3 years of initial validation was named PR 161-06, and the one currently in use is named PR 275-07.

2.3. Guinea pig positive control serum

This sample (PR 74-06) was prepared from a pool of 5 guinea pigs immunized by the intramuscular route with 2 doses of 1 ml of a monovalent vaccine containing a 107 tissue culture infectious dose fifty per milliliter ($TCID_{50}$/ml) of BoHV-1 emulsified in oil adjuvant (42.5% Marcol, 6.5% Arcalcel C and 1% $Tween_{80}$) in a 40:60 water-oil proportion. The serum was tested by virus neutralization and treated as described in Section 2.2.

2.4. Bovine serum samples

2.4.1. Reference population of positive and negative bovine serum samples

A total of 132 bovine serum samples (39 positive and 93 negative for antibodies to BoHV-1) were assayed to determine the cut-off of the assay and its associated diagnostic sensitivity and specificity. The serologically positive samples were derived from naturally infected bovines (Virology Institute Diagnosis Service, INTA) or experimentally challenged calves with proven BoHV-1 shedding, IBR disease and positivity for neutralizing antibodies to BoHV-1. The negative serum samples were obtained from cattle from BoHV-1 free herds, which did not use vaccination and were seronegative for BoHV-1 neutralizing antibodies tested with virus neutralization (1 h/37 °C) as described in Section 2.6.1.

2.4.2. Samples from vaccinated bovines to evaluate the concordance and correlation between VN and ELISA

Because the assay will be used not only for field surveys but also to evaluate the immune status of herds after vaccination, the concordance between the virus neutralization and ELISA and the correlation between the BoHV-1 antibody titer determined by the virus neutralization and ELISA were also tested in vaccinated cattle. For this study, another 193 bovine serum samples were tested. The bovine samples included in the analysis were of serum obtained at 60 days post-vaccination of groups of 5–10 animals that were vaccinated with 18 water-in-oil vaccines formulated with increasing doses of inactivated BoHV-1. The antigen concentration in the vaccines covered a range from 10^5 to 10^8 $TCID_{50}$/dose. These calibrated vaccines of known antigen concentration included potent and sub-potent vaccine batches and were used for the validation of the guinea pig model as an alternative method for vaccine potency testing (Parreno et al., 2010). Vaccination trials included BoHV-1 seronegative animals by virus neutralization (1 h/37 °C) and ELISA, from herds without a previous history of vaccination against BoHV-1 (Parreno et al., 2010).

2.5. Guinea pig serum samples

2.5.1. A reference population of positive and negative guinea pig serum samples

Guinea pigs (*Cavia porcellus*), SiS Al, around 350–400 g weight were obtained from the animal facility, of the Virology Institute, INTA, Castelar. All animals were negative for the neutralizing antibodies to BoHV-1 at arrival. A total of 135 negatives serum samples were chosen as negative references samples. Eighty-eight samples from non-vaccinated control animals and 47 samples from animals receiving placebo were all bled at 30 days post-vaccination. The

positive samples corresponded to 128 sera from guinea pigs immunized with 19 different vaccines. Animals received two doses of the vaccine 21 days apart by the intramuscular route in the hind-leg. The volume of the dose administered to the guinea pigs were 1/5 of the volume of the dose given to bovines (Kolbe and Coe Clough, 2008). Serum samples were obtained at 30 days post-vaccination. Blood extraction was conducted by cardiac puncture under anesthesia following the ECVAM recommendations for animal welfare (Leenaars et al., 1999). The protocol was approved by the Virology Institute Ethical Committee (CICUAE). The vaccines tested included 6 experimental combined vaccines (5 water-in-oil and 1 aqueous vaccine) containing 10^7 and 10^8 $TCID_{50}$/dose of inactivated BoHV-1 and 13 commercial products (8 water-in-oil and 5 aqueous vaccines applied to prevent bovine respiratory or reproductive syndrome). The immunogens were selected for the validation of the ELISA for guinea pigs, once they had demonstrated to induce antibody seroconversion to BoHV-1 by virus neutralization and ELISA in bovines.

2.5.2. Samples from immunized guinea pigs to evaluate the correlation between VN and ELISA

For the correlation analysis between the ELISA and virus neutralization antibody titers of vaccinated animals, 22 groups of 5 guinea pigs each were immunized with the same water-in-oil calibration vaccines used in the validation of the model (Parreno et al., 2010) described in Section 2.4.

2.6. Serologic assay methods

2.6.1. Virus neutralization

The virus neutralization assay used in this study was performed as described previously (Del Medico Zajac et al., 2006; Romera et al., 2000) and normalized following the recommendations provided by the American Code of Federal Regulation (CFR.113.216, 1985). Bovine and guinea pig serum samples were heat inactivated at 56 °C for 30 min. Briefly, 4 replicates of 6 serial four-fold dilutions of each sample (1:4 to 1:1024) were mixed in 96-well plates with an equal volume of the BoHV-1 reference strain Los Angeles containing 200 $TCID_{50}$; this mixing led to a final neutralization stage of 1:8 to 1:2048. In the case of the animals that remained negative for neutralizing antibodies after vaccination with sub-potent batches of calibration vaccines (containing 10^6–10^5 $TCID_{50}$/per dose, described in Section 2.5), the samples were re-tested in serial two-fold dilutions starting at 1:2 dilution for validation purposes. In all cases, an extra replicate without viruses was used as a toxicity control for the sera. To confirm that the virus neutralization assays were accurate, positive and negative control sera were also included in each run. Serum-virus mixtures were incubated for 1 h at 37 °C and then 100 μl of the Madin Derby Bovine Kidney (MDBK) cell suspension at 200,000 cells/ml ± 50,000 were added. After incubation for 2–3 days at 37 °C, plates were read microscopically for cyto-pathogenic effects. Each run of the assay was accepted if the back titration of the virus gave an infectious titer of 100 $TCID_{50}$ with a range of tolerance of 50–200 $TCID_{50}$. The positive control showed the expected antibody titer ±1 standard deviation; the negative serum showed no neutralization (monolayer with cyto-pathogenic effect), and the monolayer of control cells (cells plus medium and without virus and serum) was intact. Virus neutralizing antibody titers were calculated with the Reed and Muench method (Reed and Muench, 1938). Negative serum samples received the arbitrary value of 0.3 for calculation purposes.

