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Gema Álvarez-García, Alicia García-Culebras, Daniel Gutiérrez-Expósito, Vanesa Navarro-Lozano, Iván Pastor-Fernández, Luis Miguel Ortega-Mora. Serological diagnosis of bovine neosporosis: a comparative study of commercially available ELISA tests. **Vet Parasitol**. 2013;198(1-2):85-95.

doi: 10.1016/j.vetpar.2013.07.033.

Serological diagnosis of bovine neosporosis: a comparative study of commercially available ELISA tests

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Abstract

Bovine neosporosis control programs are currently based on herd management and serodiagnosis because effective treatments and vaccines are unavailable. Although a wide variety of serological tools have been developed, enzyme-linked immunosorbent assays (ELISAs) are the most commonly commercialized tests. Partial comparative studies have been performed in the past, and the panel of available ELISAs has notably changed in the last few years. Therefore, diagnostic laboratories are requesting updated information about the performance of these tests.

Accordingly, the aim of this study was to compare all of the commercially available ELISAs (n=10) by evaluating their performance and to re-standardize them based on TG-ROC analyses when necessary. For this purpose, a well-characterized serum panel from experimentally and naturally infected bovines and non-infected bovines (n=458) was used. Two different definitions of gold standard were considered: (i) the result of the majority of tests and (ii) pre-test information based on epidemiological, clinical and serological data. Most of the tests displayed high sensitivity (Se) and specificity (Sp) values when both gold standard criteria were considered. Furthermore, all the tests showed near perfect agreement, with the exception of the pair-wise comparisons that included the VMRD and SVANOVIR. The best-adjusted ELISAs were the HIPRA-CIVTEST, IDVET, BIOVET and IDEXX Rum (Se and Sp > 95%). After the TG-ROC analyses, higher Se and Sp values were obtained for the BIO-X, LSI Bov, LSI Rum and IDEXX Bov, though the increases were more significant for the SVANOVIR and VMRD. The Kappa values also increased with the new adjusted cut-offs. This is the first study that offers updated performance evaluations of commercially available ELISAs. Such analyses are essential for diagnostic laboratories and are valuable to the companies that develop and distribute these tests.

Key words: Bovine neosporosis, commercial ELISAs, comparative study, diagnostic performance, agreement, TG-ROC

1. Introduction

Bovine neosporosis is a parasitic disease caused by the cyst-forming coccidian parasite *Neospora caninum* which causes abortion and neonatal mortality in cattle worldwide and, consequently, significant economic losses in the cattle industry (reviewed by Dubey et al., 2007; Reichel et al., 2013).

There are numerous studies confirming the importance of bovine neosporosis. A multinational study (Bartels et al., 2006) and another recent study carried out in Spain (Eiras et al., 2011) updated the prevalence rates of *N. caninum* infection in several European countries reporting herd prevalence rates from 16% in Sweden to 80% in Spain.

At present, there is no effective treatment or vaccine for *N. caninum* infection, and control measures are based on herd management and diagnosis. The serological diagnosis of neosporosis in adult cattle and precolostral calves is an integral part of control programs because the other commonly adopted control measures include the selective culling of seropositive *Neospora*-associated aborted dams, herd replacement with seronegative cattle and testing during the quarantine period prior to herd entry (Dubey et al., 2007).

Serological techniques are primarily employed to detect specific antibodies against *N. caninum* to differentiate infected from non-infected animals. These techniques include a wide variety of enzyme-linked immunosorbent assays (ELISAs) (in-house and commercial tests), indirect fluorescent antibody tests (IFATs) and a *N. caninum*-agglutination test (NAT) (reviewed by Ortega-Mora et al., 2007). In addition, western blotting is often recommended to confirm uncertain results in valuable samples (Álvarez-García et al., 2002). Moreover, avidity tests are useful for investigating the route of *N. caninum* transmission in herds because they can differentiate between primary and chronic infections (Bjorkman et al., 2006; Aguado-Martínez et al., 2008).

In the last few years, the panel of commercially available serological kits has notably changed; new tests have been developed, several have been modified and other tests are no longer commercialized. Moreover, other in-house developed tests have been recommended for the diagnosis of bovine neosporosis (eg. Osawa et al., 1998; Wouda et al., 1998). Thus, at present, there is a paucity of up-to-date information about the performance of these diagnostic products, which is essential information for diagnostic

labs. In fact, the last comparative studies offered a fragmented picture of the diagnostic tools employed in Europe (von Blumröder et al., 2004) and the USA (Wapenaar et al., 2007) and it remains unclear if the current serological tests offer standardized interpretation of results.

Therefore, the aim of this study was to evaluate the performance of and re-standardize the commercially available ELISA tests to detect anti-*N. caninum* antibodies. Thus a sera panel that reflects an appropriate spectrum of disease was tested following the recommended procedure for validation of diagnostic tests (Jacobson, 1998; Greiner and Gardner, 2000; Gardner et al., 2010).

2. Materials and Methods

2.1. Sera and experimental design

A well-defined bovine sera panel was analyzed by ten commercial ELISA tests. This coded panel was composed of 458 bovine serum samples from both experimentally and naturally infected cattle (including aborted and non-aborted dams) as well as non-infected cattle. All the sampled animals were older than 6 months to avoid the presence of colostral antibodies.

The animals were categorized into the following groups:

2.1.1. Sera from non-infected cattle (Group a; n=125)

Heifers and cows from a dairy herd (Holstein Friesian breed) without a previous history of *N. caninum*-associated abortions tested negative in three consecutive samplings during 6-9 month intervals throughout a period of two years in order to discard antibody fluctuations below the cut-off value that may occur in chronically infected cattle. All the samples were negative using an in house *N. caninum* soluble extract antigen-based ELISA (Álvarez-García et al., 2003; Aguado-Martínez et al., 2008) that discriminates between positive and negative results. Moreover, the samples also tested negative using two recombinant antigen-based ELISAs (rNcGRA7 and rNcSAG4 ELISAs). The NcGRA7 protein is shared by both tachyzoite and bradyzoite stages, whereas NcSAG4 is the first bradyzoite stage-specific protein described. The usefulness of rNcGRA7 and rNcSAG4 ELISAs to detect acute and chronic cattle

infections, respectively, that may go undetected by conventional serological tools was previously reported (Aguado-Martínez et al., 2008).

