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A comparative study of serological tests used in the diagnosis of *Toxoplasma gondii* infection in small ruminants evidenced the importance of cross-reactions for harmonizing diagnostic performance

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ABSTRACT

Toxoplasma gondii is a major foodborne zoonotic pathogen that can be transmitted through the consumption of raw or undercooked meat of small ruminants, among others. Serology has been suggested as an epidemiological indicator and several tests are available nowadays. However, there is no comparative study with the most used ones. Therefore, the objective of this study was to develop and validate two in-house tests (Western blot -TgSALUVET WB- and ELISA -TgSALUVET ELISA 2.0-) and perform a comparative study including such tests and four commercial ELISA kits (IDScreen®, PrioCHECK®, Pigtype® and IDEXX). First, a specific pattern of recognition of immunodominant antigens by TgSALUVET WB was determined with serum panels of noninfected sheep and sheep infected with *T. gondii* or *Neospora caninum*. Next, TgSALUVET WB was used as a reference to preliminary validate TgSALUVET ELISA 2.0 using sera from sheep and goats naturally infected with *T. gondii*. Then, the abovementioned sheep serum panels were analyzed by all tests and subjected to TG-ROC analyses and agreement tests, and cross-reactivity with the anti-*N. caninum* IgGs was studied.

All the techniques were accurate enough for the cutoff values initially suggested with all serum panels (Se and Sp $\geq$ 94%), except for PrioCHECK®, which showed 83% Sp. However, a cutoff readjustment improved their diagnostic performance. Additionally, cross-reactions between anti-*N. caninum* antibodies and *T. gondii* antigens were detected with all tests. Thus, a second cutoff readjustment was carried out and the use of both readjusted cutoff values is recommended to obtain comparable data and avoid false-positive results.

1. Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is a widespread apicomplexan parasite able to infect any warm-blooded animal species, including humans. Toxoplasmosis is usually asymptomatic in immunocompetent hosts, with an estimation of one-third of the global human population chronically infected or having had past episodes of contact with the organism. However, this zoonosis is of concern since it can induce severe respiratory, neurological and ocular diseases in immunocompromised people, as well as reproductive failures in pregnant women, mainly via primary infection (WHO, and FAO, 2014). In humans, horizontal transmission of *T. gondii* mainly occurs through the consumption of sporulated oocysts that contaminate soil, water, vegetables, fruits and bivalves (environmental route) or tissue cysts contained in raw or undercooked meat (meat route) (Dubey, 2021; Pinto-Ferreira et al., 2019). Primary infections in humans and small ruminants during gestation may also lead to vertical transmission by tachyzoites that reach the fetus/es through the placenta, causing severe harm such as congenital malformation, ocular disease, hydrocephaly, abortion, reabsorption, and neonatal death, among others (Innes et al., 2009; Koutsoumanis et al., 2018; Lindsay and Dubey, 2020; Stelzer et al., 2019). In this context, *T. gondii* is a perfect paradigm of a pathogen

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whose control should be achieved through a One Health approach (Djurković-Djaković et al., 2019).

Small domestic ruminants are highly susceptible to *T. gondii* infections. Indeed, *T. gondii* has been identified as one of the most common and important reproductive transmissible agents in ewes and goats (Stelzer et al., 2019), which translates to great economic losses for producers, with approximately 1.5 million lambs lost per year in Europe (Innes et al., 2009). In addition, *T. gondii* infections in small ruminants are of public health concern since the consumption of raw or undercooked sheep/lamb meat has been identified as a primary food source of infection (WHO, and FAO, 2014) and a risk factor associated with acute toxoplasmosis in humans (Odds Ratio = 3.6–3.9 (95% CI 1.3–9.8, 1.9–8.0)) (Belluco et al., 2017; Friesema et al., 2023). Furthermore, *T. gondii* has been identified as a high priority in meat inspections based on ranking biological hazards in small ruminants (EFSA, 2013a), with global pooled seroprevalence of 33.86% and 31.78% in sheep and goats, respectively (Ahaduzzaman and Hasan, 2022).