2.6.2. Enzyme linked immunosorbent assay (ELISA) for the quantitation of total antibody to BoHV-1 in bovine

Total antibodies in bovine serum were determined using an indirect ELISA as described previously (Bratanich et al., 1990; Del Medico Zajac et al., 2006; Romera et al., 2000). Briefly, polystyrene microtiter ELISA plates (Immulon 1B, Dynatech Laboratories) were coated with 50 μl of positive (concentrated and semi-purified BoHV-1 reference strain Los Angeles containing 10^9 $DICT_{50}$/ml) or negative antigen (non-infected MDBK cells) in carbonate buffer of pH 9.6 and incubated for 12 h at 4 °C. Plates were blocked with 10% ovoalbumin in PBS-$Tween_{20}$ 0.05%, washed twice and stored at −20 °C until use. Samples were tested at 1:4, 1:10 and 1:40 dilutions for herd surveys and in 6 serial four-fold dilutions starting at 1:40 dilution for vaccine potency testing (Parreno et al., 2010). For standardization purposes, as described for virus neutralization, the samples of immunized animals with sub-potent calibration vaccines (containing 10^5 $TCID_{50}$/per dose, described in Section 2.5) that were negative at a 1:40 dilution were re-tested starting at 1:4 dilution. A peroxidase-labeled affinity purified goat anti-bovine IgG (H + L) (Kirkegaard & Perry Laboratories, KPL) in a 1:4000 dilution was used as the conjugate. After each incubation, plates were washed 4 times. The reaction was developed using hydrogen peroxide/2,2′-azino-bis 3-ethylbenzthiazoline-6 sulfonic acid (ABTS) in citrate buffer (pH 5) as the substrate/chromogen system. The antibody titer of each sample was expressed as the $\log_{10}$ of the reciprocal of the highest serum dilution with a corrected optical density OD_{405C} (OD_{405} in the positive coated wells minus OD_{405} in the negative coated wells) greater than the cut-off value of the assay.

2.6.3. Enzyme linked immunosorbent assay (ELISA) for the quantitation of total antibody to BoHV-1 in guinea pigs

The ELISA assay was adapted to detect guinea pig antibodies using the same procedure described for bovine samples, and a peroxidase-labeled affinity purified goat anti guinea pig IgG (H + L), (Kirkegaard & Perry Laboratories, KPL) was used as conjugate in a 1:4000 working dilution. As an alternative model for vaccine potency testing, samples of immunized guinea pigs were tested with 6 serial four-fold dilutions starting at 1:40 dilution (Parreno et al., 2010).

2.7. Statistical methods included in the ELISA validation

2.7.1. Feasibility studies and initial repeatability

The two positive control sera with high neutralizing antibody titer to BoHV-1 described in Sections 2.2 and 2.3 were used to evaluate the preliminary repeatability of the assay. The repeatability was expressed as the coefficient of variation of the corrected optical density of the positive control sera obtained in the runs carried out during the first year of use in Laboratory A. The control sera were assayed in duplicate in every ELISA run, and the mean of both replicates needed to fall within an established range of corrected optical density for the plate to be considered valid. Those ranges were defined also with the data obtained during the first year of the use in Laboratory A. Thereafter, both sera were assigned arbitrarily a value of 100% of positivity and used to harmonize the assay results. In the case of the bovine positive control sera, its performance was also compared to the strong positive international reference serum IBR-EU1.

2.7.2. Diagnostic sensitivity and specificity of the ELISA

The diagnostic sensitivity and specificity of the ELISA for BoHV-1 antibody detection in bovine and guinea pigs sera were calculated following the recommendations given by Greiner and Gardner in 2000 and the 2008 OIE Manual of Terrestrial animals (Greiner and Gardner, 2000a,b; OIE1.1.4, 2008). Briefly, the cut-off of the assay and its associated diagnostic sensitivity and specificity were estimated based on the frequency distribution of the ELISA values obtained after analyzing the bovine and guinea pig sera defined as reference populations. Bovine samples were tested at the 1:4, 1:10 and 1:40 dilutions, and the guinea pigs were analyzed at the

1:40 dilution. ELISA results were normalized by expressing the raw corrected optical density values as the percentage of positivity of the high-positive bovine and guinea pig reference control sera described in Sections 2.1 and 2.2. The control sera were assigned a 100% value and were included in duplicate in every plate and all ELISA runs. Additional control sera of known antibody titer were included in each assay. An ELISA run was accepted if the corrected optical density of the positive control fell within the established admissible working range as the mean of the corrected optical density plus one standard deviation and if the second standard serum sample gave the expected antibody titer. In this study, the sensitivity of the assay was defined as the probability of a positive test result given that the animal was truly positive for the antibodies to BoHV-1 (either due to infection or vaccination), while the specificity was defined as the probability of a negative test result given that the animal was truly negative for antibodies to BoHV-1 (Greiner and Gardner, 2000a).

2.7.3. ROC analysis

The cut-off selection of the assay for both species was also carried out with the aid of the receiver-operating (ROC) analysis (Greiner et al., 2000), using the MedCalc® version 11.1.1.0 statistical software.

2.7.4. Detection limit and linearity of the assay

The relative analytical sensitivity or detection limit of the ELISA was defined as the least amount of antibody to BoHV-1 that was detectable by the assay in a positive sample of known antibody titer. In the case of bovine samples, the absolute analytical sensitivity of the test was estimated by the endpoint limiting dilution analysis of the weakly positive international reference sample (IBR-EU2). In addition, to harmonize the new ELISA with existing tests, the titer of the positive control (PR 275-07) was compared to the titer obtained with the strong positive international reference sample (IBR-EU1). To determine the analytic sensitivity, linear regression curves were determined by plotting the ELISA values of those reference sera expressed as percent of positivity of the positive control of the assay versus the log-transformed dilutions (Greiner and Gardner, 2000b; OIE1.1.4, 2008; OIE2.4.13, 2008).

