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Original article

Influence of age and purpose for testing on the cut-off selection of serological methods in bovine neosporosis

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Abstract – The aim of this study was to investigate the need for different cut-off points, according to animal age and the purpose of testing, for two of the most widely used serological techniques in bovine neosporosis, IFAT and a crude antigen ELISA (Civtest®, HIPRA). Therefore, the population reference sera used were defined using a combination of multiple criteria such as epidemiological/clinical and histopathological parameters and an immunoblot test. Firstly, foetuses and breeding cattle (heifers and cows) were considered as separate subpopulations for serological evaluation. Secondly, cut-off points for each serological technique (IFAT and ELISA) according to age group (foetuses and breeding cattle) and the different practical applications (detection of infection and abortion) were calculated following the receiver operating characteristics (ROC) analysis. Cut-off points were defined, for IFAT and ELISA for aborted breeding cattle and for IFAT alone in the case of the foetuses, assuming an equivalent cost of false positive and negative results. In infected breeding cattle, for IFAT and ELISA and in foetuses for ELISA, two possible cut-off values were obtained, one for a maximum sensitivity and one for a maximum specificity and the intervals of unclear results were defined. In this case, a cut-off value for equal sensitivity and specificity was also estimated. When cut-off points for infected breeding cattle, 1:100–1:250 for IFAT and 0.306–0.451 for ELISA were applied to a target population, optimal and similar negative and positive predictive values together with similar apparent and true prevalence results were observed suggesting the possibility of using both tests interchangeably.

***Neospora caninum* / foetus / breeding cattle / serology / cut-off**

1. INTRODUCTION

Neospora caninum is a cyst-forming coccidian parasite, which has been identified in several domestic species such as cattle, dogs, horses, sheep and goats. Neosporosis has been described as a major cause of abortion in the main cattle producing countries and the infection can also be associated with neonatal mortality and

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encephalomyelitis in congenitally infected calves [10]. In bovines, different serological methods have been developed to detect parasite specific antibodies [2, 4], primarily the indirect fluorescent antibody test (IFAT) – regarded as a reference test to which other assays have been compared and calibrated with – and different enzyme-linked immunosorbent assays (ELISA) [4]. Immunoblotting has been recently used as an aid for other serological tests, rather than as a routine tool for screening cattle sera [2]. Serology in adult cattle enables the individual detection of the infection and, at the herd-level, permits neosporosis to be considered as a cause of reproductive failure by comparing the prevalence of the infection in aborting and non-aborting animals. The presence of specific antibodies in the sera from aborted cows and foetuses is indicative of exposure to *Neospora*, but examination of the foetus by histological methods is necessary for a definitive diagnosis of abortion due to neosporosis [10].

Serological diagnosis of neosporosis faces several limitations. Foetal age and the time elapsed between infection and abortion are important factors to be considered in the interpretation of foetal serology [35]. In adult cattle, specific antibody levels fluctuate with the animal's age and the state of pregnancy [8, 21, 22]. Therefore, the purposes of serological techniques can be very variable, such as the diagnosis of the infection in foetuses and in cattle that have recently aborted, determination of the infection status in individual cattle i.e. prior to purchase or entry in the breeding herd and estimation of prevalence in epidemiological studies. Because of the different purposes, different cut-off points might be necessary for different applications of testing.

The aim of our study was to investigate the need for different cut-off points, according to animal age and the purpose of testing, for two of the most widely-used serological techniques in bovine neosporosis (IFAT and ELISA). Firstly, serum samples from foetuses and aborted breeding cattle (heifers and cows) were compared in order to determine whether the distribution of serologic values differed in both populations. Secondly, a different cut-off for each serological technique (IFAT and ELISA), age group (foetuses and breeding cattle) and purpose for testing (infection and abortion) were calculated using receiver operating characteristics (ROC). The areas under the ROC curve (AUCs) were calculated for both tests in order to compare their performance. Finally, the influence of the cut-off value applied to detect *Neospora* infected cattle was studied. True prevalence was estimated in a target population and compared for both IFAT and ELISA and for the different cut-off points obtained and for those recommended in previous works [6, 24].

