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Development and Validation of a Novel ELISA for the Detection of *Neospora Caninum* Antibodies in Bovine Sera

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Abstract

Neospora caninum is a world-wide distributed apicomplexan parasite, causative agent of bovine neosporosis, which is one of the major causes of reproductive losses in cattle affecting both dairy and beef industries. Several techniques are regularly used for the diagnosis of bovine neosporosis. Amongst them, serological techniques are generally preferred, being IFAT the traditionally accepted reference method for diagnosis (gold standard). However, ELISA has become the most eligible method for large-scale screening of specific antibodies against *N. caninum* in diagnostic laboratories around the world, providing results that can be easily standardized between laboratories, within a few hours at a relative low-cost. There is no local production of commercial ELISA tests nor in Argentina neither in South America, therefore they must be purchased abroad, turning unfeasible the routinely use of ELISA tests. Here we have developed and validated accordingly to OIE specifications a novel ELISA test based on soluble antigens from *N. caninum* tachyzoites to accurately determine the presence of specific antibodies anti- *N. caninum* antigens in bovine sera samples, enabling the rapid, objective and low-cost diagnosis of bovine neosporosis in Argentina; replacing IFAT and avoiding delays and high importation taxes.

Keywords: *Neospora Caninum*; ELISA; Bovine Sera Samples; IgG

List of abbreviations: *N. caninum: Neospora Caninum; T. gondii:* Toxoplasma Gondii; IHQ: Immunohistochemical Staining; IB: Immunoblotting; DAT: Direct Agglutination Test; IFAT: Indirect Fluorescent Antibody Test; ELISA: Enzyme-Linked Immunoabsorbent Assays; OIE: World Organisation for Animal Health; sNcAg: Soluble Fraction of a Whole Tachyzoite Lysate; HRP: Horseradish Peroxidase; CV: Coefficient of Variation; OD: Optical Density; SnD: Diagnostic Sensitivity ; SpD: Diagnostic Specificity; S/P: Sample/Positive Ratio; ROC: Receiver Operating Characteristic Analysis; κ: Cohen's Kappa Coefficient; TP: True Positive; TN: True Negative; FP: False Positive; FN: False Negative; CI: Confidence Interval; PPV: Positive Predicted Value; NPP: Negative Predicted Value

Introduction

Neospora caninum is a world-wide distributed apicomplexan parasite. It has a heteroxene life cycle, in which dogs and canids in general are the definitive hosts [1]; and a wide range of domestic and wild animals (cattle, buffalos, sheeps, horses, dogs, cats, etc) are the intermediate hosts [2]. *N. caninum* is the causative agent of bovine neosporosis, which is considered one of the major causes of reproductive losses in cattle as it can lead to abortion, stillbirth, reduced milk production and weight gain and increased birth-to-conception intervals and replacement rates [3-6] induced by mid-gestational exposure to the parasite or recrudescence of latent infections [2,7]. Globally, the estimated median losses due to *N. caninum* induced abortions were valued in US $1,298.3 million per annum (range US $633.4 million to US $2,380.1 million), affecting both dairy and beef industries [8]. It is closely related to other coccidian parasites, particularly to *Toxoplasma gondii* [9-12]. Both species share many common features, but Neospora cannot infect humans and does not share the same wide host range as Toxoplasma [1,2,12,13]. It also shows a higher efficiency of vertical transmission in cattle [14,15]. Considering this, several studies have assessed the cross-reactivity between both species in serological tests, finding no or faint cross-reactions for most of the immunodominant antigens present in the whole tachyzoite antigen [16-18].

Several experimental vaccines against neosporosis have been evaluated in different animal models. However, none of them is currently marketed, and only control actions regarding to the management of the animals herds are used [19-21]. The culling of infected cattle from the herd has been proposed to be a quite efficacious control method, though it is very expensive [22]. Other options include the selective breeding from only seronegative cows to both beef and milk industries or the breeding of seropositive cows only for the beef industry, together with the culling of those cows that have actually aborted with a confirmed diagnostic of neosporosis; besides strengthening the biosecurity measures to avoid horizontal transmission. In addition to this, it has been reported that seropositivity to *N. caninum* increases the risk of abortion, with estimates for the increased risk often quoted to range between 1.7 and 7.4-fold [5,23], but decreasing over time with increasing parity [5]. In this context, the development of reliable diagnostic tools to accurately detect the spread and circulation of the parasite is paramount.