To evaluate the linearity of the assay, dose–response curves were constructed using 40 replicates of a positive bovine field sample (PR 42-06) and 14 replicates of a positive guinea pig serum (of known neutralizing antibody titer), which were assayed in four-fold dilutions (1.60–4.61) in several plates within different independent ELISA runs. The linearity of the system was verified by an analysis of variance that partitioned the residual square sum into lack of fit and pure error (Draper and Smith, 1998).

2.7.5. Intermediate precision

For full validation, the intermediate precision of the assay was estimated using the data of the bovine and guinea pig positive control sera collected from ELISA runs conducted for 3 years in the Gastroenteric virus division (Laboratory A), which has been ISO 9001-2000 certified since 2006.

To estimate the repeatability and intermediate precision of the ELISA for each species, an analysis of variance for a nested model of variance was carried out. The applied model allowed for the quantification of the relative contribution of the different sources of variation (intra assay: same plate and different plates; inter assay: different runs; and different samples: bovine or guinea pig serum) expressed as the relative coefficient of variation (Davies, 1960). In all cases, the estimations were conducted using the corrected optical density values.

2.7.6. Assay reproducibility: inter-laboratory assay

The inter-laboratory assay included data collected in four laboratories for bovine samples and three laboratories for guinea pigs samples. The laboratories included were the Gastroenteric Virus division (Laboratory A), the Herpesvirus division (Laboratory B) and the Food and Mouth Disease division (Laboratory C) from the Virology Institute, INTA, Castelar and a private veterinary laboratory (Laboratorio Azul, Azul, Buenos Aires, Laboratory D). The laboratories received the kit and coded samples including the positive and negative controls and standard sera. The laboratories were requested to perform five ELISA assays following the supplied protocol including two plates per run and testing of the samples in two replicates per plate. The reproducibility or inter-laboratory intermediate precision of the assay was calculated as the coefficient of variation (relative standard deviation) using the same nested analysis of variance already mentioned (2.6.4).

2.7.7. Relative sensitivity and specificity of the ELISA compared with virus neutralization assay

ELISA sensitivity and specificity relative to the virus neutralization (1 h/37 °C) assay was evaluated by the analysis of the serum samples described above. The agreement between the techniques was assessed by the calculation of the kappa coefficient and its 95% confidence interval.

2.7.8. Correlation between the antibody titer determined by ELISA and virus neutralization in vaccinated bovines and guinea pigs

The relationship between the antibody titer obtained by ELISA and virus neutralization in vaccinated bovines and guinea pigs was studied. For this comparative study, we selected samples obtained at 60 days post-vaccination in bovines and 30 days post-vaccination in guinea pigs. The evaluation of the kinetics of the antibody response after the vaccination showed that these selected time points showed peak antibody titers for aqueous vaccines and the plateau for oil vaccines (Fernandez et al., 2009; Parreno et al., 2010). Specifically, we evaluated (i) the correlation between the neutralizing and ELISA antibody titer to BoHV-1 of the individual serum samples and (ii) the correlation between the mean antibody titers of groups of 5–10 bovine/guinea pigs vaccinated with the same vaccine.

3. Results

3.1. Feasibility studies and initial repeatability

The initial standardization of the ELISA for bovine samples was conducted by Bratanich et al. (1990). After that, the assay was implemented for research used in three sections of the Virology Institute, INTA. In 2006, the assay was standardized for guinea pig samples. The performance of the bovine and guinea pig sera selected as positive controls of the ELISA was evaluated after the first year of ISO 9001-2000 standards implementation in Laboratory A. The analysis included 190 replicates of the bovine serum (PR 161-06) and 221 replicates of the guinea pig serum (PR 74-06). The coefficient of variation and the admissible working range of the corrected optical density established for each positive control are detailed in Table 1. As mentioned in Section 2, the positive control sera were run in duplicate in every plate. The mean of both replicates should fall within the established range of to consider the plate valid.

3.2. Cut-off, diagnostic sensitivity and specificity of the ELISA for bovine and guinea pigs: agreement with virus neutralization assay

All bovine samples ($n = 132$) were tested with virus neutralization (1 h/37 °C) and ELISA. A total of 39 samples were positive for

Table 1
IBR Ab ELISA: validation parameters.

Validation parameters	Result			Acceptance criteria
	Bovine		Guinea pig	
Cut-off	20%	40%	40%	
Diagnostic specificity	97.9%	100.0%	94.9%	
Diagnostic sensitivity	100.0%	89.8%	100.0%	
Application	Screening	Vaccine potency testing	Vaccine potency testing	
Positive control mean ODc ($\bar{y}$)	0.960		0.740	
Standard deviation (SD)	0.210		0.220	
95% confidence interval	0.926–0.985		0.710–0.770	
N	190		221	
ODc admissible working range of the positive control ($\bar{y} \pm 1$ SD)[a]	0.750–1.170		0.520–0.960	
Repeatability[b]	4.3%		9.3%	CV < 20%
Intermediate precision[b]	22.2%		23.1%	CV < 25%
Linearity	Yes, $F^c = 0.49$, $p^c = 0.69$		Yes, $F = 0.83$, $p = 0.53$	
Inter-laboratory assay				
Intermediate precision[d]				
(Intra-assay)	14.9%		8.1%	CV < 25%
(Inter-assays)	16.2%		17.1%	
Reproducibility[d]	12.4%		≈0%	CV < 30%
Inter-laboratory	(4 labs)		(3 labs)	
Total variation[d]	26.2%		10.6%	

[a] Calculated with the data of the assays run during the first year of used, in Laboratory A.
[b] Repeatability and intermediate precision calculated with the data selected from the ELISA runs carried out in Laboratory A, for 3 years (2006–2008). During this period, 4 batches of conjugate, for each species, 4 antigen batches for coating and one panel of control sera were used. ANOVA model: 10 assays, 2 plates, 2 replicates.
[c] Value of Fisher "*F*" for linearity test and the corresponding *p*-value.
[d] Repeatability, intermediate precision and reproducibility obtained in the inter-laboratory assay. ANOVA model for inter-laboratory data analysis: 5 assays, 2 plates, 2 replicates per plate conducted in 4 laboratories for bovine positive control and three laboratories for the guinea pig positive control.