2. MATERIALS AND METHODS

2.1. Experimental design and serum samples

In the present work the recommendations given by Greiner and Gardner [13] and Jacobson [16] were carried out in order to calculate the cut-off points for both serological tests, IFAT and ELISA, according to different practical situations. The experimental design was as follows:

Firstly, three different reference populations (groups 1, 2 and 3) and a target population (group 4) were defined:

Group 1: Negative reference sera consisted of two age groups: aborted foetuses (n = 21) and breeding cattle (heifers, n = 21; cows, n = 21). Foetal fluids and serum samples were recovered from each category from animals belonging to herds with no history of reproductive failure caused by *Neospora*, and a lack of recognition of any tachyzoite immunodominant antigen (IDA) either in foetal fluids or adult sera by an immunoblotting technique [1].

Group 2: Positive reference sera from *Neospora* aborted foetuses (n = 13) and *Neospora* aborted heifers-cows (n = 33). In

both cases, samples were collected from animals with a *Neospora* confirmed diagnosis of abortion based on the finding of specific or compatible lesions in the brains of aborted foetuses as described by others [11] and the identification of at least one specific immunodominant antigen (IDA) either in the foetal fluids or sera by an immunoblotting technique. There were no paired samples in this study i.e. the negative and positive reference samples from aborted foetuses did not come from their corresponding aborted cows.

The sera from the aborted foetuses and aborting breeding cattle were collected within a month of abortion.

Group 3: Positive (n = 22) and negative reference sera (n = 27) from heifers and cows without any recent individual abortion problem belonging to herds with a previous diagnosis of *Neospora* abortion as described previously. All samples were analysed by immunoblotting and positive sera detected at least one IDA.

Group 4: The target population was composed of infected and non-infected heifers and cows (n = 372) from 11 herds with a previous history of *Neospora* abortion problems in the last three years. An individual diagnosis of the infection using immunoblotting was also carried out.

After blood recovery, all sera and foetal fluids used in this study were aliquoted and stored at –80 °C prior to testing for specific antibodies against *N. caninum*.

Secondly, two different age groups were analysed (foetuses and breeding cattle belonging to groups 1 and 2) in order to investigate the distribution of their serologic values and justify the use of different cut-off points for the IFAT and ELISA according to the age of the animal.

Thirdly, cut-off points were calculated for IFAT and ELISA according to the purpose of testing. In order to diagnose abortion, the cut-off points were estimated for foetuses and breeding cattle (groups 1 and 2). A cut-off point to diagnose infection was also calculated (group 3).

Finally, the cut-off points obtained to diagnose infection were revalidated. In this way, the cut-off points obtained for group 3 were applied to a target population (group 4) and their effect on the tests performance characteristics and prevalence estimates were investigated.

2.2. Parasite and antigen preparation

N. caninum tachyzoites (Nc-1 isolate) were obtained by continuous passage in Vero cell culture following previously described standard procedures [19]. The parasites were harvested from tissue culture and washed three times in sterile 0.3 M PBS, pH 7.4 and separated from host cell debris by passaging the mixture through a 25-gauge needle, following a passage through a 5 μm polycarbonate filter. Nc-1 purified tachyzoites were pelleted and stored at –80 °C until use. Tachyzoites for IFAT were resuspended in phosphate-buffered saline (PBS) to a final concentration of approximately 10^7/mL. Soluble antigen extract, used in western blot, was prepared as follows: to obtain *N. caninum* soluble proteins, purified tachyzoites (2×10^9) were suspended in 1 mL of 10 mM Tris hydrochloride containing 2 mM of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo, USA), disrupted by ultrasonic treatment (Branson mod. Sonifier 450, Branson Ultrasonic Co., USA) in an ice-bath, and centrifuged at 10 000 × g for 20 min at 4 °C. Protein content was determined using the Micro BCA protein assay method (Pierce, Rockford, USA) [29], and the supernatant was aliquoted and cryopreserved at –80 °C.