Several techniques are regularly used for the diagnosis of bovine neosporosis, including histology and immunohistochemical staining (IHQ), nested-PCR [24], immunoblotting (IB), direct agglutination test (DAT), direct fluorescent antibody test (IFAT) and different enzyme-linked immunoabsorbent assays (ELISAs) [25-27]. Serological assays are preferred, being IFAT generally considered as the "gold standard" test [28,29]. However, due to the fact that it is easier and less time-consuming to obtain rapid results from large cattle herds with ELISAs than with IFAT, this assay has become the most eligible method for high-throughput screening of specific antibodies against *N. caninum* in diagnostic laboratories around the world [3]. ELISAs can provide results within a few hours at relatively low-cost do not rely on cell cultures or live parasites and do not need expensive specialized equipment, thus can be performed in almost any laboratory. ELISAs are also useful for determining the route of *N. caninum* transmission as avidity tests enable to differentiate between acute and chronic infections [30-34]. Furthermore, discrete results obtained by this assay allow the operator to minimize the bias introduced by the visual subjective analysis of the data achieved by IFAT. This is particularly relevant when comparing results obtained from different laboratories and countries, as the lack of harmonization between protocols involved in the diagnosis of neosporosis may interfere with the accurate estimation of its prevalence and with the management strategies described above [35,36].

Despite different commercial ELISA tests are available in many countries [37], there is no local production of those kind of products in Argentina; therefore they must be purchased abroad [36]. This implies high costs and long waiting periods, turning unfeasible the routinely use of ELISA tests in the diagnosis of bovine neosporosis. The aim of this study was to develop and validate, a novel ELISA test based on soluble antigens from *N. caninum* tachyzoites to accurately determine the presence of specific antibodies in bovine serum samples, following the World Organization for Animal Health´s (OIE) guidelines [38]. This would enable the rapid, objective and low-cost diagnosis of bovine neosporosis in Argentina; replacing IFAT and avoiding delays and high importation taxes.

Materials and Methods

Reference Sera Samples

A total of 634 serum samples gently provided by Dr. Dadin P. Moore from the Animal Health Group (EEA INTA Balcarce) were involved in this study. The sample size, level of confidence and error range were determined following OIE recommendations. This theoretical number of samples from animals whose serostatus is known enables to determine the Diagnostic Sensitivity and Specificity (SnD and SpD, respectively) with an error range of 2% and a confidence level of 90% [38].

They correspond to two different panels. One of them consisted of 374 samples from 27 pregnant heifers from a herd with high prevalence of the disease at different time points; the other panel comprised 260 samples corresponding to 130 heifers at parturition and their calves before colostrum intake. Their serostatus was monitored by IFAT.

Indirect Fluorescent Antibody Test (IFAT)

The presence of specific antibodies anti-*N. caninum* antigens was assessed by IFAT using a fluorescein isothiocyanate labeled affinity-purified rabbit anti-bovine IgG antibody (Sigma, St. Louis, USA), as it was previously described [39-41]. Briefly, *N. caninum* specific antibodies were measured in serum samples using dilutions starting at 1:25. Positive and negative control sera were used. Slides were examined with an epifluorescence microscope (Olympus Bx 51, Olympus Inc., Tokyo, Japan). Antibody titers were expressed as the reciprocal of the highest serum dilution that showed complete peripheral fluorescence of tachyzoites [42]. A titer greater than 1:25 was indicative for *N. caninum* infection [39].

ELISA Procedures

Purification of the Antigen: The soluble fraction of a whole tachyzoite lysate (sNcAg), purified as previously described [9,43] was used as capture antigen. Briefly, tachyzoites (Nc1 strain) were partially purified using sephadex columns (Sephadex TM G-25 Medium, GE Healthcare, Sweden); disrupted by ultrasonic treatment and centrifuged. Soluble antigens (sNcAg) were recovered from the supernatant and quantified using a commercial protein assay method (Micro BCA Pierce, Rockford, USA).

Feasibility and Standardization: To standardize and optimize the ELISA protocol positive and negative control samples were generated by pooling sera samples from three positive or negative cattle, respectively, according to IFAT titers. Those controls were diluted 1:10 in stabilizing buffer and were used throughout the different steps of the validation process. Polystyrene microtiter plates

(Grenier Bio One, Austria) were coated with different amounts of sNcAg per well (500, 750 and 1000 ng) diluted in coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6). Different dilutions of the sera (1:40; 1:60; 1:120) and the antibody anti-bovine IgG conjugated to horseradish peroxidase (HRP) (1:2500, 1:5000; Jackson ImmunoResearch, PA, USA) and two different presentations of the substrate (revealing solution) were evaluated. To reduce the background level, different blocking solutions based on casein and different washing conditions (in PBS-tween 0.05%) were also assessed. Plates were incubated with different blocking solutions for 60 and 90 minutes at 37 ºC and for 16 hours at 4 ºC. Several conditions related to the incubation of samples and conjugate were also assessed, ranging from 21 to 37 ºC and from 10 to 60 minutes.