Serology is a valuable strategy for monitoring the infection with a public health commitment (EFSA, 2013b). Accordingly, serological techniques are the most commonly used tools for the diagnosis of *T. gondii* infections in small ruminants (Dubey et al., 2020a, 2020b). However, there are several issues that hamper the interpretation of serological results and that could lead to misdiagnosis. First, diagnostic performance and analytical specificity data are not always available, and the scarce number of comparative studies hinders access to updated information. Furthermore, cross-reactivity with closely related parasites is of major concern in small ruminants. For example, there is increased evidence of the relevance of *Neospora caninum* infection in sheep and the coexistence of *T. gondii* and *N. caninum* infections in flocks (Gharekhani et al., 2018; Gondim et al., 2017; González-Warleta et al., 2014; Sun et al., 2020; Villagra-Blanco et al., 2019). Second, a few limitations have been identified concerning the comparative studies carried out to date: a) the initial diagnostic performance of the evaluated techniques was not always specified, b) the majority of the studies arbitrarily defined an in-house technique as a reference test, c) the serum panel consisted of a low number of sera, initially characterized with a limited set of serological tests (Glor et al., 2013; Mainar-Jaime and Barberán, 2007; Mangili et al., 2009; Opsteegh et al., 2010), and d) commercial ELISA tests have barely been evaluated (within comparative studies) despite being very frequently used since not all laboratories are specialized in the development and validation of serological tests (Glor et al., 2013; Opsteegh et al., 2010).

Therefore, the objective of this study was to compare the performance of a wide panel of serological techniques routinely used for the detection of anti-*T. gondii* IgGs in small domestic ruminants. For this, a total of 840 well-characterized serum samples from experimentally and naturally infected animals were employed. Initially, two in-house tests, a Western blot (WB) and a lyophilized tachyzoite-based ELISA, were standardized. Second, the abovementioned in-house tests and the most frequently used and/or known commercial ELISA kits (IDScreen,