2.1.2. Sera from N. caninum naturally infected cattle (Group b; n=169)

Serum samples from seropositive non-aborted dams:

Serum samples (n=136) were collected from dams from a dairy herd (Holstein Friesian breed) with a history of *N. caninum*-associated abortions. The herd comprised 200 cows and had an intra-herd seroprevalence of 85% and a 9.2% annual abortion rate. The *N. caninum*-associated abortions were diagnosed using histopathology and PCR. Moreover, an endogenous transplacental transmission rate of 80% was estimated based on an equal distribution of seropositive animals across the different age groups and a significant association between seropositive dams and their daughters. One hundred and thirty serum samples came from mother-daughter pairs (n=65 pairs), and 6 serum samples came from only mothers or daughters. All the samples tested positive using an in house *N. caninum* soluble extract antigen-based ELISA.

Serum samples from seropositive aborted dams and/or dams at risk of abortion:

Sera from a herd with an endemic pattern of N. caninum-associated abortions

The sera were collected from aborted cows (n=21) belonging to the previously mentioned herd. The samples were collected 2 months prior to or after the abortion when an increase in specific antibody levels is expected (Quintanilla-Gozalo et al., 2000). All the samples tested positive using an in house *N. caninum* soluble extract antigen-based ELISA.

Sera from a herd with an epidemic pattern of N. caninum-associated abortions

The sera were collected from animals from a dairy herd (Holstein Friesian breed) of 1080 cows, which did not have a history of reproductive failure and had a *N. caninum* seroprevalence of less than 5% prior to an abortion storm. During this event all abortions were concentrated in a two month period, and the abortion rate during this period was 5.1%. In addition, 81.82% of the abortions occurred in seropositive animals that displayed low avidity anti-*N. caninum* antibodies (Rojo-Montejo et al., 2009). The intra-herd seroprevalence increased to 19.5% after the abortion storm. The tested

samples belonged to the seropositive aborted cows or the cows at risk of abortion during the abortion storm (n=12). These animals were located in the same yard according to the gestation period and lactating age.

2.1.3. Sera from N. caninum experimentally infected cattle (Group c; n=150)

All experimentally infected animals were seronegative prior to inoculation using an in house *N. caninum* soluble extract antigen-based ELISA and rNcGRA7 and rNcSAG4 ELISAs.

Bulls

Three bulls of the Asturiana de los Valles breed were intravenously infected (i.v.) with 10^8 live *N. caninum* tachyzoites of the Nc-1 isolate. Sequential serum samples were collected weekly and fortnightly for approximately 8 months (Serrano-Martínez et al., 2007). Later, these samples were assayed using an avidity ELISA and an ELISA based on the recombinant proteins NcGRA7 and NcSAG4. The results were representative of primary infection until 8–10 weeks p.i. (low IgG avidity values) and chronic infection from 10 weeks p.i. to the end of the experiment (high IgG avidity values). Similar antibody kinetics were observed during primary and chronic infections with the rNcGRA7 and *N. caninum* soluble extract antigen-based ELISAs, but the experimentally infected bovines did not show specific anti-rNcSAG4 antibodies with the rNcSAG4 ELISA. The most feasible explanation for the absence of specific anti-rNcSAG4 antibodies in the study was a low efficiency of tachyzoite–bradyzoite conversion in an experimental bovine model of neosporosis (Aguado-Martínez et al., 2008). For the present study, a total of 55 serum samples were selected (n=24 samples for the first bull, n=11 for the second bull and n=20 for the third bull), and they were collected at 24 different time points throughout the 270 days of the experiment (Fig. 2). These sera were representative of primary and chronic infections.

Heifers

Three heifers of less than 24 months old were inoculated i.v. with 10^7 live *N. caninum* tachyzoites of the Nc-1 isolate at 70 days of gestation. Sequential serum samples were collected fortnightly prior to and after the inoculation and until the delivery or abortion (Rojo-Montejo et al., under review). For the present study, a total of 18 sera were selected at 7 different time points throughout the 87-day experiment

(n=5 sera samples for the first heifer, n=7 sera samples for the second heifer and n=6 sera samples for the third heifer). Additionally, three heifers of less than 24 months old were i.v. inoculated with $4x10^8$ live *N. caninum* tachyzoites of the Nc-1 isolate at 135 days of gestation. Sequential serum samples were collected fortnightly prior to the date of inoculation and after the inoculation up until the delivery or abortion (Rojo-Montejo el al., 2013 submitted). Of these samples, a total of 44 were selected at 16 different time points throughout the experiment (n=16 sera samples for the first heifer, n=13 sera samples for the second heifer and n=15 sera samples for the third heifer). Moreover, three additional non-infected heifers were employed as negative controls (inoculated with PBS) (n=14 sera samples from the first negative control heifer, n=9 sera samples for the second negative control heifer and n=10 sera samples for the third negative control heifer). All these samples were assayed using an in house *N. caninum* soluble extract antigen-based ELISA.

In all animals from group "c" all samples remained seropositive (between 2-3 weeks post-infection) once they had seroconverted.

2.1.4. Sera from animals infected with closely related apicomplexan parasites (Group d; n=14)

Nine sera from seropositive cows of Brown Swiss breed naturally infected with *Besnoitia besnoiti* were analyzed. *B. besnoiti* infection was confirmed using an in-house ELISA developed by the SALUVET group (García-Lunar et al., 2013). Five sera from heifers of Holtein Friesian breed with natural *Sarcocystis* spp. infections also were analyzed. *Sarcocystis* infection was detected using the visualization of tissue cysts in the heart via histological examination and *B. besnoiti* infection was discarded by an in house ELISA mentioned above. All samples tested negative using an in house *N. caninum* soluble extract antigen-based ELISA. These sera were included in the experiment to study cross-reactivity with other apicomplexan parasites.