Accuracy Assessment: Repeatability, Reproducibility and Precision: The repeatability of the assay was evaluated by selecting a strong, mid and weak positive sample (IFAT titers 1:1600, 1:800 and 1:400, respectively) and a negative control (IFAT titer <1:25). They were run by ELISA in triplicate in eight independent series within the same plate. The reproducibility was evaluated by the analysis of 20 randomly selected samples by two operators from different laboratories (Virology Institute and Experimental Agricultural Station Salta, Argentina). These samples were run 8 times by each operator in independent assays. A Man-Whitney test was developed for each sample to assess significant differences between operators. The precision of the positive and negative controls was determined running them 20 times in independent assays. For each determination Coefficient of Variation (CVs) were determined. Accuracy tests were approved when CVs between optical density (OD) values were 30% or lower (according to OIE specifications [38]).

Performance characteristics: To assess the SnD and SpD and to determine the optimal threshold value a positive and negative serum samples (according to IFAT titers, considering this technique as the gold standard) were run in different dilutions (1:40, 1:60 and 1:120). Results were normalized as sample/positive control ratios (S/P%). Frequency distribution curves and a receiver operating characteristic analysis (ROC) of positive and negative samples were constructed to determine the area under the curve and positive and negative predicted values. Test agreement, SnD and SpD were calculated using GraphPad Prism 7 software. The level of agreement between the different tests was expressed as Kappa values (κ). The agreement was considered low, moderate and high, i.e., $0.4 \leq \kappa < 0.6$; $0.6 \leq \kappa \leq 0.8$ and $0.8 \leq \kappa < 1$, respectively. Mann-Whitney test was used to evaluate whether differences between tests were significant or not.

High-Yield Screening in Pooled Samples

In order to assess whether this protocol could be applied to the quick high-yielded screening of bovine neosporosis, different pooled samples were generated. For this purpose, a strong, mid and weak positive sample (IFAT titers 1600, 800 and 400, respectively) was mixed with either 25, 50, 75 or 100 negative samples. Samples were run at different dilutions (1:40 and 1:120) and both the limit of detection and the performance characteristics were determined as described above.

Results

IFAT

A total of 634 bovine sera samples were analyzed by IFAT. This technique was considered as the gold standard. From those, 327 samples were classified as negative (titers lower than 1:25) and 307 as positive; with titers ranging from 1:25 to 1:12800.

Feasibility and Standardization

In a first attempt to determine the feasibility of the technique, different concentrations of capture antigen (500, 750 and 1000 ng/well), different dilutions of the sera (1:40, 1:60, 1:120) and two different presentations of the revealing solution were evaluated following a standard protocol. To standardize the final conditions of use sera samples at the optimized dilution were incubated 30 or 40 minutes either at 37 ºC or at room temperature. Different dilutions of the secondary antibody (peroxidase conjugated) were assessed (1:2500 or 1:5000), whether incubated 20 or 30 minutes at 37 ºC or at room temperature. Optimal conditions finally stablished are summarized on Table 1. Briefly, plates are coated with 750 ng of sNcAg/well and incubated for 6h at 4 ºC. After washing 3 times, 125 µl/well of a casein-based blocking solution is added and plates are incubated ON at 4 ºC. Then samples are added (50µl/well) diluted 1:120 in blocking solution and incubated for 30 min at room temperature. Plates are washed 3 times and an anti-bovine IgG diluted 1:5000 in blocking solution is added and incubated for 20 min at room temperature. Plates are washed again and the substrate (50µl/well) is incorporated. Plates are incubated at room temperature in a dark place for 10 min. Then stop solution (H_2SO_4, 50µl/well) is added and plates are read immediately at 450nm. The obtained results are normalized calculating the S/P(%). Samples with S/P(%) greater than 40% are considered positive, and those with S/P(%) values smaller than 30% are negative. S/P(%) values between 30 and 40 correspond to doubtful samples that should be confirmed by running them again diluted 1:40 in blocking solution.

