BRIEF REPORT

TÍTLE: "Update in the serological diagnosis of bovine neosporosis: A comparative study of all commercial ELISA tests available in the market"

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1. INTRODUCTION

Bovine neosporosis is a parasitic disease caused by the cyst-forming coccidian parasite *Neospora caninum* that causes abortion and neonatal mortality in cattle worldwide (Dubey et al., 2007; Dubey and Schares, 2011).

A supranational study carried out by Bartels et al. (2006) updated prevalence rates of *N. caninum* infection in several European countries. Prevalence rates for dairy herds were estimated to be 16% in Sweden, 49% in Germany, 63% in Spain and 76% in The Netherlands and, for beef herds, 41% in Germany, 46% in Spain and 61% in The Netherlands (Bartels et al., 2006). Recently, Eiras et al. (2011) calculated herd and individual seroprevalence rates in diary, beef and mixed cattle in Galicia (Spain) reporting 80.6% true herd seroprevalence and 23.2% true animal seroprevalence. Regarding the presence of *N. caninum* in aborted fetuses several studies carried out in the United States, New Zealand, The Netherlands and Germany have estimated that between 12% and 42% of aborted fetuses from dairy cattle were infected with *N. caninum* (Dubey, 2003).

Regarding control, at present, there is not effective treatment or vaccine and control measures are based on herd management coupled to diagnosis to reduce *N. caninum* infection (Dubey et al., 2007). In fact, the serological diagnosis of neosporosis in adult cattle and precolostral calves is an integral part of control programs because one of the most commonly adopted measures include selective culling of seropositive *Neospora*-associated aborted dams and herd replacement with seronegative cattle (Jenkins et al., 2002; Dubey et al., 2007).

Serological techniques available to detect specific antibodies anti *N. caninum* in order to differentiate infected from non infected animals 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) (Dubey and Schares, 2006; Ortega-Mora et al., 2006). In addition, western blot is usually recommended to confirm doubtful results in valuable samples (Álvarez-García et al., 2002). Moreover avidity and recombinant antigen based ELISAs (rNcGRA7 ELISA and rNcSAG4 ELISA) are useful tools in investigating the mode of *N. caninum* transmission in herds since they permit to differentiate mainly between primo-infection and chronic infection (Björkman et al., 1999, 2006; Aguado-Martinez et al., 2005, 2008).

In the last few years the panel of commercial serological kits available has notably changed since new tests have been developed, others already available in the marked have been modified and several ones are not available anymore. Thus at present there is not updated information about the performance of all these diagnostic products, which is an essential information demanded by diagnostic reference labs. In fact, last comparative studies were done in the past offering a fragmented picture of diagnostic tools employed in Europe (Von Blumröder et al., 2004) and in USA (Wapenaar et al., 2007).

Therefore the aim of this study was to study the performance and re-standardized all commercial ELISA tests available in the market worldwide to detect anti-*N. caninum*-specific antibodies for control purposes and epidemiological studies.

2. MATERIALS AND METHODS

2.1. Sera and experimental design

A well reference 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 sampled animals were older than 6 months in order to avoid the presence of colostral antibodies.

The animals analyzed were categorized into the following groups:

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

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

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

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

2.2. Tests

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

The first gold standard was defined by the decision of the majority of the test (`Majority of tests´). If equal numbers of tests retuned positive and negative results, the sample was regarded as doubtful and 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, clinical and serological data (absence or presence of clinical signs compatible with disease and was seropositive by one or two reference tests: *N. caninum* soluble extract antigen-based ELISA and by recombinant protein based ELISAs). Group "a" and "d" was regarded as negative reference sera *versus* groups "b", and "c" that were regarded as positive reference sera.

TG-ROC analyses were carried out with respect to the gold standard `Majority of tests´ (Greiner et al., 1995) and SPSS 17.0 for Windows (SPSS Inc.) was used.

3. RESULTS

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

See results in Table 2.

3.2. TG-ROC analysis

TG-ROC analysis, based on the 'Majority of tests', were conducted to check the accuracy of the cut-offs suggested by manufacturers. These analyses were conducted for the ELISA tests that showed Se and/or Sp values less than 95%. Fortunately TG-ROC analysis was not performed for BIOVET because this test showed Se and Sp values higher than 95%.

3.3. Test agreement (K-statistics)

Test agreement results are presented in Table 4.

K-values were calculated again using the adjusted cut-offs obtained by the TG-ROC analysis on the basis of the gold standard 'Majority of tests' (Table 5).

3.4. Cross-reactions

BIOVET ELISA only yielded one false positive result with a *B. besnoiti* positive serum.

4. REFERENCES

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Table 1. ELISA tests used in the comparative study

Trademark (ID Test)	Antigen	Type	Cut-off value	References
CIVTEST Bovis Neospora (CIVTEST)	Sonicate lysate of tachyzoites	iELISA	>10/6[a] RIPC=(ODs -ODnc/ODpc- ODnc)x100	Rebordosa et al., 2001 Álverez-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)	
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 Wouda., 1998 Wu et al., 2002
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 Frössling et al., 2003 Frössling et al., 2006
Biovet-*Neospora Caninum* (BIOVET)	Sonicate lysate of tachyzoites	iELISA	≥ 0,60 R=(mODs-mODwsc)/(mODpc mODwsc)	Paré et al., 1995 Wu et al., 2002 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)
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)
BIOVET	≥ 0,60	99.2 (98.2-100)	98.4 (96.6-100)	98.5 (97.1-100)	98.8 (97.3-100)

[a] doubtful cut-off

*re-calculated cut-off

Table 4. Test agreement before TG-ROC analysis

Test	K-Values (95%CI)								
	CIVTEST	IDVET	LSI Bov	LSI Rum	BIO-X	VMRD	IDEXX Bov	IDEXX Rum	SVANOVIR
BIOVET	0.96 (0.93-0.99)	0.98 (0.96-1.00)	0.92 (0.88-0.96)	0.92 (0.88-0.96)	0.95 (0.92-0.98)	0.66 (0.58-0.73)	0.92 (0.88-0.96)	0.95 (0.92-0.98)	0.84 (0.79-0.89)

Table 5. Test agreement after TG-ROC analysis

Test	K-Values (95%CI)								
	CIVTEST	IDVET	LSI Bov	LSI Rum	BIO-X	VMRD	IDEXX Bov	IDEXX Rum	SVANOVIR
BIOVET	0.96 (0.93-0.99)	0.98 (0.96-1.00)	0.96 (0.93-0.99)	0.96 (0.93-0.99)	0.95 (0.92-0.98)	0.88 (0.83-0.92)	0.98 (0.96-1.00)	0.95 (0.92-0.98)	0.87 (0.82-0.91)