2.2. Tests

The samples were analyzed using nine commercial indirect enzyme-linked immunosorbent assays (iELISA) and one commercial competitive enzyme-linked immunosorbent assay (cELISA) (Table 1). The tests were performed, and the cut-off values were applied according to the manufacturer's instructions.

2.3. Analysis of data

Sensitivity (Se), specificity (Sp) and test agreement (expressed as Kappa-values; κ), including 95% confidence intervals (95% CI), were calculated using WinEpiscope 2.0 (http://www.clive.ed.ac.uk).

Two different definitions of a gold standard were used to calculate the diagnostic characteristics of the tests because a perfect reference test is not available for the diagnosis of bovine neosporosis (Ortega-Mora et al., 2007).

The first gold standard was defined by the result of the majority of the tests (`Majority of tests´). If equal numbers of tests retuned positive and negative results, the sample was regarded as doubtful and was discarded.

The second gold standard was defined according to the pre-test information (`Pre-test information´). A sample was considered positive or negative based on epidemiological (previous history of endemic or epidemic *N. caninum*-associated abortions), clinical (aborting or non-aborting cattle) and serological data (seropositive or seronegative using one or two reference tests: *N. caninum* soluble extract antigen-based ELISA and recombinant protein-based ELISAs). Groups "a" and "d" were regarded as negative reference sera as compared to groups "b" and "c", which were regarded as positive reference sera. Results of the tests were evaluated blinded.

TG-ROC analyses were carried out with respect to the gold standard `Majority of tests´ (Greiner, 1995), and SPSS 17.0 for Windows (SPSS Inc.) was used. In addition, the Student's t-test was employed to compare specific antibody levels between serum samples from seropositive aborted dams from a herd with an endemic pattern of *N. caninum*-associated abortions (n=21) and samples from seropositive non-aborted dams (n=136). Both groups were described above (group b: sera from *N. caninum* naturally infected cattle) for each ELISA test. Moreover, the repeated measures ANOVA with Tukey's multiple comparison test was used for the comparison of antibody levels at different days p.i. in the serum of experimentally infected animals for each ELISA test. A *P*-value of less than 0.05 was considered statistically significant. These statistical analyses were carried out using GraphPad Prism 5 v.5.01 (San Diego, CA, USA) software.

3. Results

3.1. Sensitivity (Se) and specificity (Sp) of tests according to the `Majority of tests'and `Pre-test information´ gold standards

Se and Sp values were calculated for each ELISA based on the cut-offs recommended by each laboratory (Table 2). When "Majority of tests" was regarded as the gold standard only one sample was discarded since equal numbers of tests retuned positive and negative results. Irrespective of the chosen gold standard, the Se was high (>95%) for most tests except for the SVANOVIR test (85.9% relative to the gold standard "Majority" and 87.2% relative to the gold standard "Pre-test info"). All the tests showed high Sp values (93–100%) except for the VMRD test (65.1% relative to the gold standard "Majority of tests" and 66.5% relative to the gold standard "Pre-test info").

3.2. TG-ROC analysis

TG-ROC analysis, based on the 'Majority of tests', was conducted to confirm the accuracy of the suggested cut-off values. These analyses were conducted for the ELISA tests that showed Se and/or Sp values of less than 95%. Thus, the cut-offs were recalculated for the LSI Bov, LSI Rum, BIO-X, VRMD, IDEXX Bov and SVANOVIR ELISAs (Table 2, Fig. 1).

According to the Sp and Se values, the tests that were notably modified after TG-ROC analysis were the VRMD and SVANOVIR ELISAs. In the case of the SVANOVIR ELISA, the new suggested cut-off was percent positivity (PP) $\geq$ 15 for Se and Sp values of 94.0% and 93.7%, respectively. In the case of the VMRD ELISA, the new suggested cut-off was percent inhibition (% I) $\geq$ 65 for Se and Sp values of 94.8% and 91.6%, respectively (Table 3).

This study also permitted slight readjustments of other tests, including the LSI Bov ELISA (readjusted cut-off $\geq$ 52; Se=98.9% and Sp=97.9%), LSI Rum ELISA (readjusted cut-off $\geq$ 53; Se=99.3% and Sp=97.4%), BIO-X ELISA (readjusted cut-off $\geq$ 23/13; Se=98.1% and Sp=96.2%) and IDEXX Bov ELISA (readjusted cut-off $\geq$ 1; Se=98.5% and Sp=97.9%) (Table 3).

TG-ROC analysis was not performed for the HIPRA-CIVTEST, IDVET, IDEXX Rum and BIOVET ELISAs because these tests showed Se and Sp values higher than 95%.

Interestingly, all the ELISAs that employed cut-off values with a range of doubtful results yielded a low percentage of doubtful results (3.2% for HIPRA-CIVTEST, 2.1% for IDVET, 3.9% for BIO-X and 2.4% for IDEXX Rum).

3.3. Test agreement (K-statistics)

First, K-values were calculated between ELISAs and both gold-standard criteria prior to and after TG-ROC analyses. HIPRA-CIVTEST, IDVET, IDEXX Rum and BIOVET ELISA showed in both cases perfect agreement (K-values higher than 0.95). K-values of LSI Bov, LSI Rum, BIO-X, IDDEX-Bov and SVANOVIR ELISAs were close to or higher than 0.90 and remained similar or slightly increased after TG-ROC re-adjustment of cut-offs. The lowest K-value corresponded to VMRD ELISA, which significantly increased after TG-ROC analysis (Table 4).

When all ELISAs were compared to each other all the tests showed near perfect agreement (K = 0.8–0.9) with the exception of the pair-wise comparisons, which included the VMRD and SVANOVIR ELISAs (Supplementary Table 1).

The K-values were recalculated using the adjusted cut-offs obtained using TG-ROC analysis based on the gold standard ‘Majority of tests’ (Supplementary Table 2).

As expected, there was a substantial increment of K-values in the pair-wise comparisons, including the VMRD and SVANOVIR ELISAs, reaching K-values between 0.8 and 0.9 (Supplementary Table 2).