Step	Optimal condition
Coating	750ng/well in coating buffer. Incubate 6h at 4 °C
Washing steps	Wash 3x with PBS-tween 0.05%
Blocking	Block ON at 4°C with 0.5% of the blocking agent

Step	Optimal condition
Sera samples	Dilution of use 1:120 (1:40 in the confirmatory assay). Incubate 30 min at room temperature
Conjugate	Dilution of use 1:5000. Incubate 20 min at room temperature
Revealing solution	Incubate 10 min at room temperature in a dark place
Stop solution	Add quickly and read immediately at 450 nm

Table 1: Optimal conditions selected for the ELISA protocol after feasibility and standardization was assessed

Accuracy Assessment

A strong (Pos+++), mid (Pos++) and weak (Pos+) positive sample and a negative control (Neg) were run by triplicate in eight independent series in order to determine the intra-plate variability (repeatability). Within each series (triplicate) of the same operator the coefficient of variation for each sample was less than 10% for the 3 out of 4 samples analyzed. Higher variation (10-14%) was observed in 4 series of the weak positive control (Table 2 and Figure 1). Variation lower than 10% was observed between the 8 series for each sample (Table 3), within the same plate.

Series	Sample ID	Media (OD450nm)	SD	CV(%)
1	Pos+++	1.347	0.062	4.602
	Pos++	1.062	0.034	3.206
	Pos+	0.917	0.081	8.788
	Neg	0.292	0.009	2.926
2	Pos+++	1.291	0.072	5.608
	Pos++	1.026	0.018	1.769
	Pos+	0.793	0.082	10.293
	Neg	0.279	0.015	5.321
3	Pos+++	1.221	0.079	6.484
	Pos++	0.977	0.008	0.828
	Pos+	0.804	0.072	8.946
	Neg	0.219	0.007	2.678
4	Pos+++	1.131	0.047	4.112
	Pos++	0.903	0.070	7.745
	Pos+	0.765	0.048	6.226
	Neg	0.245	0.008	3.341
5	Pos+++	1.223	0.050	4.112
	Pos++	0.952	0.019	.1971
	Pos+	0.796	0.084	10.571
	Neg	0.221	0.010	4.355
6	Pos+++	1.212	0.029	2.418
	Pos++	0.964	0.018	1.869
	Pos+	0.806	0.083	10.303
	Neg	0.257	0.008	3.026
7	Pos+++	1.241	0.061	4.880
	Pos++	1.018	0.010	1.026
	Pos+	0.730	0.100	13.625
	Neg	0.266	0.009	3.212
8	Pos+++	1.291	0.020	1.567
	Pos++	0.992	0.049	4.973
	Pos+	0.786	0.034	4.475
	Neg	0.265	0.013	4.712

Table 2: The repeatability or intra-operator variability was assessed by running a strong, mid and weak positive sample and a negative control in eight independent series within the same plate. The average OD(450nm) values, SD and coefficient of variation (CV%) within each serie are depicted

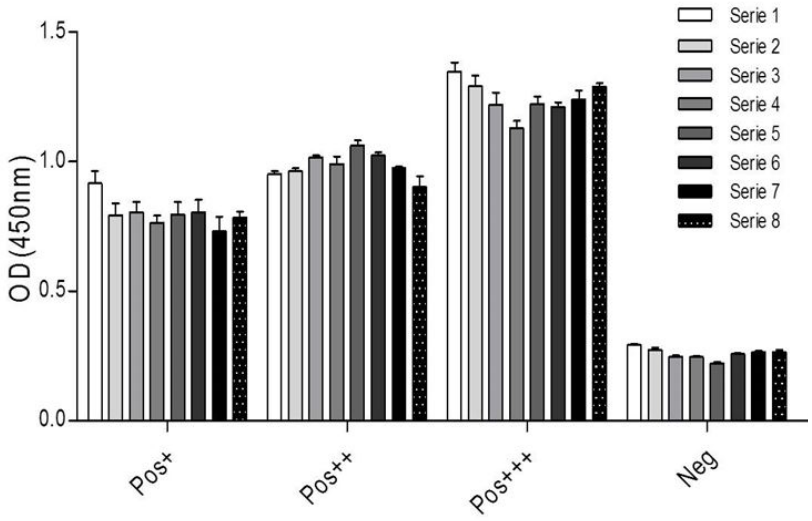

Figure 1: Repeatability was assessed evaluating a strong, mid and weak positive sample and a negative control (according to IFAT titers). Samples were run by triplicate in eight independent series within the same plate. Average values for each sample are shown, different colors indicate different series

Sample ID	Media (Series 1-8)	SD (Series 1-8)	CV(%) between series
Pos+++	1.245	0.053	4.223
Pos++	0.978	0.028	2.868
Pos+	0.800	0.073	9.123
Neg	0.258	0.010	3.693

Table 3: Repeatability between independent series for the same operator, indicated with the coefficient of variation (CV%)

To assess the reproducibility of the technique, 20 samples were run in 8 independent assays by two operators from different laboratories. The coefficient of variation intra-operator was determined for each sample using raw data (OD values). The mean variation for the operator one was 22.52% and for the operator two was 8.59%, both acceptable levels according to OIE [38] (<30%, Tables 4a and b).