2.3. IFAT technique

The procedure was carried out basically as described by others [33]. The cattle sera were diluted at two-fold serial dilutions starting at a 1:25 dilution in PBS to the end point titre. Unbroken tachyzoite membrane fluorescence was considered as a positive reaction.

2.4. ELISA technique

A crude antigen ELISA (Civtest® Hipra Laboratories S.A., Gerona, Spain) [24], was run as recommended by the manufacturer and positive and negative controls were provided with the kit. The test results were expressed as O.D. values.

2.5. SDS-PAGE and western blot analysis

Electrophoresis was performed according to a previous work [18] in 12.5% polyacrylamide gels. Low molecular weight standards (Bio-Rad Laboratories; California, USA) were subjected to electrophoresis concurrently so that the rates of migration (Mr) of the different antigens recognised by the sera could be estimated. Proteins were electrophoretically transferred to a nitrocellulose membrane for western blot (Mini Trans-Blot Cell). The cow sera and foetus fluid dilutions corresponded to 1:100 and 1:50 respectively. As a secondary antibody, a mouse monoclonal anti-bovine antibody IgG1 and IgG2 (1:200) (Hipra Laboratories S.A.; Gerona, Spain) was used and antigen-antibody reactions were developed using 4-chloro-1naphtol (Bio-Rad Laboratories; California, USA) as the substrate. Intense recognition of at least one of the following 4 immunodominant proteins (17–18, 34–35, 37 and 60–62 kDa) by aborted and infected cows as well as by foetuses was regarded as a positive result [1].

2.6. Analysis of data

The non-parametric Mann Whitney *U*-test was employed to investigate the distribution of serologic values in both bovine populations.

ROC analysis was applied to estimate cut-off points by employing two different softwares, AccuROC for Windows 95/98/NT 2.0 and TG-ROC CMDT [12]. The areas under the ROC curve (AUC) together with the standard error of the AUC (SE_{AUC}) were calculated to compare the overall diagnostic performances of both IFAT and ELISA [13]. The cut-off points obtained by ROC analysis using AccuROC were selected for either maximal diagnostic sensitivity or maximal diagnostic specificity values for IFAT and ELISA and in the absence of a cut-off point for both maximal sensitivity and specificity an interval of unclear results was established. The TG-ROC CMDT approach was employed to confirm the selection of different ELISA cut-off points (d_o) for equal sensitivity and specificity (θ_o) for the different groups. A non-parametric approach for correlated samples was employed to compare the AUC [9] for IFAT and ELISA for the different groups considered. True prevalence values obtained in a target population for the different cut-off points considered for infected cattle were compared by a contingency tables analysis. Diagnostic sensitivity and specificity were recalculated by ROC analysis and apparent prevalence, negative (NPV) and positive predictive values (PPV) were also estimated and considered in the true prevalence calculation [16, 31].

The STAT-VIEW v.4.0. package (Abacus Concepts, Inc. Berkeley, California, USA) was used to calculate the contingency tables and Mann Whitney *U*-test analysis.

The precision of both serological techniques was also measured by their repeatability including interassay and operator-to-operator variations [16], which were calculated in triplicate for seven positive samples and seven negative samples.

3. RESULTS

3.1. Distribution of serologic values in different bovine populations

No significant differences were found between the heifers and cows in group 1 when tested by IFAT and ELISA. When negative reference sera from foetuses and

Table I. IFAT and ELISA values for reference sera sample populations of *Neospora* infected and non-infected bovine foetuses and breeding cattle.

		Foetuses			Heifers-cows			P value
		Median	Upper Quartile	Lower Quartile	Median	Upper Quartile	Lower Quartile	
IFAT*	Negative	0	0	0	0	0	0	0.2755
	Positive	1:128	1:500	1:32	1:500	1:1000	1:250	0.0012
ELISA**	Negative	0.077	0.082	0.072	0.111	0.137	0.095	0.0001
	Positive	0.728	0.907	0.102	1.060	1.642	0.805	0.0008

P value based on the Mann-Whitney rank test.* IFAT titres. ** O.D. values.

breeding cattle (heifers and cows) were processed by the IFAT technique, no significant differences were found since none of the samples showed positive IFAT titres from a starting 1:25 dilution. However, significant differences were found when these two groups of samples were processed by the ELISA (Tab. I). For group 2, heifers and cows (breeding cattle) also had different distribution of serologic values from foetuses when positive reference samples from both bovine populations were tested by both techniques.