3.4. Cross-reactions

Nine of the 14 sera that were positive against *Sarcocystis* spp. and *Besnoitia besnoiti* infections were positive according to four of the 10 evaluated ELISAs. The BIO-X test showed the highest number of cross-reactions (4/14), and all were with sera positive against *B. besnoiti*. The VMRD ELISA showed cross-reactions (3/14) to *Sarcocystis* spp. positive sera, and the LSI Bov and BIOVET ELISAs only yielded one false positive result with *B. besnoiti*-positive serum.

358 *3.5. Antibody titers in aborted vs. non-aborted cattle using Student´s t-test analysis*

359 Student's t-test comparisons showed significant differences between aborted and
360 non-aborted dams for the HIPRA-CIVTEST (*P*= 0.0055), Bio-X (*P*= 0.0023) and
361 BIOVET (*P*= 0.0012).

362

363 *3.6. Kinetics of antibody responses using ANOVA analysis*

364 Antibody kinetics were examined in experimentally infected bulls and cows
365 (Fig. 2) throughout the sampling period. The samples included in the statistical analyses
366 comprised 27 sera samples from 3 experimentally infected bulls collected at 9 different
367 sampling points post infection (1, 2, 3, 4, 5, 6, 7, 8 and 9 weeks post-infection) and 33
368 sera samples of 3 experimentally infected cows at 135 days of gestation collected at 11
369 different sampling points post infection (2, 3, 4, 6, 7, 9, 11, 13, 15, 17 and 19 wpi). Both
370 the groups were described above in detail (section 2.1 section, sera and experimental
371 design; group c). In summary, all the kits behaved similarly.

372 All the sera obtained from experimentally infected bulls were negative at 1 wpi
373 irrespective of the kit employed. Seroconversions were mostly detected at 2 wpi, and all
374 the animals remained positive from 3 wpi onwards. Antibody levels peaked at 4wpi or
375 even later depending on the test. Seroconversions (values above the cut-off) were
376 detected in all the animals at 2 wpi with the BIO-X and IDVET tests, whereas the
377 IDEXX Bov and VMRD displayed positive and negative results at 2 wpi.
378 Seroconversions were observed at 3 wpi for the CIVTEST, LSI Bov, LSI Rum, IDEXX
379 Rum, SVANOVIR and BIOVET. Accordingly, the specific antibody levels differed
380 throughout the experiment depending on the test, as evidenced using an ANOVA with
381 Tukey's multiple comparison test analysis. Thus the HIPRA-CIVTEST, IDVET, LSI
382 Bov, IDEXX Bov, IDEXX Rum and BIOVET behaved similarly, and significant
383 differences were observed in the pair-wise comparisons that included 1 and 2wpi.
384 Conversely, significant differences for LSI Rum and BIO-X were detected when all the
385 time points were compared to 1 and 3 wpi, respectively. The VMRD and SVANOVIR
386 showed significant differences between 1 wpi and 4 wpi onwards because large standard
387 deviations were observed at different dpi.

388 When serum antibody levels of experimentally infected cows were analyzed, all
389 the cows were seropositive using the BIO-X, LSI Rum, IDEXX Bov and VMRD,
390 whereas the HIPRA-CIVTEST, IDVET, LSI Bov, IDEXX Rum and BIOVET displayed
391 positive and negative results at 2wpi. Seroconversions were observed at 3 wpi for the

SVANOVIR. The antibody level kinetics throughout the experiment were more variable in cows than in bulls. The most relevant results were as follows: significant differences were observed in the 2 wpi pair-wise comparisons between the HIPRA-CIVTEST, IDVET, LSI Bov and BIOVET or the 3 wpi comparisons between the IDVET and BIO-X. For the LSI Rum, VMRD, IDEXX Bov, IDEXX Rum and SVANOVIR, significant differences were only observed for several pair wise comparisons, including 2 wpi. Finally, all samples from negative control heifers were seronegative by all ELISAs employed.

4. Discussion

This is the first study to compare commercially available ELISA tests for the serological diagnosis of bovine neosporosis. The results in the present work showed generally higher test agreement and performance compared to previous works, which suggests that the serodiagnosis of bovine neosporosis is currently accurate. However, the results also indicate that further refinements are required.

Few comparative studies of commercial tests have been performed. Several studies have compared two commercial ELISAs (Wu et al., 2002; Hall et al., 2006) or three tests (two commercial ELISAs and one agglutination test; Waldner et al., 2004). However the most complete studies have compared the serological tests employed in Europe and North America. In particular von Blumröder et al. (2004) studied the performance of six commercial and five in-house serological tests and an in-house IFAT used in Europe, and they reported a high level of agreement among them. Despite this positive result, the authors offered an *a posteriori* adjustment of all the tests to obtain more comparable results irrespective of the test employed. Wapenaar et al. (2007) later studied the performance of serological tests employed in North America (Canada and USA) (three commercial ELISAs, an in house IFAT, an agglutination test and a commercial IFAT that was regarded as the reference test), and they showed several significant discrepancies among the tests.

In general, most of the available tests for the diagnosis of bovine *N. caninum* infection have shown strong performances (reviewed by Dubey and Schares 2006). However, reference diagnostic laboratories have noted the need to work with accurately validated commercial ELISAs because discrepancies between tests still exist

(previously noted by Aguado-Martínez et al., 2006), and the results obtained from these tests are often employed in control programs. A low number of discrepancies among tests is acceptable because they often correspond to values near the cut-off and are difficult to avoid. However, no information about test performance is available from previous comparative studies for several tests as mentioned below. Furthermore, the performance reported by the manufacturer is either based on the analysis of a short panel of reference sera, is outdated or the target population has changed.