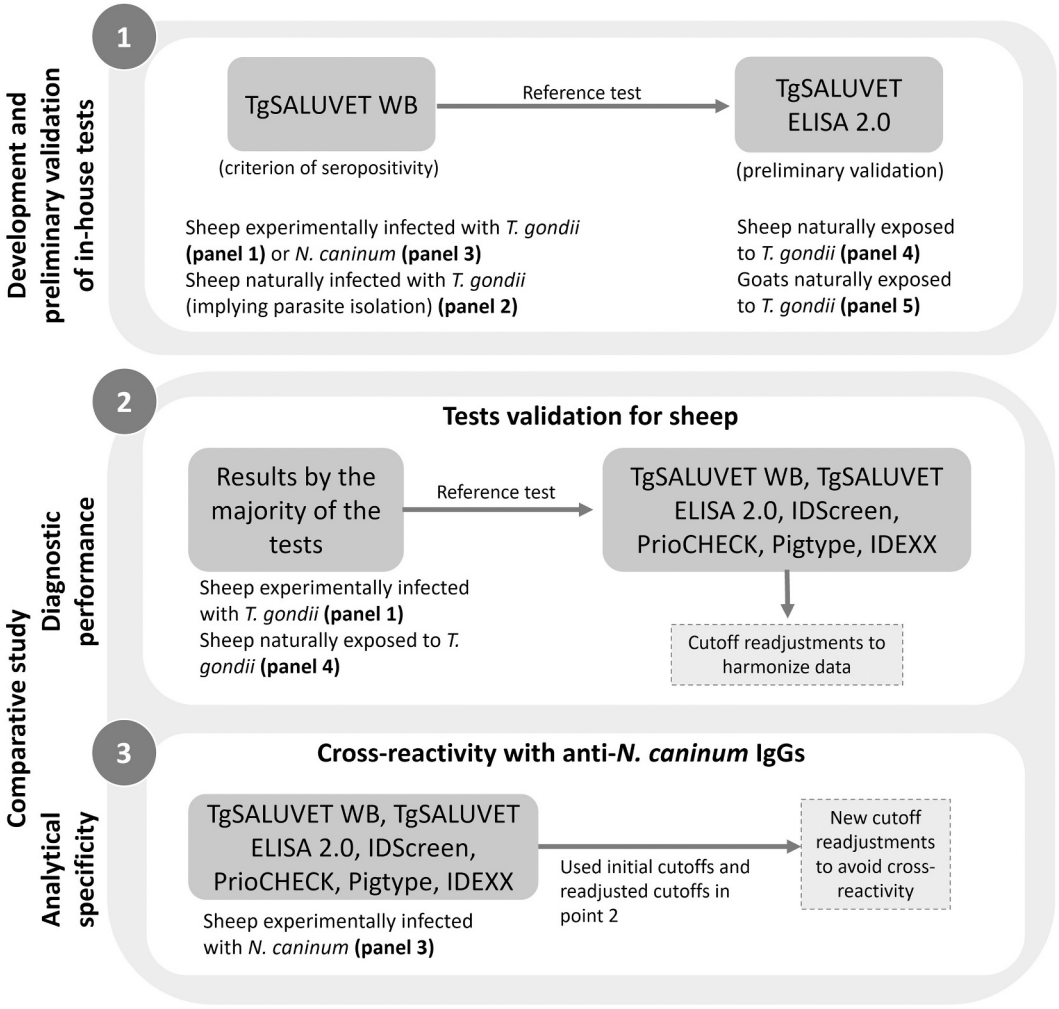

Fig. 1. Experimental design followed in this comparative study. A checklist of the items included in the standards for reporting diagnostic accuracy studies (Kostoulas et al., 2017) has been provided as Supplementary Table 1.

Table 1
Serological techniques used for the detection of anti-*Toxoplasma gondii* IgG in small domestic ruminants included in this comparative study.

Technique	Antigen	Host species	Sample type	Sample dilution	Cutoff	Diagnostic performance*	Reference
TgSALUVET WB	Tachyzoites	Sheep and goats	Serum	1/20	To be determined in this study	To be determined in this study	This study
TgSALUVET ELISA 2.0	Lyophilized tachyzoites	Sheep and goats	Serum	1/100	To be determined in this study	To be determined in this study	This study
IDScreen	P30	Ruminants, swine, dogs, cats	Serum, plasma and meat juice	Serum and plasma: 1/10, meat juice:1/2	S/P% ≤ 40, ≥ 50	Se = 100%, Sp = 100%[a]	Basso et al., 2022; Gazzonis et al., 2020; Mangili et al., 2009; Villagra-Blanco et al., 2018, 2019
PrioCHECK	Tachyzoite**	Small ruminants	Serum, plasma and meat juice	Serum and plasma: 1/100, meat juice: 1/10	PP ≥ 20	Not specified	Glor et al., 2013; Martínez-Rodríguez et al., 2020
Pigtype	Tachyzoite**	Cattle, sheep, goats, cats, dogs, foxes, pigs and wild boars	Serum, plasma (meat juice for pigs and wild boars)	Serum and plasma: 1/100, meat juice: 1/10	S/P ≥ 0.3	Not specified[b]	None
IDEXX	Not specified***	Small ruminants	Serum and plasma	1/400	S/P%<20, ≥ 30 (weak positive), ≥ 100 (positive)	Not specified	Mainar-Jaime and Barberán, 2007; Opsteegh et al., 2010

Se: sensitivity, Sp: specificity. S/P%, PP and RIPC = ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100, S/P = ([sample OD- negative control OD]/[positive control OD- negative control OD]).

* According to the information provided within the kit.

** No additional details provided.

*** Only specified that the microtiter plates are coated with inactivated antigen. The short protocol described by the manufacturer was followed for Pigtype.

[a] Based on 33 positive samples from France and 300 negative samples tested (the origin species was not specified). Intra-plate repeatability= 3–4%, inter-plate reproducibility= 4–6%.

[b] Microtiter plate coefficient of variation= 6.8%.

PrioCHECH, Pigtype and IDEXX) were compared. Finally, cross-reactivity with the anti-*N. caninum* IgGs was investigated in all tests.

2. Materials and methods

2.1. Experimental design and panels of sera

The experimental design that was followed and the tests that were evaluated in this study are shown in Fig. 1 and Table 1, respectively. The sera panels employed in each step of the workflow are detailed below:

2.1.1. Pattern of recognition of immunodominant antigens and criterion of seropositivity for TgSALUVET WB

Panel 1 ($n = 124$) consisted of samples from 14 Rasa Aragonesa breed sheep that had been orally inoculated with 10 *T. gondii* oocysts (TgSpSh1, genotype II) at 90 days of gestation, as well as sera from 6 noninoculated pregnant sheep. Sampling was carried out at −2, 3, 8, 12, and 14 days post-infection (dpi). In addition, serum samples from 9 of these animals (six infected and three noninfected sheep) were also collected at 21 and 27 dpi (Vallejo-Blanco et al., 2023). Samples collected prior to the infection tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a; Vallejo-Blanco et al., 2023) and were kept at −80 °C until analysis. Negative ($n = 20$, prior to the infection) and positive ($n = 9$, from 27 dpi) serum samples from this panel were used to define the pattern of recognition of *T. gondii* tachyzoite immunodominant antigens (IDAs) by TgSALUVET WB and the criterion of seropositivity. All the serum samples were also included in the comparative study of all the serological tests (see Section 2.3).