ID	Operator 1 – Independent assays								Media	SD	CV(%)
1	0.481	0.573	0.354	0.646	0.299	0.366	0.401	0.432	0.444	0.117	26.378
2	0.466	0.547	0.356	0.605	0.286	0.379	0.403	0.393	0.429	0.105	24.350
3	0.690	0.772	0.504	0.801	0.485	0.589	0.571	0.675	0.636	0.118	18.495
4	0.779	0.905	0.618	0.996	0.488	0.645	0.721	0.705	0.732	0.162	22.072
5	1.090	1.211	0.787	1.334	0.676	0.919	0.997	1.119	1.017	0.218	21.456
6	1.221	1.343	0.905	1.449	0.723	1.005	1.032	1.077	1.094	0.236	21.556
7	1.534	1.655	1.181	1.656	1.164	1.294	1.296	1.326	1.388	0.199	14.371
8	1.552	1.633	1.269	1.904	1.129	1.232	1.320	1.215	1.407	0.265	18.817
9	0.246	0.315	0.218	0.371	0.181	0.231	0.258	0.241	0.258	0.059	23.061
10	0.516	0.554	0.347	0.709	0.366	0.464	0.489	0.494	0.492	0.113	22.927
11	0.276	0.338	0.207	0.361	0.203	0.228	0.242	0.262	0.265	0.058	22.025
12	1.811	2.046	1.608	2.193	1.217	1.328	1.403	1.116	1.590	0.394	24.793
13	0.183	0.225	0.150	0.276	0.123	0.135	0.158	0.223	0.184	0.053	28.754
14	1.293	1.420	0.926	1.547	0.791	0.926	0.981	1.081	1.121	0.269	24.006
15	0.902	1.101	0.663	1.151	0.651	0.748	0.801	0.891	0.864	0.187	21.617
16	0.289	0.349	0.237	0.364	0.195	0.258	0.275	0.289	0.286	0.062	21.830
17	0.303	0.346	0.220	0.517	0.209	0.262	0.279	0.309	0.306	0.097	31.677
18	0.271	0.313	0.198	0.186	0.195	0.214	0.253	0.258	0.236	0.045	18.974
19	0.305	0.369	0.248	0.434	0.243	0.277	0.305	0.325	0.313	0.064	20.359
20	0.287	0.350	0.224	0.414	0.214	0.258	0.277	0.274	0.287	0.066	22.982

ID	Operator 2 - Independent assays								Media	SD	CV(%)
1	0.381	0.344	0.389	0.375	0.356	0.374	0.362	0.356	0.37	0.02	4.10
2	0.356	0.352	0.411	0.454	0.328	0.331	0.324	0.471	0.38	0.06	15.57
3	0.399	0.457	0.433	0.412	0.461	0.431	0.471	0.485	0.44	0.03	6.71
4	0.535	0.509	0.598	0.551	0.525	0.537	0.545	0.537	0.54	0.03	4.78
5	0.785	0.689	0.724	0.710	0.760	0.742	0.768	0.777	0.74	0.03	4.59
6	0.893	0.892	0.829	0.798	0.863	0.842	0.811	0.830	0.84	0.04	4.17
7	1.119	1.101	1.093	0.990	1.129	1.091	1.078	0.980	1.07	0.06	5.26
8	1.056	1.153	1.144	1.101	1.156	1.128	1.119	1.028	1.11	0.05	4.20
9	0.242	0.214	0.269	0.290	0.256	0.255	0.257	0.253	0.25	0.02	8.50
10	0.404	0.364	0.321	0.400	0.402	0.391	0.371	0.375	0.38	0.03	7.34
11	0.242	0.278	0.245	0.276	0.238	0.232	0.216	0.219	0.24	0.02	9.54
12	1.435	1.341	1.348	1.412	1.404	1.366	1.352	1.426	1.39	0.04	2.73
13	0.111	0.190	0.118	0.111	0.131	0.120	0.127	0.128	0.13	0.03	19.75
14	0.798	0.763	0.880	0.812	0.852	0.813	0.840	0.847	0.83	0.04	4.43
15	0.625	0.685	0.635	0.715	0.643	0.674	0.628	0.699	0.66	0.03	5.24
16	0.269	0.289	0.266	0.312	0.255	0.317	0.286	0.281	0.28	0.02	7.63
17	0.255	0.311	0.287	0.221	0.219	0.231	0.266	0.282	0.26	0.03	12.98
18	0.301	0.251	0.222	0.401	0.246	0.266	0.262	0.317	0.28	0.06	19.90
19	0.255	0.301	0.301	0.212	0.245	0.233	0.245	0.271	0.26	0.03	12.22
20	0.271	0.219	0.211	0.282	0.260	0.271	0.276	0.305	0.26	0.03	12.16