The panel of commercially available ELISAs has notably changed in the last few years. Indeed, some of the previously validated tests are either no longer available or have been modified. The ELISA kits that are currently on the market and have been used in different diagnostic and/or epidemiological studies are: the HIPRA-CIVTEST (Álvarez-García et al., 2003; von Blumröder et al., 2004), IDEXX Bov (Pare et al., 1995; Bien et al., 2012), SVANOVIR (Bjorkman et al., 1997; von Blumröder et al., 2004; Malmsten et al., 2011), BIOVET (Pare et al., 1995; Waldner et al., 2004) and VMRD (Kyaw et al., 2004; Wapenaar et al., 2007). For example, only the in-house ELISA on which the SVANOVIR was based, not the SVANOVIR itself, has previously been compared in a multi-centered study (von Blumröder et al., 2004). Other ELISAs are relatively new, and their performances need to be corroborated. Such tests include the IDVET (Spilovska et al., 2009; De Craeye et al., 2011), BIO-X (Ghalmi et al., 2009), IDEXX Rum (Pare et al., 1995), LSI Bov (Bartels et al., 2005) and LSI Rum. On the contrary, previously employed commercial ELISAs, such as the Pourquier (Institut Pourquier, Montelellier, France), Cypress (Cypress Diagnostics CV), p38 (AFOSA), Chekit Bommeli/ Intervet and Mastazyme (Mastazyme^{TM} MAST Diagnostics) (von Blumröder et al., 2004) are no longer marketed.

Most of the tests used in this study showed high levels of agreement and high Se and Sp values irrespective of the gold standard considered, indicating good or excellent performance. This result was expected based on the similarity of the tests (antigen, principles and technical aspects) and according to previous reports (von Blumröder et al., 2004). The best-adjusted ELISAs were HIPRA-CIVTEST, IDVET, BIOVET and IDEXX Rum that showed excellent Se and Sp values (>95%). A slight increase in agreement and more balanced Se and Sp values were obtained with the BIO-X, LSI Bov, LSI Rum and IDEXX Bov when the adjusted cut-offs from the TG-ROC analysis were applied. However, the most significant adjustment was performed for the SVANOVIR and VMRD after TG-ROC analysis, indicating that the tests were not

459 inferior, but they were improved notably after the validation study. Indeed, in a previous
460 study, the in-house ELISA version of the SVANOVIR that employed ISCOM
461 tachyzoite extract as an antigen showed 98% Se and Sp values after the "majority of
462 tests"-based adjustment (von Blumröder et al., 2004). Moreover previous VMRD results
463 showed 89% Se and 99% Sp values (Wapenaar et al., 2007). The contradictory results
464 obtained with this test may be explained by the different gold standard employed (based
465 on the IFAT test results) and the composition of the panel of sera. In general, the
466 differences observed in the pair wise comparisons of all the tests are likely due to the
467 validation processes conducted by the manufactures. This is evidenced by an increase in
468 the Se and Sp values upon re-adjustment based on TG-ROC analysis. Although most of
469 the ELISAs studied here were indirect assays based on whole or soluble tachyzoite
470 extract, there were three tests based on single antigens or a mixture of purified antigens.
471 These tests showed variable results, with either high Se and Sp (BIO-X), high Se and
472 low Sp (VMRD) or high Sp and low Se values (SVANOVIR). Moreover, cut-off values
473 for the different tests to differentiate between positive and negative results were
474 similarly adjusted for both gold standards employed in the present work. These results
475 may be explained by the restrictive criteria to classify animals as infected or non-
476 infected. There is no consensus on the most appropriate gold standard for this type of
477 study. However, it seems reasonable that results may be less biased with "the majority
478 of the tests" than by relying on a single gold standard based on previous comparative
479 studies that included assays with varying principles (von Blumröder et al., 2004).
480 A similar validation study is highly recommended for commercially available
481 IFAT assays (IFAT VMDR Inc.), taking into account a certain degree of subjectivity
482 inherent to such tests that rely on visualizing results under a fluorescence microscope
483 (reviewed by Bjorkman and Uggla, 1999).
484 Other in-house ELISAs have been suggested for the serological diagnosis of
485 neosporosis. Validation of these tests with a well-referenced sera panel would be
486 similarly recommended prior to considering them as routine diagnostic tools. This issue
487 is of particular relevance for recombinant protein-based ELISAs that may not provide
488 Se and Sp values as high as tachyzoite extract-based ELISAs (Aguado-Martínez et al.,
489 2008) or ELISAs that have been standardized only with sera from experimental
490 infections (Hiasa et al., 2012; Yin et al., 2012). These ELISAs can provide additional
491 information about the route or time of infection, but at present they cannot replace the
492 commercial ELISAs validated herein for conventional serodiagnosis (Aguado-Martínez

et al., 2008). The same is true for the agglutination tests (NAT), which are very useful techniques for seroprevalence studies in several species that do not have available secondary antibodies (reviewed by Bjorkman and Uggla, 1999). However, they are presently not recommended for the diagnosis of bovine neosporosis by reference diagnostic laboratories. In this sense, recent studies have shown a preference for the competitive ELISA VMRD for the detection of anti-*N. caninum* antibodies in the sera of species other than cattle, such as dogs (Sharma et al., 2008), cats (Millán et al., 2009), wild ruminants and sheep (Panadero et al., 2010), wild boars (Bartova et al., 2006), horses (Bartova et al., 2010a), hares (Bartova et al., 2010b) and pigs (Bartova and Sedlak, 2011).

We included three multi-species commercial tests in our study (IDEXX Rum for bovines, caprines and ovines, and LSI Rum and IDVET for ruminants). Further validations should be conducted with the appropriate reference sera to confirm their performance for species other than bovines. This recommendation should also apply to the VMRD test for any use other than cattle sera. In the absence of positive and negative reference sera, it is highly recommended to check the sera employed in the validation study using another *a posteriori* and confirmatory diagnostic tool, such as the western blot (Malmsten et al., 2011).

Another important issue is the study of cross-reactions with closely related apicomplexan parasites with relevance to cattle, such as *Sarcocystis* spp. and *Besnoitia besnoiti*. It is well known that 100% of cattle are infected with *Sarcocystis* spp. (Dubey et al., 1989). However, bovine besnoitiosis is a re-emerging disease in Europe (EFSA, 2010). Therefore, these organisms frequently co-exist with *N. caninum* infections. Thus, cross-reactions should be avoided for an accurate diagnosis. In the present study, most of the tests did not yield false positive results, and only three tests showed 7-28% false positive results. Cross-reactions may be responsible for a high percentage of false positive results when using a panel of bovine sera from a different origin (Nasir et al., 2012). Thus, it would be desirable to discard cross-reactions with these three tests by employing a wide panel of appropriate sera.