Panel 2 consisted of 17 sheep naturally infected that tested positive for *T. gondii* by a soluble antigen-based ELISA. In all these animals, *T. gondii* was isolated by mouse bioassay from myocardial tissue (Fernández-Escobar et al., 2020). These samples tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a) and were kept at −80 °C until use. This panel of sera was employed to define the pattern of recognition of *T. gondii* tachyzoite IDAs by TgSALUVET WB and the criterion of seropositivity.

Panel 3 was composed of 26 serum samples from sheep inoculated intravenously with *N. caninum* tachyzoites (Sánchez-Sánchez et al., 2018, 2021b). All of them tested positive for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a), with low (RIPC = 35–49, $n = 8$), medium (RIPC = 50–79, $n = 7$) and high (RIPC≥80, $n = 11$) IgG levels. This sera panel was used to test cross-reactivity between anti-*N. caninum* IgGs and *T. gondii* tachyzoite IDAs in TgSALUVET WB to define the criterion of seropositivity for TgSALUVET WB. It was also used in the comparative study to evaluate cross-reactivity in all the ELISA tests.

2.1.2. Development and initial validation of TgSALUVET ELISA 2.0

Panel 4 ($n = 239$) was composed of serum samples from sheep naturally exposed to *T. gondii*. These samples were collected at two slaughterhouses in Spain (Fernández-Escobar et al., 2020). All sera tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a) and were kept at −80 °C. This panel was used to develop and validate TgSALUVET ELISA 2.0. This panel was also included in the comparative study. The precision of TgSALUVET ELISA 2.0 was tested using TgSALUVET WB-positive (n = 11) and -negative (n = 11) serum samples from Panel 1.

Panel 5 ($n = 434$) was composed of serum samples from goats naturally exposed to *T. gondii*. These samples were collected from different flocks with a previous history of *T. gondii*-associated abortions. These samples tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a) and were kept at −80 °C until use. This panel was used to test the suitability of TgSALUVET ELISA 2.0 for goats, using TgSALUVET WB as a reference.

2.1.3. Comparative study of all serological tests

The sera from sheep experimentally and naturally infected with *T. gondii* were analyzed separately in the comparative study (Panels 1 and 4, respectively). In addition, the sera from sheep experimentally infected with *N. caninum* were included in this step to test cross-reactivity in all the ELISA tests (Panel 3).

2.2. Parasite culture and antigen production

Toxoplasma gondii tachyzoites of the ME49 strain were cultured in the VERO-81 cell line with a multiplicity of infection (MOI) of 4:1, parasite:cell, with DMEM (Dulbecco's Modified Eagle Medium – high glucose, Sigma®, Ref.6429) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Lonza, Ref. H317-745E). The FBS used tested negative for *T. gondii, Besnoitia besnoiti* and *N. caninum* to avoid cross-reactivity (García-Lunar et al., 2015). After 72 h, the culture was syringed 3 times through a 25G (0.5 × 16 mm) needle, and tachyzoites were purified using 3-μm Whatman® filters (Millipore, Ref. TSTP02500), quantified and centrifuged at 1350 xg for 15 min at 4 °C. Pellets of 1×10^8 tachyzoites were kept at −80 °C until use for TgSALUVET WB. For TgSALUVET ELISA 2.0, glass vials with 1×10^8 tachyzoites in 4 mL of phosphate-buffered saline (PBS) were stored at −80 °C until being lyophilized as specified in a previous study (García-Lunar et al., 2017; López-Ureña et al., 2023).

2.3. Serological techniques

The main characteristics of the serological tests evaluated in this comparative study are shown in Table 1.