Table 4: Reproducibility test. Twenty randomly selected samples were run in eight independent assays by two operators from different laboratories. The mean OD(450nm) values, SD and CV% for each sample obtained by operator 1 (Table 4a) and operator 2 (Table 4b) is depicted

A Mann-Whitney test was carried out to compare the OD values obtained for each sample from operator one (laboratory A) and operator two (laboratory B). No significant differences were observed for 10 out of 20 of the analyzed samples ($p>0.05$). All of them, however, exhibited acceptable CVs (<30%) (Figure 1).

To assess the precision of the technique the dispersion observed between different series of the negative and positive control samples in 20 independent assays was determined. Results obtained for each sample were very precise, as low level of dispersion was observed, particularly from series 4 to 20 (Figure 2).

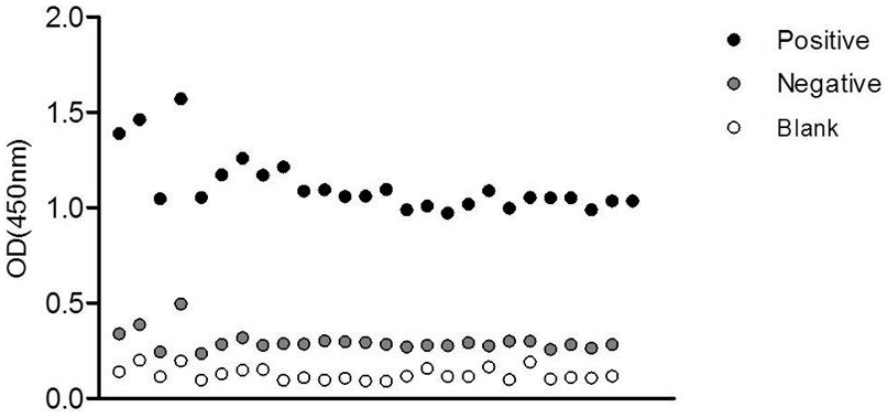

Figure 2: Accuracy test. The dispersion of the positive (black circles) and negative (grey circles) control and blank values (white circles) from twenty independent assays is depicted

Performance characteristics

Cut off values: The identity of the samples was unknown to the laboratory operators to avoid any testing bias. Relative frequency distribution curves (percentages) of positive (n=307) and negative (n=327) samples (final dilution 1:120) were constructed. Two histograms were built, and their intercept point was considered as the cut-off value (Figure 3). A S/P ratio of 40% was defined as the appropriate cut-off threshold, but as certain overlapping of both curves was observed, samples with S/P values between 30 and 40% were considered doubtful and were run again in a final dilution of 1:40. The cut off value of this confirmatory test was defined as 30% (Figure 4).

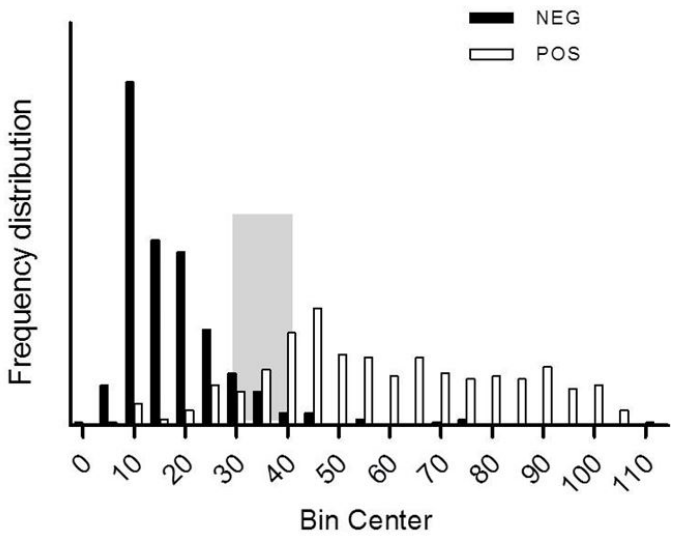

Figure 3: Frequency distribution of the reference positive and negative sera samples (according to IFAT titers) is shown. The intercept region is depicted in grey