3.2. Determination of cut-off values

The cut-off values obtained by ROC analysis (Fig. 1) for IFAT and ELISA with reference sera from either aborted cows and foetuses belonging to groups 1 and 2 or infected cattle from group 3 are summarised in Table II. In aborted breeding cattle the cut-off point obtained by ROC analysis for IFAT and ELISA gave 100% diagnostic sensitivity and specificity as well as for IFAT in the case of the foetuses. In infected breeding cattle for both techniques and in foetuses for ELISA, two possible cut-off values were obtained, one for a maximum sensitivity and one for a maximum specificity (Tab. II). When equal sensitivity and specificity values were considered following the TG-ROC CMDT approach, we also obtained different ELISA cut-off values (d_o) for the different groups (0.381 for aborting breeding cattle, 0.373 for infected breeding cattle and 0.093 for foetuses) with a θ_o value equal or higher than 0.9.

3.3. ROC curve comparison

Comparison of the ROC curves for correlated samples obtained with IFAT and ELISA for the different groups considered demonstrated a negligible difference in the overall accuracy of both tests. For aborted breeding cattle and foetuses from groups 1 and 2, the AUC values obtained were as follows: $AUC_{IFAT} = 1$; $AUC_{ELISA} = 1$, and $AUC_{IFAT} = 1$; $AUC_{ELISA} = 0.99$, respectively. Significant differences were only found for AUC in infected breeding cattle from group 3 ($P < 0.05$) with an AUC of 0.9 and 1 for IFAT and ELISA, respectively. In all cases, their corresponding standard error values were always smaller than 0.05.

3.4. Diagnostic characteristics and prevalence as a function of the cut-off point

Prevalence and diagnostic characteristics for the different cut-off points considered for infected breeding cattle are summarised in Table III. Significant differences in the diagnostic characteristics depending on the cut-off point and technique considered were observed. The cut-off points with better diagnostic values and