Commercial ELISAs are employed not only for conventional diagnoses by reference diagnostic laboratories but also by research laboratories with varying purposes. Interestingly, only three tests (HIPRA-CIVTEST, Bio-X and BIOVET) showed significant differences in specific antibody levels between aborted and non-aborted cows. It has been reported that aborting cows develop higher specific antibody

levels than non-aborting cows (Pereira-Bueno et al., 2000). Therefore, the results of this study show the ability of these ELISA kits to be used in observational and experimental studies

As expected, all the tests showed similar antibody level kinetics for the experimental infections. Because experimental infections are performed under controlled conditions (isolate, dose, route of inoculation, management measures), seroconversions occur in a similar manner. However, more discrepancies were observed when the sera from cows were analyzed. In this case, the antibody level kinetics varied depending on the kit employed. This finding may be explained by the fact that the sera came from pregnant cows, and variations in antibody levels during pregnancy could influence the performance of the kits. Indeed, individual variations in antibody levels were more evident in cows compared to bulls. Although sera from experimental infections are convenient for initial validations of serological tests, field sera are always recommended for further refinements; results observed with sera from experimental infections do not necessarily correlate to results extended to a target population.

In summary, we have evaluated the performance of all the commercially available ELISA tests through the analysis of a well-defined panel of sera and subsequent refinement based on TG-ROC analyses. Serological assays are essential tools for control programs, they are preferred by diagnostic laboratories and they are also used in many epidemiological studies. The information obtained here increases the robustness of the tests, which is essential for providing confidence in assay performance to the reference diagnostic laboratories. Moreover, the results obtained here may help assay developers to follow the guidelines for validation and certification of diagnostic assays elaborated by the OIE (Wright et al., 2007).

According to Jacobson (1998), the validation of diagnostic assays, rather than relying on a small number of experiments that are based on limited reference samples, is a process involving constant surveillance and readjustment of performance characteristics for each target population, an observation that has been corroborated by the present work. Moreover, accredited diagnostic laboratories are highly encouraged to conduct multi-centered studies with validated assays (eg. Dargatz et al., 2004). This is the best way to assess reproducibility and to provide consistent results among laboratories according to the principles and methods of diagnostic test validation. This is of great importance because reproducibility may significantly influence results.

Acknowledgements

We thank private companies for providing the kits for this study. This work was partially funded by CYTED (113RT0469).

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Tables

Table 1: ELISA tests used in the comparative study.

Trademark (ID Test)	Antigen	Type	Cut-off value	References
CIVTEST Bovis Neospora (HIPRA-CIVTEST)	Sonicate lysate of tachyzoites	iELISA	>10/6[a] RIPC=(ODs -ODnc/ODpc- ODnc)x100	Álvarez-García et al., 2003
IDVET ID Screen (IDVET)	Sonicate lysate of tachyzoites	iELISA	≥50/41[a] S/P=ODs -ODnc/ODpc- ODnc	-
LSIVet Bovine (LSI Bov)	Sonicate lysate of tachyzoites	iELISA	≥30 RIPC=(ODs-ODnc/ODpc-ODnc)x100	-
LSIVet Ruminant (LSI Rum)	Sonicate lysate of tachyzoites	iELISA	≥30 RIPC=(ODs-ODnc/ODpc-ODnc)x100	-
Bio-X Diagnostics (BIO-X)	NcSRS2 purified protein	iELISA	>15/10[a] Val=(Delta ODs)x100/(Delta ODp)	Ghalmi et al., 2009
VMRD Inc. (VMRD)	Surface protein antigen (GP65) captured using a monoclonal antibody	cELISA	≥30 %I=100-[(ODsx 100)/(ODmnc)]	Baszler et al., 1996 Baszler et al., 2001
IDEXX Neospora X2 (IDEXX Bov)	Sonicate lysate of tachyzoites	iELISA	≥ 0.50 S/P=ODs-ODnc/ODpc-ODnc	Paré et al., 1995
IDEXX Chekit Neospora (IDEXX Rum)	Detergent lysate of tachyzoites	iELISA	≥ 40/30[a] RIPC=(ODs -ODnc/ODpc- ODnc)x100	Paré et al., 1995
Nc iscom ELISA. Svanovir (SVANOVIR)	Tachyzoite proteins incorporated into iscoms	iELISA	≥ 20 PP=[(mODs or nc)/mODpc)]x100	Björkman et al., 1997
Biovet-*Neospora caninum* (BIOVET)	Sonicate lysate of tachyzoites	iELISA	≥ 0.60 R=(mODs-mODwsc)/(mODpc mODwsc)	Paré et al., 1995 Waldner et al., 2004

a, doubtful cut-off; i, indirect; c, competitive; OD, optical density; IRPC, relative index per cent; S/P, sample/positive; Val, validation; %I, percent inhibition; PP, percent positivity; R, ratio; s, sample; pc, positive control; nc, negative control; m, mean; wsc, wash solution control.

Table 2: Se and Sp values relative to gold standard criteria on the basis of the cut-offs suggested by manufacturers.

Test ID	Majority of tests		Pre-test information	
	Se 95%(CI)	Sp 95%(CI)	Se 95%(CI)	Sp 95%(CI)
HIPRA-CIVTEST	96.1 (93.9-98.4)	100 (100-100)	95.7 (93.2-98.2)	100 (100-100)
IDVET	99.6 (98.9-100)	98.9 (97.4-100)	98.9 (97.6-100)	98.3 (96.3-100)
LSI Bov	99.3 (98.2-100)	94.1 (90.7-97.5)	98.9 (97.6-100)	98.9 (97.3-100)
LSI Rum	99.6 (99.0-100)	93.0 (89.3-96.7)	98.9 (97.6-100)	97.2 (94.7-99.6)
BIO-X	98.9 (97.6-100)	94.9 (91.7-98.2)	98.5 (97.0-100)	94.0 (90.4-97.6)
VMRD	98.9 (97.6-100)	65.1 (58.2-71.9)	98.5 (97.0-100)	66.5 (59.5-73.5)
IDEXX Bov	100 (100-100)	93.0 (89.3-96.7)	99.3 (98.2-100)	96.6 (93.9-99.3)
IDEXX Rum	95.8 (93.3-98.2)	100 (100-100)	96.1 (93.8-98.5)	100 (100-100)
SVANOVIR	85.9 (81.8-90.1)	99.5 (98.5-100)	87.2 (83.2-91.2)	100 (100-100)
BIOVET	98.9 (97.6-100)	98.9 (97.4-100)	98.5 (97.0-100)	98.8 (97.3-100)

Table 3: Se and Sp relative to gold standard criteria on the basis of the re-calculated cut-offs after TG-ROC analysis.