2.3.1. TgSALUVET WB

Antigen preparation, as well as the electrophoresis and electrotransfer of proteins were carried out following the procedure described by Sánchez-Sánchez et al. (2019b), with a few changes mentioned below. Aliquots of 2×10^7 tachyzoites were subjected to a cold wet ultrasonic bath for 15 min, followed by a wet bath at 100 °C for 5 min, in loading buffer under reducing conditions (10% glycerol, 6.8 pH 50 mM TRIS, 2% SDS, 0.05% bromophenol blue and 100 mM DTT final concentration). Then, the content was transferred to a 15% polyacrylamide gel and then to a 0.2-μm nitrocellulose membrane (Bio Rad laboratories). Membranes were cut into strips (1–2 mm each) and placed on stands with individual rails. Samples diluted at 1/20 in blocking solutions (5% powdered skim milk 0.05% tris-buffered saline-Tween 20 (TBS-T)) were added in separate rails and incubated for 1.5 h at room temperature. After that, three washes with TBS-T, each for 5 min, were performed, and the secondary antibody was added diluted at 1/1000 in TBS-T (monoclonal anti-goat/sheep IgG antibody conjugated with peroxidase, Sigma, Ref. A9452). It was incubated and washed under the same conditions, including one additional wash with TBS. The bounded antibodies were revealed using 4-chloro-1-naphthol solution (Thermo Scientific, Ref. 34010) and stopped with ultra-pure water based on the reaction developed in the positive controls. The strips were scanned with the GS-800 Calibrated Densitometer (Bio-Rad) for further analysis. The positive and negative controls used were obtained from Panel 1.

The analysis of the pattern of recognition of immunodominant antigens (IDAs) was performed by two experienced operators to avoid bias. Herein, the antigens of 9–10, 18–20, 24–26, 30 and 37–40 kDa were considered IDAs based on previous studies performed with small ruminant serum samples and *T. gondii*-based Western blot tests (Wastling et al., 1994; Conde et al., 2001). The criterion of seropositivity was established based on the IDAs frequency and intensity of recognition (see Section 2.4).

2.3.2. TgSALUVET ELISA 2.0

The assay was carried out following a previous described procedure with a few modifications (López-Ureña et al., 2023). Ninety-six-well microtiter plates (Thermo Scientific, Fisher Brand Maxisorp®, MA, USA, Ref. 10554831) were coated with 1×10^5 lyophilized tachyzoites per well in 0.1 M carbonate buffer (pH 9.6) (100 μL/well) overnight at 4 °C. The plates were then washed three times with 0.05% PBS-Tween 20 (PBS-T) and blocked with 5% powdered skim milk PBS-T for 2 h at room temperature (300 μL/well). After that, 100 μL per well of sera controls and samples diluted at 1/100 in blocking solution was placed per well and incubated at 37 °C for 1 h. Three additional washes were performed under the same conditions, and 100 μL of diluted secondary antibody at 1/10000 in PBS-T was dispensed per well (monoclonal anti-goat/sheep IgG antibody conjugated with peroxidase, Sigma Ref. A9452) and incubated at 37 °C for 1 h. For the detection of bound antibodies, the plates were washed again, and 100 μL of ABTS® (Roche, 11684302001) was added per well. The reaction was stopped with 100 μL per well of 0.3 M oxalic acid, when the OD of the positive control was between 1 and 1.1. Data were normalized as relative index percentage (RIPC) with the following formula: ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100. Here, the same positive and negative controls employed for TgSALUVET WB were used.

2.3.3. Commercial ELISA tests

Four commercially available ELISA tests used to detect anti-*T. gondii* IgGs in small ruminants were included in this comparative study: ID Screen® Toxoplasmosis Indirect Multispecies (Innovative Diagnostics, France); PrioCHECK® Toxoplasma Ab SR (Prionics, Switzerland); Pigtype® Toxoplasma Ab (Indical Bioscience, Germany) and IDEXX Toxotest Ab (IDEXX Laboratories, Inc., the United States), named IDScreen, PrioCHECK, Pigtype and IDEXX, respectively. The manufacturers' protocols were followed, and the characteristics of the ELISA tests are summarized in Table 1.

2.4. Data analysis

The frequency and intensity of IDAs recognition were used to define the criterion of seropositivity for TgSALUVET WB. The intensity of recognition was classified as high (+++), medium (++) or low (+). The frequency of the recognition of each IDA corresponding to the different panels of sera employed was compared by a contingency test (Fisher's exact test). This analysis was performed with GraphPad Prism, version 8.0.1.