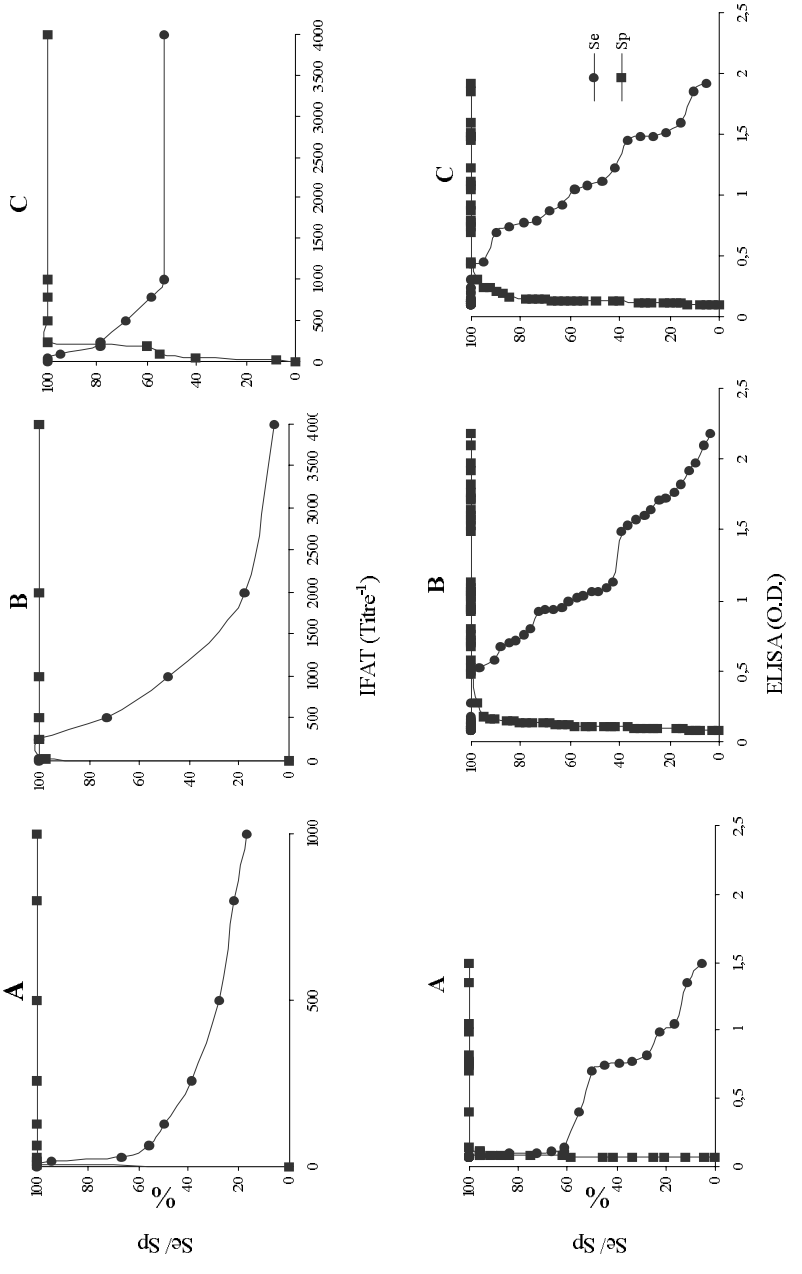

Figure 1. Sensitivity and specificity values as a function of the different cut-off points obtained in the ROC analysis for IFAT (top) and ELISA (bottom).
A: foetuses, B: aborted breeding cattle; C: infected breeding cattle.

Table II. Cut-off values suggested by ROC analysis for IFAT and ELISA as a function of animal age and technique purpose.

	Aborted foetuses	Breeding cattle	
		Aborted cattle	Infected cattle
IFAT*	1:16[a,b]	1:250[a,b]	1:100[a] 1:250[b]
ELISA**	0.098[a] 0.401[b]	0.484[a,b]	0.306[a] 0.451[b]
	0.093[c]	0.381[c]	0.373[c]

[a] Cut-off values for a maximum sensitivity. [b] Cut-off values for a maximum specificity. [c] Cut-off values for equal sensitivity and specificity ($\theta > 0.90$). * IFAT titres; ** O.D. values.

similar apparent and true prevalence results corresponded to the cut-off points estimated in this work, 1:250 for IFAT and 0.306–0.451 for ELISA.

When true prevalence values were compared, significant differences were obtained for 1:100 and 1:250, as well as for 1:250 and 1:640 IFAT cut-off points ($P < 0.05$). The differences observed in the true prevalences for the ELISA cut-off points turned out to be non-significant despite the lower prevalence obtained when the cut-off point suggested by the manufacturer was considered. When true prevalence rates corresponding to IFAT and ELISA cut-off points for maximum sensitivity were compared, significant differences were obtained ($P < 0.01$), whereas for maximum specificity the differences observed were not significant.

3.5. Precision of IFAT and ELISA

As shown in Table IV, most of the coefficients of variation for ELISA and IFAT were lower than 10%.

4. DISCUSSION

A few attempts have been made to compare and critically evaluate the different serological methods currently in use for bovine neosporosis. It has been recommended that several issues, such as appropriate selection of the reference population and sampling strategies, should be taken

Table III. IFAT and ELISA diagnostic characteristics and prevalences obtained in a target population according to the different cut-off points considered for infected breeding cattle.