Test ID	Cut-off employed	Majority of tests		Pre-test information	
		Se 95%(CI)	Sp 95%(CI)	Se 95%(CI)	Sp 95%(CI)
HIPRA-CIVTEST	>10/6[a]	96.1 (93.7-98.5)	99.5 (98.4-100)	95.7 (93.6-98.3)	100 (100-100)
IDVET	≥50/41[a]	99.6 (98.9-100)	97.3 (94.9-99.6)	98.9 (97.7-100)	98.3 (96.3-100)
LSI Bov	>52 *	98.9 (97.6-100)	97.9 (95.9-99.9)	98.2 (96.7-99.8)	99.4 (98.3-100)
LSI Rum	>53 *	99.3 (98.2-100)	97.4 (95.1-99.6)	98.6 (97.2-100)	98.9 (97.3-100)
BIO-X	>23/13[a] *	98.1 (96.4-99.7)	96.2 (93.4-99.0)	97.8 (96.0-99.5)	97.0 (94.5-99.6)
VMRD	>65 *	94.8 (92.1-97.4)	91.6 (87.6-95.5)	94.3 (91.6-97.0)	94.3 (90.9-97.7)
IDEXX Bov	>1 *	98.5 (97.1-100)	97.9 (95.9-99.9)	97.9 (96.2-99.6)	98.3 (96.4-100)
IDEXX Rum	≥40/30[a]	96.5 (94.3-98.7)	99.5 (98.4-100)	96.1 (94.1-98.6)	100 (100-100)
SVANOVIR	>15 *	94.0 (91.2-96.9)	93.7 (90.2-97.1)	94.0 (91.2-96.7)	94.3 (90.9-97.7)
BIOVET	≥ 0.60	99.2 (98.2-100)	98.4 (96.6-100)	98.5 (97.1-100)	98.8 (97.3-100)

[a] cut-off with a range of doubtful results

*re-calculated cut-offs after TG-ROC analysis

Table 4: Test agreement between ELISAs and gold-standard criteria.

Gold standard criteria		K-values (95%CI)*					
		LSI Bov	LSI Rum	BIO-X	VMRD	IDEXX Bov	SVANOVIR
Majority of tests	Prior to TG-ROC	0.94 (0.91-0.97)	0.94 (0.90-0.97)	0.94 (0.91-0.97)	0.67 (0.60-0.74)	0.94 (0.91-0.97)	0.83 (0.78-0.88)
	After TG-ROC	**0.97** (0.95-0.99)	**0.97** (0.95-0.99)	**0.95** (0.92-0.98)	0.88 (0.83-0.92)	**0.97** (0.94-0.99)	0.87 (0.82-0.92)
Pre-test information	Prior to TG-ROC	**0.98** (0.96-1)	**0.96** (0.94-0.99)	0.93 (0.89-0.97)	0.69 (0.61-0.76)	**0.96** (0.94-0.99)	0.85 (0.79-0.89)
	After TG-ROC	**0.97** (0.95-0.99)	**0.97** (0.95-0.99)	0.94 (0.91-0.97)	0.88 (0.83-0.92)	**0.95** (0.93-0.98)	0.87 (0.83-0.92)

* Almost perfect agreement is marked in bold (K-values > 0.95)