To determine the precision of TgSALUVET ELISA 2.0, four replicates of each serum sample were run within a plate, and three different plates were analyzed in parallel. The following formula was used to determine the intraplate coefficient of variation (CV): mean ([standard deviation of the three replicate ODs/mean of the three replicate ODs] x 100). The interplate CV was determined as follows: mean ([standard deviation of the OD mean of each sample from each plate/mean of the OD mean of each sample from each plate] x 100). Coefficients of variation below 20% were interpreted as acceptable (Jacobson, 1998). Diagnostic performance and preliminary cutoff were estimated by a nonparametric two-graph receiver operating characteristic (TG-ROC) analysis with SigmaPlot 12.0 Software, and the WinEpi platform (Thrusfield et al., 2001) (http://www.winepi.net/), using TgSALUVET WB as reference test.

For TG-ROC analyses, the result obtained by the majority of the tests (four out of six techniques) was regarded as a reference in the comparative study. If a serum sample was positive by three tests and negative by the other three tests, it was considered doubtful and was excluded from the analysis. After that, an agreement test, expressed as *kappa* values, was performed using the WinEpi platform with a confidence level of 95%.

The kinetics of anti-*T. gondii* IgGs was also studied for all tests using serum Panel 1. Significant differences between noninfected and infected sheep within sampling days were analyzed with a mixed-effects analysis, followed by Sidak's multiple comparison test if applicable, using GraphPad Prism, version 8.0.1. Sphericity was not assumed, and the

Geisser-Greenhouse correction was automatically applied when necessary.

Fisher's exact test was performed for each ELISA to determine if the presence or absence of anti-*N. caninum* IgGs had a significant influence on the number of false-positive results in *T. gondii*-based ELISA tests.

Significant differences were considered when *P* values were lower than 0.05.

3. Results

3.1. Pattern of recognition of immunodominant antigens and criterion of positivity for TgSALUVET WB

The 18–20, 24–26, 30 and 37–40 kDa IDAs were recognized with high intensity for 100% of sheep experimentally infected with *T. gondii* at 27 dpi (Panel 1), as well as for all the sheep naturally infected with *T. gondii* (Panel 2), while the 9–10 kDa IDA was recognized with high and medium intensity for 100% and 82.4% of the sheep from Panel 1 (at 27 dpi) and Panel 2, respectively (Table 2, Fig. 2). Remarkably, the 30 and 37–40 kDa IDAs were recognized with medium and low intensity for 90% and 40% of the noninfected sheep, respectively. Furthermore, for all sheep experimentally infected with *N. caninum* (Panel 3) the 30 and 37–40 kDa IDAs were recognized with high intensity, and, respectively, for 30.8% and 11.5% of sheep experimentally infected with *N. caninum* the 18–20 and 24–26 kDa IDAs were recognized with low intensity, whereas the 9–10 kDa IDA were recognized for none of the sheep experimentally infected with *N. caninum*. Nonsignificant differences were observed in the frequency of recognition of the 30 kDa antigen between the noninfected sheep and the sheep infected with either *T. gondii* or *N. caninum*. Moreover, the 9–10, 18–20 and 24–26 kDa IDAs showed significant differences in the frequency of recognition between sheep naturally infected with *T. gondii* and sheep experimentally infected with *N. caninum* (Table 2). Thus, based on these results, the recognition of the 9–10 and/or 24–26 kDa antigens together with two other IDAs (18–20, 30 and 37–40 kDa) with medium-high intensity was established as a criterion of seropositivity. Accordingly, none of the sheep experimentally infected with *N. caninum* tested positive by TgSALUVET WB (Table 2, Fig. 2). Seroconversion was recorded from 14 dpi onward in Panel 1 based on TgSALUVET WB.

3.2. TgSALUVET ELISA 2.0 initial validation

The mean CV values for the intra and interplate repeatability for TgSALUVET ELISA 2.0 were 4.59 (standard deviation (SD) = 0.02) and 9.48 (SD = 0.05), respectively. This ELISA test showed a high performance with an AUC of 0.99 for the preliminary cutoff defined, RIPC≥19.18, with 93% Se and 96% Sp based on the TG-ROC analysis from sheep naturally infected with *T. gondii* (Panel 4) and using TgSALUVET WB as a reference test. Seroconversion was recorded from 21 dpi onward in Panel 1 when applying the defined cutoff. Furthermore, TgSALUVET ELISA 2.0 was suitable for goats (Panel 5), showing 100% Se and 98% Sp when using the abovementioned cutoff value and TgSALUVET WB as a reference test.