Technique	Cut-off values	Se	Sp	PPV	NPV	Apparent prevalence	True prevalence
IFAT (titre)	1:100[a]	95	55	15.1	99.4	48.9	7.8
	1:250[b]	78.9	100	100	92	23.1	29.2
	1:640[c]	57.8	100	100	92	8.8	16.4
ELISA	0.306[a] (O.D.)	100	97.5	91.3	100	24.7	22.7
	0.451[b] (O.D.)	94.7	100	100	98	23.9	25.2
	5[c] (IRCP)*	100	85	53	100	27.6	14.8

Se: Diagnostic sensitivity. Sp: Diagnostic specificity. PPV: Positive predictive value. NPV: Negative predictive value. IRCP: Relative index × 100.
[a] Cut-off value selected for a maximum sensitivity. [b] Cut-off value selected for a maximum specificity. [c] Cut-off value suggested in the literature for IFAT [6] and ELISA [24].

$$* \text{ IRCP} = \frac{(\text{O.D.}_{405}\text{S} - \text{O.D.}_{405}\text{NC})}{(\text{O.D.}_{405}\text{PC} - \text{O.D.}_{405}\text{NC})} \times 100$$

S = sample, NC = mean negative control, PC = mean positive control.

Table IV. Interassay and operator-to-operator precision of ELISA and IFAT.

Sample No.	ELISA (O.D.) #				IFAT titre (log_2) #			
	Mean		%CV*		Mean		%CV*	
	a	b	a	b	a	b	a	b
Positive samples								
1	1.729	1.618	4.0	6.3	10.23	10.23	5.6	5.6
2	1.741	1.756	1.3	6.6	10.63	10.63	5.4	5.4
3	1.612	1.651	3.3	5.3	9.97	9.30	0.0	6.2
4	0.909	0.898	12.3	2.5	9.30	9.97	6.2	0.0
5	1.718	1.719	3.4	5.7	10.30	9.30	5.6	6.2
6	1.061	1.039	9.4	13.9	10.63	10.63	5.4	5.4
7	2.004	1.907	9.0	5.8	11.97	10.97	0.0	9.1
Negative animals								
1	0.115	0.119	1.0	8.6	6.64	6.31	0.0	9.1
2	0.100	0.097	10.2	14.9	6.31	5.98	9.1	9.7
3	0.123	0.125	3.7	5.6	6.64	5.31	0.0	10.9
4	0.129	0.130	5.1	8.0	5.64	4.98	0.0	11.6
5	0.104	0.117	11.5	9.0	5.98	4.98	9.7	11.6
6	0.122	0.130	4.2	8.7	5.98	4.98	9.7	11.6
7	0.134	0.125	6.0	2.0	5.64	4.98	17.7	11.6

The given values are the means of three independent measures. * CV, coefficient of variation. [a] Values for interassay precision. [b] Values for operator-to-operator precision.

into consideration when validating a veterinary diagnostic test. Diagnostic sensitivity and specificity are test performance parameters that could be highly influenced by factors such as stage of disease and animal age [13]. In the present work, we applied a validation procedure, as proposed recently [13, 16, 17], to IFAT and an indirect ELISA, two of the techniques most used world-wide for the diagnosis of neosporosis in cattle. Therefore, restrictive criteria for selection of the reference population, selection of the cut-off point as a function of animal age and disease status were considered as crucial steps in the validation process. The combination of multiple tests, such as epidemiological, clinical, histopathological and serological measures, is considered as a gold standard to define the reference populations used in our study [16]. Three main approaches have been previously followed to establish the reference populations: the serological status of the new-born calf as an indicator of the dam status assuming a high efficiency of vertical transmission [8], the simultaneous use of two serological techniques to confirm the status of the animal [27] and the use of the IFAT test as a gold standard [4, 25]. In our opinion, the first approach introduces a source of error mainly due to a not very high efficiency of

vertical transmission observed in natural conditions in some endemically infected herds – from 48% [21] to 95% [8] – and the fact that experimentally infected model animals infected before pregnancy, which showed consistent specific antibody titres during gestation, did not transmit the infection to their descendants [15]. On the contrary, the use of IFAT as a gold standard does not appear to be accurate enough in a validation study since there is still some uncertainty concerning standard cut-off values either for adults or foetuses, since cut-off titres in IFAT differ between laboratories and are often set at 1:160–1:640 [4] or 1:25 to 1:640 [6, 28] for adult bovines and from 1:25 to 1:80 for foetal serology [3, 5, 35]. Besides, the IFAT test cannot resolve non-specific or suspicious positive reactions, whereas western blot is more specific [30] and allows unequivocal serological diagnosis even in cases that are problematic for IFAT testing.