Figures

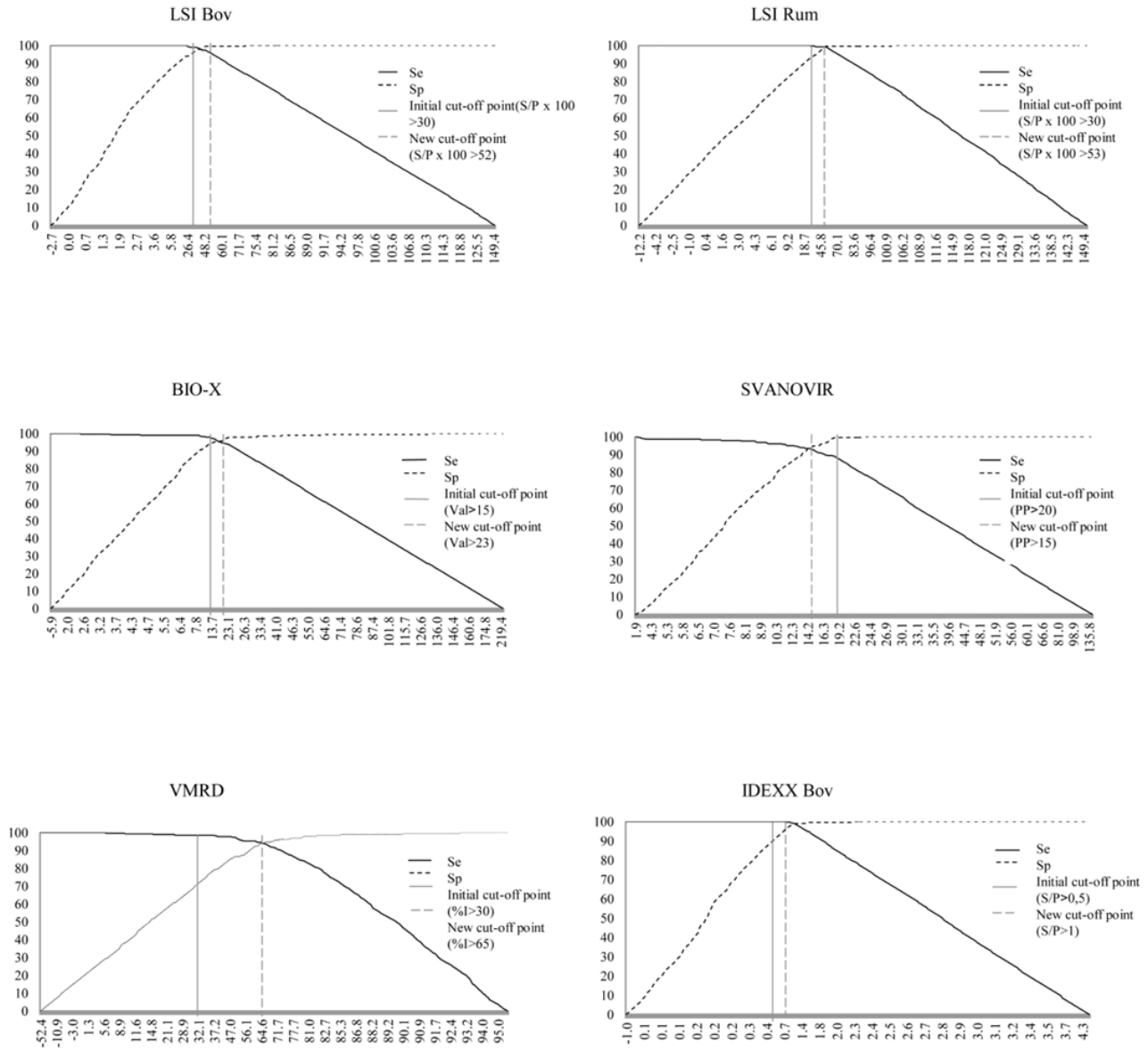

Figure 1: TG-ROC analysis of 6 commercial ELISA tests based on the gold standard 'Majority of tests'.

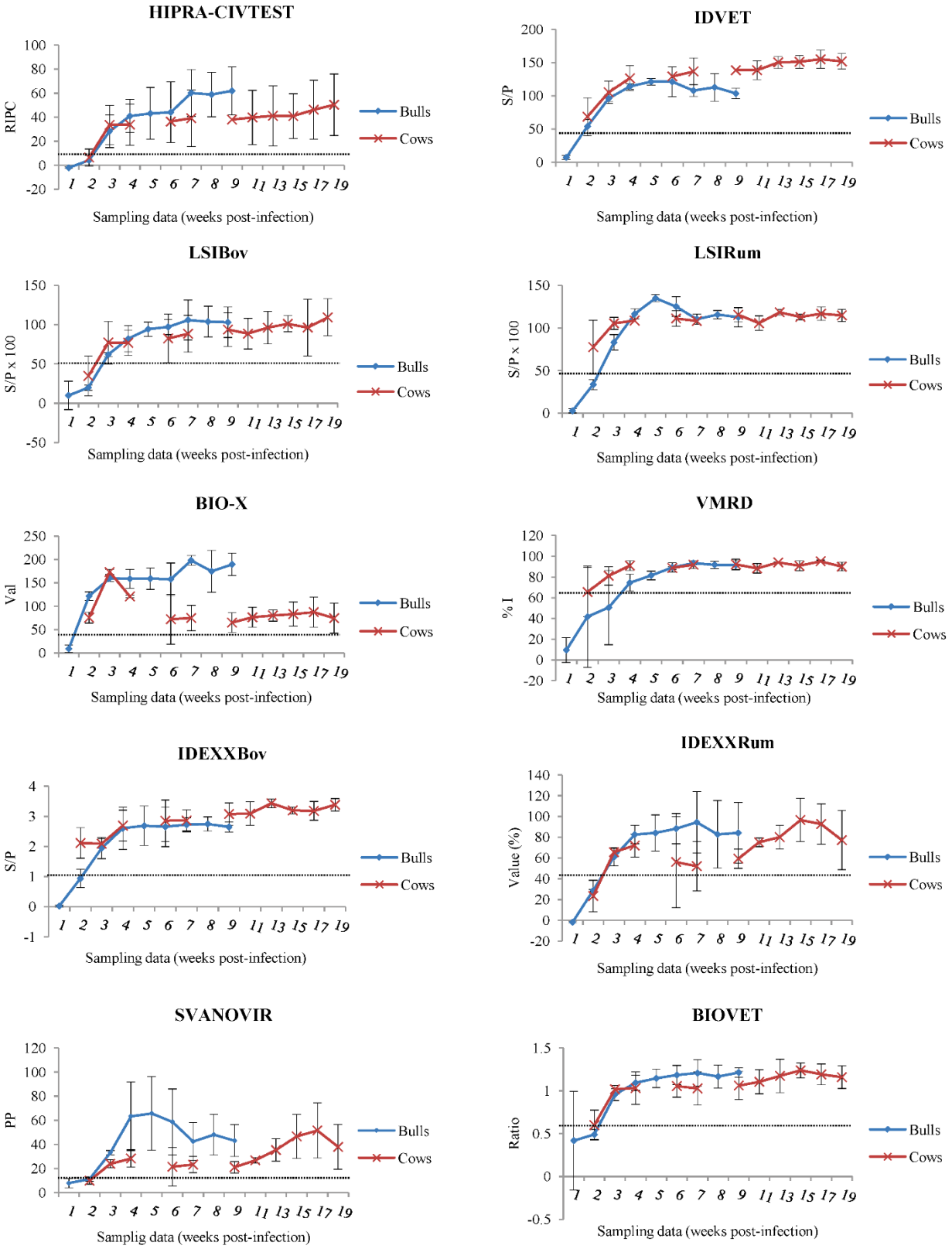

Figure 2: Specific anti-*N. caninum* antibodies developed over time by experimentally infected bulls and cows at 135 days of gestation. Recalculated cut-offs were employed for the LSI Bov, LSI Rum, BIO-X, VRMD, IDEXX Bov and SVANOVIR ELISAs.