3.3. Comparative study

All the ELISA tests showed high Se and Sp values when sera from experimentally infected sheep were analyzed (Panel 1) (Se and Sp values equal to or higher than 94%, AUC = 1; Fig. 3A, no doubtful results were observed by the majority of the tests). TgSALUVET WB showed 100% Se and 94% Sp. The highest diagnostic performance corresponded to IDScreen and TgSALUVET ELISA 2.0, followed by PrioCHECK. In general terms, moderate to perfect agreements were also recorded (k = 0.78–1.00), and after cutoff readjustments, Se, Sp and *kappa* values reached almost perfect values (Fig. 3A; Table 3).

Seroconversion was recorded from 21 dpi when the initial ELISA test cutoff values were considered (Fig. 4). Seroconversion was detected earlier when the mean levels of anti-*T. gondii* IgGs were compared between noninfected and infected sheep during the experimental assay, with a significant increase in IgG levels starting at 12 dpi with PrioCHECK and TgSALUVET ELISA 2.0, 14 dpi with IDScreen and Pigtype, and 21 dpi with IDEXX (Fig. 4).

All the ELISA tests also showed excellent diagnostic performance when analyzing sera from sheep naturally infected (Panel 4) (Se and Sp values equal to or higher than 95%, AUC = 1; Fig. 3B, and only 4 out of 239 samples that resulted doubtful based on the results obtained by the majority of the tests), except for PrioCHECK, which showed 83% Sp. Pigtype was the test that showed the best diagnostic performance, followed by IDScreen and TgSALUVET ELISA 2.0. TgSALUVET WB presented 94% Se and 97% Sp. The *kappa* values are shown in Table 3 (k = 0.73–0.98), with PrioCHECK presenting the lowest values (k = 0.73–0.80). Both the performance and agreement of the tests improved when their cutoff values were readjusted based on the TG-ROC analyses, with 98–100% Se and Sp and 0.84–1.00 *kappa* values (Fig. 3B, Table 3), except for TgSALUVET WB with 93% Se. The cutoff, Se, Sp and AUC values are specified for each ELISA in Fig. 3B.

All the ELISA tests showed false-positive results with the anti-*N. caninum* IgGs (Panel 3) when using the cutoff values suggested by the manufacturers, as follows: IDScreen (14/26), PrioCHECK (13/26), Pigtype (11/26), IDEXX (6/26) and TgSALUVET ELISA 2.0 (6/26) (Table 4). False-positive results remained when the readjusted cutoff values were applied (Table 4). There was a significant association between the number of false-positive results and the presence of anti-*N. caninum* IgGs when using both the initial and readjusted cutoff values (Table 4). Consequently, new cutoff readjustments were required for all the ELISA tests to avoid false-positive results, as detailed in Table 4.

When both readjusted cutoff values were applied to TgSALUVET ELISA 2.0 using the goat serum panel (Panel 5), whether it was the one meant to obtain comparable data among tests or the one targeted to avoid cross-reactivity with the anti-*N. caninum* IgGs, TgSALUVET ELISA 2.0 maintained excellent performance, with 100% Se and 94% Sp and 99% Se and 99% Sp, respectively.

Table 2
Frequency and intensity of recognition of *Toxoplasma gondii* tachyzoite immunodominant antigens (IDAs) by TgSALUVET WB.

IDAs	Noninfected sheep	*T. gondii* infected sheep		*N. caninum* infected sheep	C vs. D Significance
	(A) n = 20 Frequency/intensity	(B) n = 9 Frequency/intensity/significance	(C) *n* = 17 Frequency/intensity/significance	(D) *n* = 26 Frequency/intensity/significance	
37–40	40/ +	100/ +++/ **	100/ +++/ ****	100/ +++/ ****	ns
30	90/ ++	100/ +++/ ns	100/ +++/ ns	100/ +++/ ns	ns
24–26	0	100/ +++/ ****	100/ +++/ ****	11.5/ +/ ns	****
18–20	0	100/ +++/ ****	100/ +++/ ****	30.8/ +/ **	****
9–10	0	100/ +++/ ****	82.4/ ++/ ****	0/ ns	****

A: sera collected prior to the infection (Panel 1). B: sera collected at 27 days post-infection (Panel 1). C: sera from Panel 2. D: sera from Panel 3. Frequency: percentage (%). Intensity: +++ (high), ++ (medium), + (low), mean within each group. For statistical analyses, columns B, C, D were compared to column A or column C to column D. ns: no significant differences. Significant differences are represented as follows: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$.