One of the most important biological factors that could affect the distribution of the test value, as well as the diagnostic sensitivity and specificity, is animal age [13]. Bovine foetuses are only able to develop a serologically detectable response from month 4–5 of gestation onwards [20]. On the contrary, specific antibody titres observed in the 7–12 month-old calves and in heifers [21] or seroprevalence in the 13 to 24 month-age group [7] are lower than in other age groups, probably related to the decline in maternal antibodies in congenitally infected cattle [34]. Although they are not surprising, our results confirm the use of different cut-off points for foetuses and breeding cattle, and the homogeneity of serologic values for a *N. caninum* infected breeding cattle population composed of heifers and cows.

With regards to the use of the tests, different approaches have been followed. Whereas the traditional calculation of cut-off points has been followed by most authors, recently a new approach based on Bayes theorem was reported for the serologic diagnosis of *Neospora* infection [32]. In the present work, the calculation of cut-off points was carried out depending on the intended uses of the tests. Therefore a combination of ROC analysis and utility-based decision theory was followed to calculate the cut-off points for the two main independent bovine populations defined in this study: foetuses and breeding cattle and for two purposes of testing. The cut-off points obtained for *Neospora* aborted foetuses and *Neospora* aborted breeding cattle corresponds to an improvement in serology to diagnose the cause of abortion, since cut-off points for both techniques were obtained following the same criteria and using a well characterised panel of reference sera. This approach permits more precise cut-off points to be defined for a desired sensitivity and specificity according to the different practical applications for which a serological test is supposed to be used. In both cases, lower IFAT cut-off points were obtained either for maximal sensitivity or specificity compared to those described by others [4, 28] without an increment of false positive results. On the contrary, the cut-off values obtained for aborted dams were higher than for infected animals in agreement with previously described higher antibody titres in *Neospora*-aborted cattle compared with seropositive non-aborted cattle [21]. In this case, cut-off points estimated for by two different ROC analyses were very similar and confirmed the necessity of using different cut-off points. TG-ROC CMDT software determines a cut-off point for equal sensitivity and specificity values, which might not correspond to any of the cut-off points obtained in the practical field, whereas the AccuROC software provides sensitivity and specificity values for the different cut-off points obtained in the experiment.

From a practical viewpoint and in the absence of a perfect cut-off point for infected breeding cattle, the use of a value for maximum sensitivity could be

interesting to investigate the status of individual cattle prior to purchase or entry into a *Neospora*-free herd. On the contrary, a cut-off for maximum specificity could be of use when evaluating the presence of the disease in an area. A final possibility could be the use of both cut-off points obtained by the AccuROC approach or the cut-off point obtained by TG-ROC CMDT assuming an equivalent cost of false positive and negative results. This decision implies the use of a confirmatory test for intermediate results [2], which could be western blot based on the identification of a/some tachyzoite immunodominant antigens [1, 27] by the problematic sera.

The agreement between IFAT and ELISA has been studied using the kappa index [23, 26], and moderate and good results have been obtained in different studies. On the contrary, different ELISAs have also been compared by the kappa index [36] and by correlation analysis using paired results [27] obtaining results with good agreement and high coefficients of determination, respectively. In the present work, the comparison of ROC curves suggests the possibility of using IFAT or ELISA interchangeably. The area under the ROC curve (AUC) has been suggested as a global statistic summary of the overall performance of a test [14], and the high AUC values for both tests indicated a good positive-negative classification. This assumption is confirmed by the similar negative and positive predictive values and similar true prevalence results obtained in the prevalence study carried out in a target population with a cut-off point of 1:250 for IFAT and 0.306 or 0.451 for ELISA. However, when a greater diagnostic sensitivity is required, ELISA offers more accurate diagnostic characteristic values compared to the 1:100 IFAT cut-off point.

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