neutralizing antibodies, while 93 were negative. For the ELISA, all of the positives samples gave percent of positivity that were higher than 20% at the 1:40 dilution, and 91 out of 93 negative samples showed values that were lower than 20% (when tested at 1:4; 1:10 and 1:40 dilutions). The frequency of the percent of positivity distribution obtained for the positive and negative reference samples run at 1:40 dilution is depicted in Fig. 1a. Using a 20% cut-off, the assay showed a 100% diagnostic sensitivity and 97.85% diagnostic specificity (Fig. 1b) and was considered suitable to use as a screening method for field surveys of infected animals. This cut-off was confirmed by ROC analysis conducted by MedCalc® software as the threshold that gives the highest accuracy (Table 1 and Fig. 1d). The value obtained for the area under the ROC curve (AUC = 0.99, $p = 0.0001$) indicated that the test has the ability to distinguish between the positive and negative samples with 95% confidence in the 99.9% of the times that a random sample is tested. To have the sensitivity required by the OIE for international trade purposes, samples must be tested at a starting dilution of 1:4.

A second cut-off at 40% provided 89.74% diagnostic sensitivity and 100% diagnostic specificity. This threshold was selected when the assay was used for vaccine potency testing in the target species (Table 1 and Fig. 1c).

Whether the cut-off was used for screening (20%) or for vaccine testing (40%), the agreement between the ELISA and virus neutralization was almost perfect (Fig. 1b and c; kappa: 0.964 and 0.925, respectively). The estimated sensitivity and specificity values shown in Fig. 1b and c also express the relative sensitivity and specificity of the ELISA regarding the virus neutralization assay (1 h/37 °C), using each cut-off.

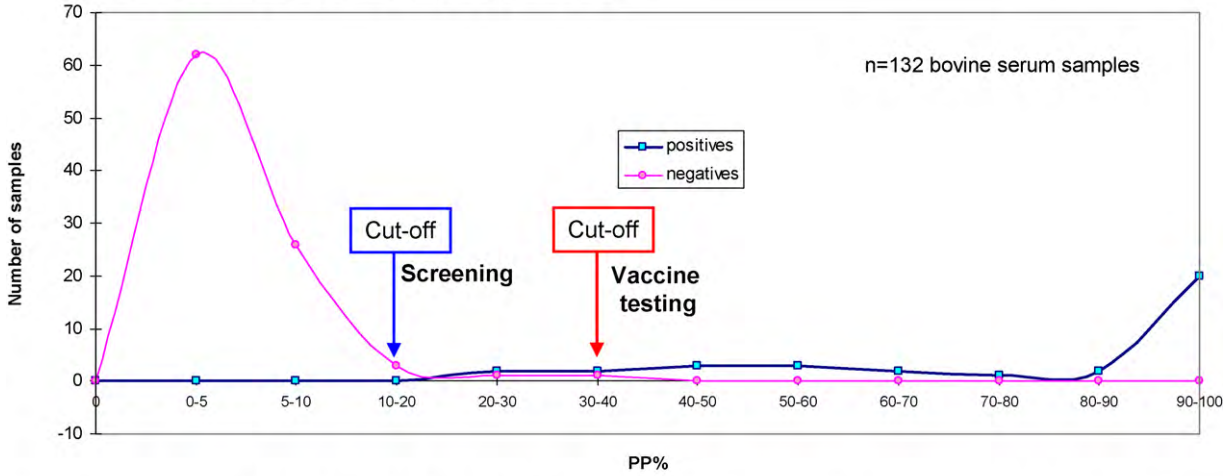
For guinea pig samples, the frequency analysis of the percent of positivity value distribution of the reference sera showed that a cut-off between 37 and 39% gave the highest levels of diagnostic sensitivity and specificity (99%; data not shown). However, because the assay was designed to evaluate vaccine potency, a higher cut-off value of 40% was selected, giving 95% sensitivity and the maximum specificity (100%) (Fig. 1a). This value was also obtained when the cut-off was calculated as the mean plus three standard deviations of the negative reference population and confirmed by ROC analysis as the cut-off of the highest specificity (Table 1 and Fig. 2b). Similarly to the ROC curve obtained for bovine samples, the ELISA has the ability to score properly a guinea pig sample 99.8% of the times that is tested, with 95% confidence. Using a 40% cut-off the ELISA showed an almost perfect agreement with the virus neutralization assay (kappa: 0.943) and a relative sensitivity and specificity of 99.21% and 94.95%, respectively (Fig. 2c).

3.3. Detection limit and linearity of the assay

In the case of the ELISA for bovine samples, the detection limit or absolute analytical sensitivity of the test was estimated by the limiting dilution of the OIE international reference samples (Perrin et al., 1994). The weakly positive sample (IBR-EU2) tested in the four-fold serial dilutions was detected as positive only in the 1:4 dilution of the reconstituted material using the screening cut-off (PP = 20%) (Fig. 3a). The strong positive serum (IBR-EU3) gave an ELISA antibody titer of 4.01 using the same cut-off, or 3.4 with a 40% threshold. To harmonize the assay, the internal positive control was titrated in parallel with the external reference, and its titer was also 3.4 (Fig. 3b). Because this bovine control serum has a neutralizing antibody titer determined by virus neutralization 1 h/37 °C of 2.4, the ELISA titer was 20 times higher than the neutralizing antibody titer.