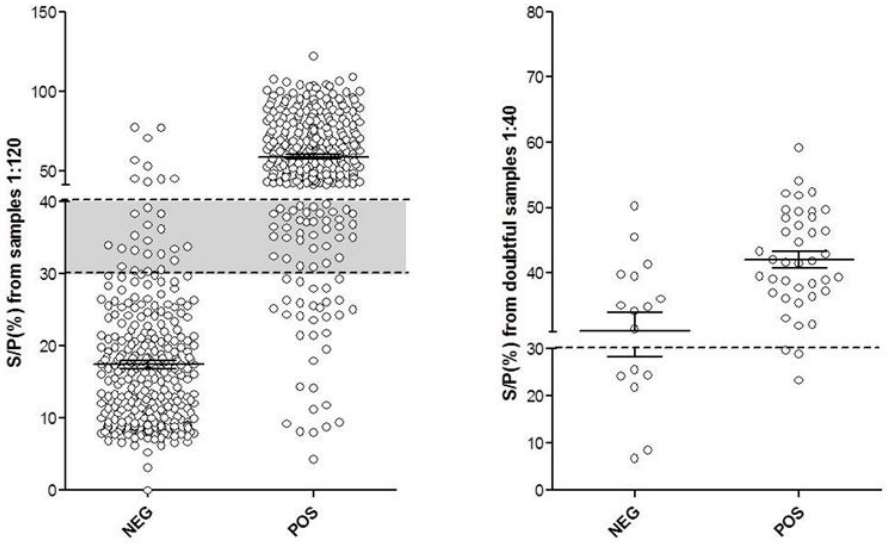

Figure 4: Sensitivity (Sn) and Specificity (Sp) was assessed by a ROC analysis. The doubtful range (grey) and the confirmatory test cut off is shown

Receiver Operating Characteristic (ROC) Curve Analysis: Samples were run in the conditions above described. Starting from 307 positive and 327 negative samples (according to IFAT titers), a total of 285 true positive (TP), 291 true negative (TN), 22 false positive (FP, type I error) and 36 false negative (FN, type II error) samples were detected. A ROC analysis was assessed to determine the diagnostic sensitivity (Sn) and specificity (Sp) of the technique. The area under the curve was 0.9499 (SD=0.009159; p=0.0001; 95% confidence interval, CI). Considering a prevalence of the disease of 30%, the estimated Sn and Sp were 92.50 and 96.81, respectively; with a positive predicted value (PPV) of 0.928 and a negative predicted value (NPP) of 0.889.

Concordance Analysis: The estimated accuracy of the technique was 0.908, depicting very good discrimination between *N. caninum* infected and non-infected cattle. With a 90% of confidence, the Cohen's kappa coefficient was 0.817, thus showing an almost perfect concordance between ELISA and IFAT. A significant correlation between both techniques was observed (p<0.0001; IC; 95%; Spearman r=0.7903; data not shown).

High-Yield Screening in Pooled Samples

Pooled samples containing one strong (Pos+++), mid (Pos++) or weak (Pos+) positive serum within 25, 50, 75 or 100 negative samples were run at different dilutions (1:120 and 1:40) following the protocol described in Table 1. One strong or mid positive serum (with IFAT titers ranging from 1:1600 to 1:800) pooled with up to 100 negative samples was detected when samples were diluted 1:40 (Table 5). To assess the SpD and SnD and determine the most suitable cut off value, the total panel of 634 samples was run at this dilution and a ROC analysis was carried out. Under these conditions, pooled samples with S/P values greater than 30% were considered positive, with a SnD of 90.09% and a Sp of 90.10 (95% CI, Figure 5). The area under the curve was 0.9433 (95% IC, 0.9243-0.9623).

Pool ID	OD(450nm)		S/P(%)	
	1:120	1:40	1:120	1:40
Neg 25	0.265	0.414	15.134	20.126
Neg 50	0.396	0.538	22.616	26.155
Neg 75	0.346	0.503	19.760	24.453
Neg 100	0.571	0.547	32.610	26.592
Pos+++ 25	0.687	1.001	39.235	**48.663**
Pos+++ 50	0.568	0.779	32.439	**37.871**
Pos+++ 75	0.524	0.776	29.926	**37.725**
Pos+++ 100	0.569	0.848	32.496	**41.225**
Pos++ 25	0.362	0.825	20.674	**40.107**
Pos++ 50	0.386	0.752	22.045	**36.558**
Pos++ 75	0.356	0.795	20.331	**38.649**
Pos++ 100	0.351	0.707	20.046	**34.370**
Pos+ 25	0.255	0.471	14.563	22.897
Pos+ 50	0.348	0.587	19.874	28.537
Pos+ 75	0.319	0.601	18.218	29.217
Pos+ 100	0.117	0.607	6.682	29.509