To evaluate the linear behavior of the assay and to estimate the detection limit relative to the virus neutralization test, serial four-fold dilutions of a bovine serum sample and a guinea pig serum sample, both with a neutralizing antibody titer of 2.4 determined by VN 1 h/37 °C were assayed. The bovine serum was tested in 40 plates distributed in 5 assays (Fig. 3c), and the guinea pig serum was tested in 14 plates within 6 ELISA runs (Fig. 3d). In both cases, the regression analysis of the obtained dose–response curves indicated that the assays showed a range of linear behavior. The linearity of the system was verified in both cases by analysis of variance, demonstrating that the lack of fit of the model was not significant, for bovine and guinea pigs, respectively (Table 1). At a 40% cut-off,

(a) Bovine Reference population frequency distribution



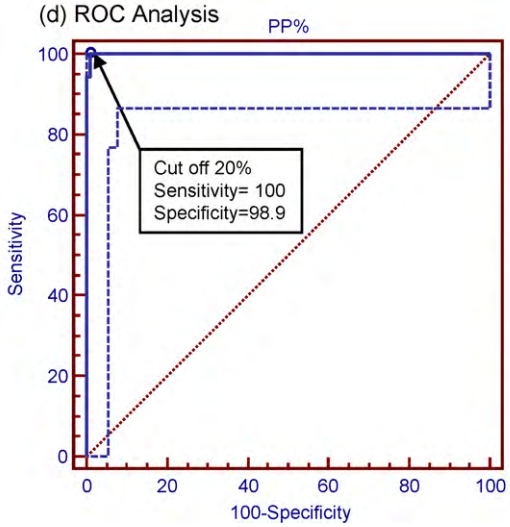
(b) Diagnostic sensitivity and specificity for screening

ELISA Cut-off 20%	BOVINE Reference population		
	positive	negative	Total
positive	39	2	41
negative	0	91	91
Total	39	93	132
Kappa 0.964 p<0.0001	**DSe= 100%**	**DSp= 97.6%**	

(c) Diagnostic sensitivity and specificity for vaccine testing

ELISA Cut-off 40%	BOVINE Reference population		
	positive	negative	Total
positive	35	0	35
negative	4	93	107
Total	39	93	132
Kappa 0.925 p<0.0001	**DSe= 89.7%**	**DSp= 100%**	

(d) ROC Analysis



Area under the ROC curve (AUC)	0.999
Standard Error	0.000765
95% Confidence Interval	0.970 to 1.000
z statistic	652.525
Significance level P (Area=0.5)	0.0001

MedCalc sofware version 11.1.1.0

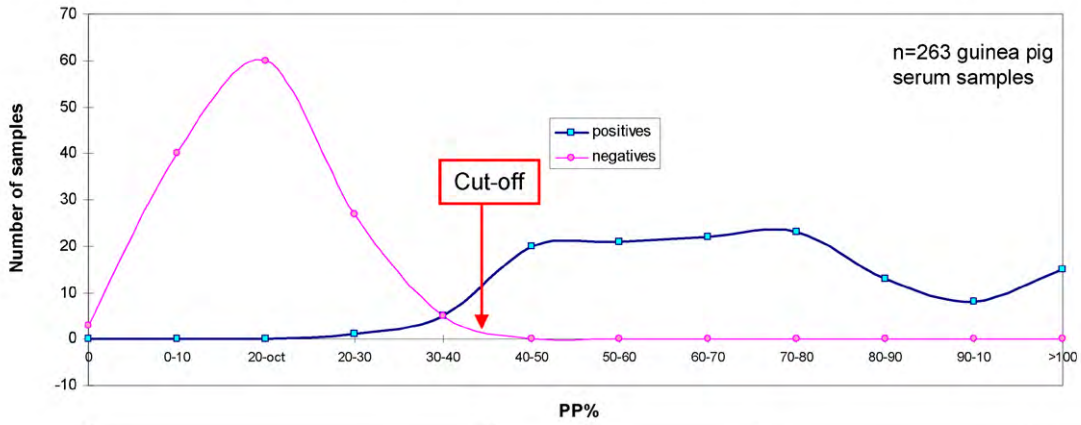
Fig. 1. Cut-offs of the assay for bovine samples and their associated diagnostic sensitivity (DSe) and specificity (DSp). (a) Frequency distribution of the ELISA values obtained after analyzing the bovine reference population at a 1/40 dilution. ELISA values were expressed as the percentage of positivity (PP%) of the high-positive bovine reference control sera as described in Section 2.2. (b) Diagnostic sensitivity and specificity using the cut-off selected for screening of cattle. (c) Diagnostic sensitivity and specificity using the cut-off selected for vaccine potency testing in the host species. (d) ROC analysis, MedCalc® version 11.1.1.0 statistical software.

the ELISA antibody titers of the samples were 3.4 and 3.9, for the bovine and the guinea pig serum, respectively. Again, the ELISA titer of the bovine sample was 20 times higher that the titer obtained by virus neutralization, while for the guinea pig serum the ELISA was able to detect a positive sample at 1/50 higher dilution than the virus neutralization.

3.4. Intermediate precision

To calculate the intermediate precision of the assay, data selected from the ELISAs run in Laboratory A over a 3-year period (2006–2008) was analyzed using a nested analysis of variance. During this period, a total of 4 conjugates antibodies for each species,

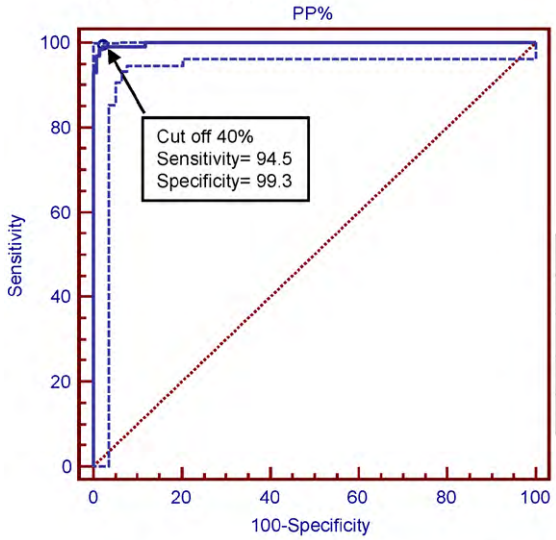
(a) Guinea pig Reference population frequency distribution and associated Diagnostic Sensitivity and Specificity



ȳ + 3 SD = 14.84 + 3 x 8.36 = 39.97

ELISA Cut-off 40%	GUINEA PIG Reference population		
	positive	negative	Total
positive	122	0	122
negative	6	135	141
Total	128	135	263
	DSe 95%	**DSp 100%**	

(b) ROC Analysis



Area under the ROC curve (AUC)	0.998
Standard Error	0.00113
95% Confidence Interval	0.983 to 1.000
z statistic	440.044
Significance level P (Area=0.5)	0.0001

MedCalc sofware version 11.1.1.0

(c) Concordance analysis ELISA vs. Virus Neutralization

ELISA Cut-off 40%		Virus neutralization		Total
		positive	negative	
IBR ELISA	positive	127	6	133
	negative	1	113	114
Total		128	119	**247**
Kappa = 0.943 p=0.0001		**RSe 99.2%**	**RSp 94.9%**	

Fig. 2. Cut-offs of the assay for guinea pig samples and their associated diagnostic sensitivity (DSe) and specificity (DSp). (a) Frequency distribution of the ELISA values obtained after analyzing the guinea pig reference population at a 1/40 dilution. ELISA values were expressed as the percentage of positivity (PP%) of the high-positive guinea pig reference control sera as described in Section 2.3. (b) ROC analysis, MedCalc® version 11.1.1.0 statistical software. (c) Agreement between ELISA and virus neutralization using a 40% cut-off value. Only 247 out of 263 serum samples were available for testing by virus neutralization.

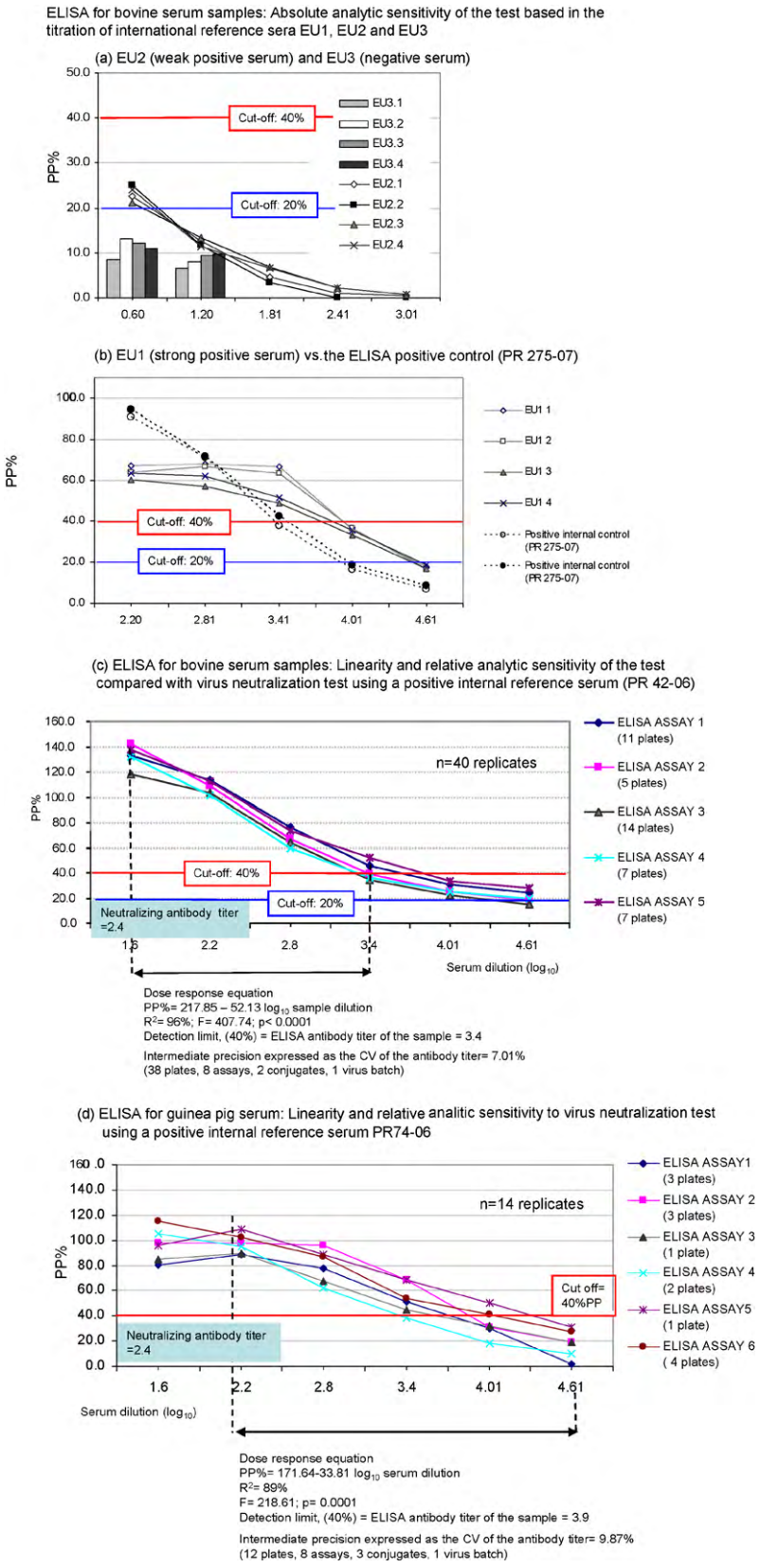


Fig. 3. Dose–response curves limiting dilution analysis of: (a) IBR-EU2 (external weakly positive serum) and IBR-EU3 (external negative serum), serial four-fold dilutions starting at 1:4; (b) IBR-EU3 (external strong positive bovine serum) vs. the ELISA positive control (PR 275-07), serial four-fold dilutions starting at 1:160; (c) a BoHV-1 specific bovine standard serum (PR 42-06) of known neutralizing antibody titer; (d) a BoHV-1 specific guinea pig serum (PR 74-06) of known neutralizing antibody titer. The response detected by ELISA was expressed as the PP% of the positive control sera. Titers were expressed as the $\log_{10}$ of the highest dilution with a PP% higher than the cut-off of the assay (20% and/or 40%). Each line represents the mean PP% value obtained for the different replicates of the diluted sample in each plate of every independent ELISA run. Linearity of the ELISA was evaluated by ANOVA of the regression analysis, partitioning the residual square sum into lack of fit and pure error (Table 1).