Table 5: OD(450nm) and S/P values obtained in pooled samples diluted 1:120 or 1:40 are shown. The highlighted values indicate the detection range

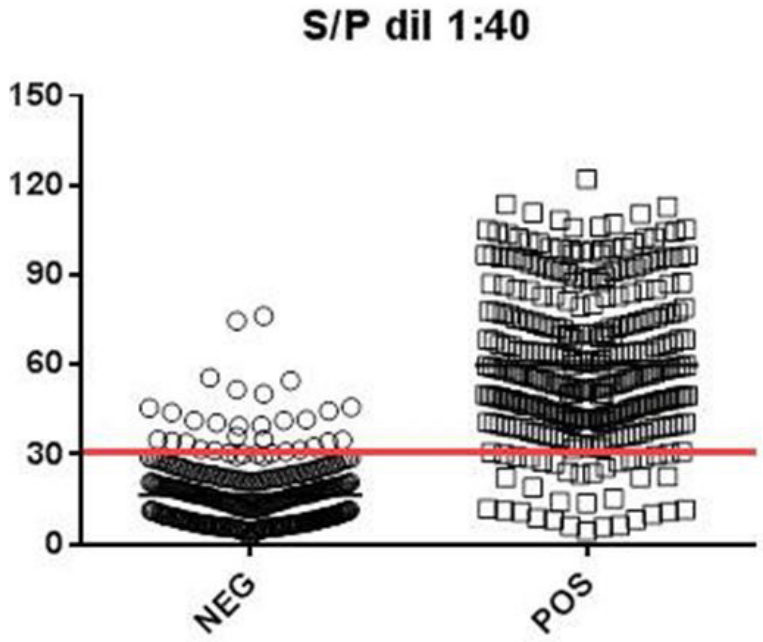

Figure 5: ROC analysis of the high-yield screening assay. The full line indicates the cut off value

Discussion

Amongst serological diagnostic tools, ELISAs have become the most preferable technique to detect the presence of specific antibodies against *N. caninum* in bovine serum samples. When correctly validated, they can provide reliable information allowing the screening of large herds in few hours. Unfortunately, national commercial kits are not available in Argentina and they must be purchased abroad, leading to very high costs due to importation taxes. In this context, local farmers tend to delay the diagnose, underestimating the prevalence and local impact of this disease. What is more, the lack of accuracy in the diagnosis of bovine neosporosis also impacts globally, as there is no a standardized method to assess the global prevalence of this disease [10].

One of the main problems in the validation of *N. caninum* diagnostic tests is the lack of an appropriate gold standard as; in general, no perfect tests are available for the detection of *N. caninum* infection [27,44]. In the absence of routine direct tests in adult cows enabling accurate results, and in the absence of a large panel of fully characterized samples, a no gold standard (e.g. Bayesian) analysis is sometimes advised [45]. However, IFAT has generally been used as a reference test in the evaluation of other diagnostic

tests and different versions of IFATs have routinely been used in both diagnostic and research laboratories, despite its subjective reading and substantial between-laboratory variation [44]. In addition to this, the accuracy of the serological diagnosis of *N. caninum* by measuring tachyzoite-specific antibodies in cattle was confirmed by contrasting the performance of ten commercial ELISAs based on two different definitions of the gold standard (majority of tests and pre-test information) [46]. To establish appropriate control measures for bovine neosporosis is essential to understand the performance of the different available ELISAs. The differences between these serological tests, as well as the multiple versions of test protocols in use, make it difficult to compare test results from different studies. Therefore, it is essential that proper evaluations are made for the tests used.

Sometimes, due to financial and logistical issues, validation assays may be developed with a smaller sample size than the statistically required. In this case, the calculated SeD and SpD will indicate less diagnostic confidence in the results. The size of the sample may also be limited by the fact that the reference populations and the OIE reference standards are not available for *N. caninum*. Therefore, initially it may be necessary to use a suboptimal number of samples. However, in future studies it would be highly desirable to enhance the confidence level and reduce the allowable error range in SeD and SpD estimations obtained in the present study by increasing the sample size.

Here we have developed and validated an ELISA kit locally produced; which enables the quick, objective and economic diagnosis of bovine neosporosis; avoiding delays and high importation taxes. This technique also shows objective results; as the lecture of the row data does not depend on the operator. Our results showed an almost perfect level of concordance with the gold standard technique; with a very good SeD and SpD. It is also very useful to perform high-yielded screening of large herds quickly; by pooling up to 100 samples.

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