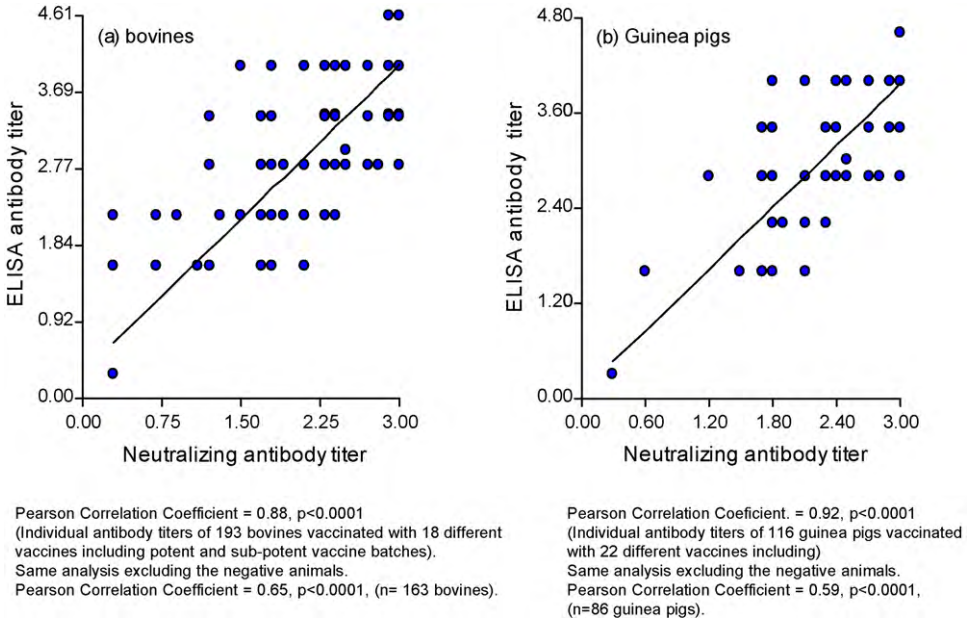


Fig. 4. Relevance of the ELISA defined as the correlation between individual ELISA and neutralizing antibody titers induced by killed IBR vaccines in groups of 5–10 (a) bovine calves, sampled at 60 days post-vaccination, (b) guinea pigs sampled at 30 days post-vaccination.

4 batches of BoHV-1 antigen for coating and one set of positive and negative control samples were used. The analysis of variance for the proposed nested model (3 years, 5 assays, 6 plates per assay and 2 replicates per plate) indicated that the assay had very good values of repeatability (intra plate variation). The coefficient of variation for the bovine and the guinea pig positive controls were 4.3% and 9.3%, respectively. The intermediate precision of the assay given by the overall relative variation for both species was also acceptable (CV_{bovine} = 22.2% and $CV_{guinea\ pigs}$ = 23.1%) and met the OIE requirements (CV < 25%) (Table 1).

3.5. Correlation between the antibody titer obtained by ELISA and virus neutralization in vaccinated animals

Because the ELISA will be used not only for field surveys, but also to evaluate antibody response to vaccination in cattle and guinea pig, the relationship between the antibody titer obtained by ELISA and virus neutralization in vaccinated bovines and guinea pigs was investigated. For this purpose, the data obtained from animals immunized with sub-potent to highly immunogenic vaccines (10^5 to 10^8 $TCID_{50}$ of BoHV-1 per dose) were analyzed by correlation analysis. The analysis was conducted at specific time points selected to be the most appropriate for vaccine quality control: 60 days post-vaccination for bovines and 30 days post-vaccination for guinea pigs. There was a significant correlation between the ELISA and the neutralizing antibody titers to BoHV-1 determined in the same serum sample for both species. Some of the animals receiving the sub-potent vaccines (10^6–10^5 TCD_{50} of BoHV-1 per dose) did not develop a detectable antibody response by virus neutralization and ELISA. Excluding the negative animals, the Pearson correlation coefficient were lower, but still statistically significant ($p < 0.001$). The scatter plots, where both coefficients are reported, are depicted in Fig. 4.

The correlation between the mean antibody titer of groups of 5–10 bovines/guinea pigs vaccinated with the same vaccine was also evaluated, and again a significant correlation between the antibody titers determined by virus neutralization and ELISA was obtained for both species (0.95 and 0.97, respectively, $p < 0.0001$; data not shown).

3.6. Assay reproducibility: inter-laboratory assay

As part of the inter-laboratory assay, the repeatability and intermediate precision of the ELISA were estimated in all participating laboratories, using an analysis of variance model of data from 10 assays, 2 plates and 2 replicates. With this model, the repeatability of the assay for the bovine positive control was 5.2% for Laboratory B and 6.9% for Laboratory C, and the intermediate precisions were 19.9% and 21.3%, respectively. For the guinea pig positive serum control, the repeatability and intermediate precisions of the assay obtained in Laboratory D were 9.1% and 14%, respectively.

The inter-laboratory study data were analyzed with an analysis of variance with 5 ELISA runs, 2 plates and 2 replicates and were collected in four laboratories (A, B, C and D) for the bovine samples and three laboratories (A, B and D) for the guinea pig samples. The overall mean for the bovine and guinea pig positive controls were $\bar{y} = 0.894$ and $\bar{y} = 0.556$, both falling within the range of acceptance (Table 1).

The reproducibility (inter-laboratory-precision) of the assays expressed as the coefficient of variation was 12.4% for bovine, while it was close to zero for the guinea pig positive control (Table 1). The unexpected extremely high reproducibility obtained for guinea pigs was double checked.

4. Discussion

Two indirect ELISAs for BoVH-1 antibody detection in bovine and guinea pigs sera were validated to meet 17025 International Standards Organization (ISO) requirements (Greiner and Gardner, 2000a; Jacobson and Romatowski, 1996; OIE1.1.4, 2008; Wright, 1998).

The statistical validation included the estimation of the reliability of the assays using certain cut-off values and their associated diagnostic sensitivity and specificity established in both of the species to fit the purpose of the assay (Greiner and Gardner, 2000b).

A 20% cut-off and 100% sensitivity was adopted to use the assay as a screening method for a field survey of infected cattle. Using this threshold, the test was able to score the EU1 and EU2 sera as positive, while the EU3 was scored as negative, complying with the OIE requirements (Perrin et al., 1994). It is important to note that serum

samples should be tested at a starting dilution of 1:4 to achieve the sensitivity required by the OIE. Several BoHV-1 ELISAs were developed that showed similar performance and demonstrated to be more suitable than the virus neutralization for herd surveys (Cho and Bohac, 1985; Kramps et al., 1994, 2004; Perrin et al., 1994). In this regard, in the protocol of the neutralization assay recommended by the OIE for the selection of BoHV-1 negative animals for international trade, it is highly important that the serum-virus mix is incubated for 24 h at 37 °C (24 h/37 °C). This assay has a higher sensitivity than the one used in the present study (1 h/37 °C) (Bitsch, 1978). In addition, it is well known that the sensitivity and specificity of an assay varies either with the stage of the infection or with the immune status of the host (Kramps et al., 2004); thus, in the positive reference population we included naturally infected bovines from endemic farms, samples from vaccinated animals and samples from experimentally challenged animals (Del Medico Zajac et al., 2006; Parreno et al., 2010; Romera et al., 2000). The ELISA was able to detect positive animals from 11 days post challenge and beyond (Romera et al., 2000). Taking together the latter result and the detection limit obtained with the IBR-EU2 weakly positive international sample, the ELISA developed herein has a sensitivity similar to the virus neutralization method proposed by the OIE (24 h/37 °C), with the advantage that the results can be obtained in a few hours instead of several days.

Because the entire virus is used in the assay, the sera containing antibodies to other alphaherpesviruses will also be recognized. In fact, sera containing antibodies against the other two alphaherpesviruses reported in Argentina, BoHV-5 and BoHV-4, showed strong cross-reactivity (Del Medico Zajac et al., 2006; Verna et al., 2008). On the other hand, guinea pig serum samples from animals vaccinated with bovine viral diarrhea virus, bovine parainfluenza type 3 virus and rotavirus showed no cross reaction in the assay (data not shown).

It is important to highlight that BoHV-1 as well as BoHV-5 infections are endemic in Argentina (Campero et al., 2003; Del Medico Zajac et al., 2006; Moore et al., 2003; Odeón et al., 2001). To retain international trade markets, vaccination and serology surveys of the herds will be mandatory in the near future. The ELISA developed herein represents the first technique that was validated using serum samples representing the epidemiology of the disease in the region and international reference samples, that will be commercially available in the country. The used of this ELISA to detect antibodies in serum, in combination with BoHV-1/5 multiplex PCR to detect virus genome in semen samples, represent a suitable method of high sensitivity and minimal risk of false negative results for the selection of bulls as semen donors' for exporting to BoHV-1-free countries. The technique also constitutes an important method for the implementation of BoHV-1 eradication programs in the region.

A 40% cut-off was selected in both species when the ELISA was applied to vaccine potency testing to obtain the maximum specificity and to produce the best agreement with the virus neutralization test, which is the official test used by the international regulatory agencies for vaccine quality control (CFR.113.216, 1985; OIE2.4.13, 2008). The virus neutralization using 1 h incubation for the serum-virus mix (1 h/37 °C) was the method of choice for this purpose (animal selection to be included in field trials for vaccine potency testing as well as the method to evaluate the antibody response to vaccination). Thus, the performance of the ELISA was compared with the viral neutralization (1 h/37 °C).

A very good correlation was obtained between the ELISA and neutralizing antibody titers in vaccinated animals analyzed at specific time points after vaccination (60 days for bovines and 30 days for guinea pigs), indicating the relevance of the developed ELISAs to be applied for batch to batch vaccine potency testing in both species. It is important to highlight that this correlation was specific for the selected time points, which corresponded to the peak antibody titer after vaccination with aqueous vaccines and the plateau of the antibody response to oil vaccines in each species. The analysis was conducted to evaluate the ELISA assay as a suitable method to replace or complement the virus neutralization for vaccine potency testing in cattle and in the guinea pig model (Parreno et al., 2010). It is well known that neutralizing antibody titers to BoHV-1 after vaccination and natural infection are always lower than the antibody titers determined by ELISA because neutralizing antibodies represent only a fraction of the total IgG antibody to the whole virus detected by ELISA. In addition, neutralizing antibodies to BoHV-1 decrease over time, reaching undetectable levels after several months post infection or vaccination. However, primed animals remain seropositive for antibodies to BoHV-1 detected by ELISA for life. Then the ELISA and neutralizing antibody titers no longer are correlated (Romera et al., 2000).

As part of the validation of the ELISA for vaccine quality control, the detection limit of the assay was calculated in terms of the lower antigen dose included in an oil-in-water vaccine that can induce detectable levels of antibodies in bovine and guinea pigs, resulting in both cases in 1×10^6 $TCID_{50}$ of BoHV-1 per dose. Finally, the relationship between ELISA antibody titers induced by killed IBR vaccines and protection against infection was also established (Parreno et al., 2010). The concordance obtained between the ELISA antibody titers of immunized calves and guinea pigs indicated that this assay applied in serum from immunized guinea pig represents an excellent alternative method for BoHV-1 vaccine potency testing, which is line with the 3Rs initiative of refining, reducing and replacing animal experimentation (Hendriksen, 2009).

The ELISA showed very good intermediate precision and reproducibility in both species. Surprisingly, for guinea pigs samples, the ELISA gave almost perfect reproducibility among laboratories. Further studies are under way, including a larger number of laboratories to confirm this finding.

After conducting a survey to evaluate the quality of IBR killed vaccines available in the local market during 2008 and 2009, The Argentinean Sanitary Authority (SENASA) has decided to adopt the guinea pig model (Parreno et al., 2010) together with the virus neutralization assay (1 h/37 °C) and the present ELISA as the official method to control the potency for BoHV-1 of the vaccine batches to be released to the market in Argentina.

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