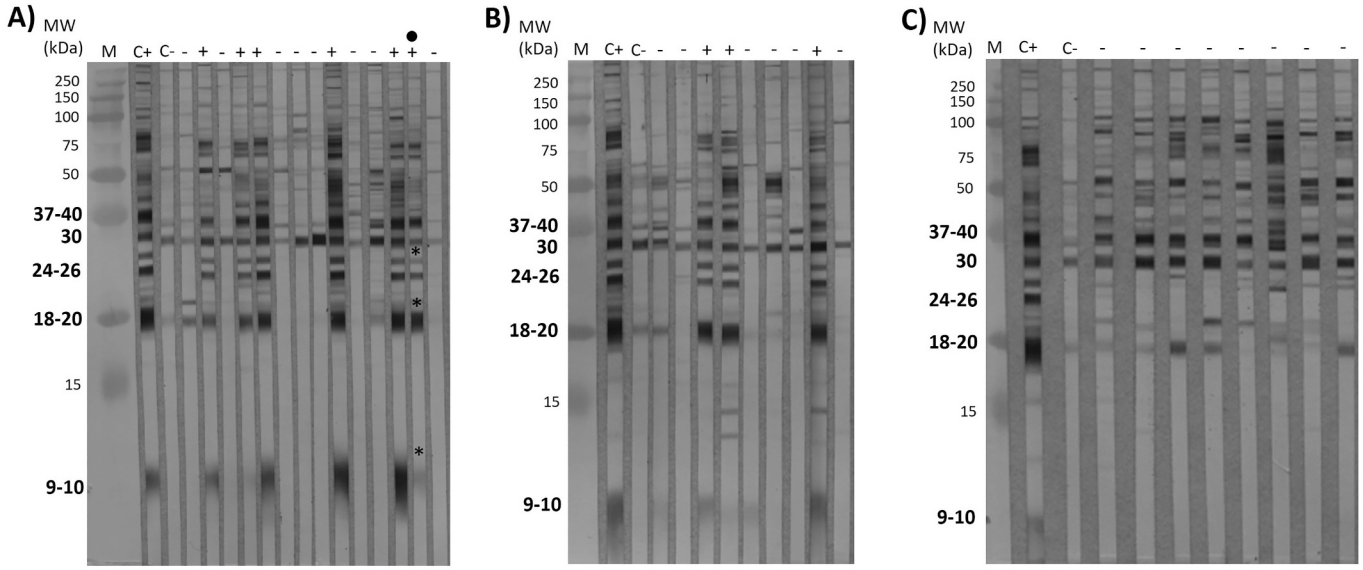

Fig. 2. Recognition of *Toxoplasma gondii* tachyzoite antigens by TgSALUVET WB. **(A)** Sera from *T. gondii* experimentally infected sheep; **(B)** Sera from *T. gondii* naturally infected sheep. *Toxoplasma gondii* was isolated by mouse bioassay from myocardial tissue from all positive samples; **(C)** Sera from *Neospora caninum* experimentally infected sheep; MW: molecular weight. IDAs are marked in bold letters. M: molecular weight marker, C+: *T. gondii* positive control serum. C-: *T. gondii* negative control serum. Strips with a "+" or "-" were classified as positive or negative for *T. gondii*, respectively. In the strip identified with "●", antigens with a "*" above show low intensity for the 10 kDa band, medium intensity for the 28 kDa band and high intensity for the 18–20 kDa band.

4. Discussion

A comprehensive comparative study was carried out in terms of the number of serological tests included and the range of reference serum panels employed. We have developed and validated two in-house serological techniques that were later included in the comparative study together with four commercially available ELISA tests. All the tests showed good diagnostic performance and agreement when using sera from both experimental and natural infections, although further readjustments improved Se and Sp values. However, all the ELISA tests showed a high number of false-positive results when *N. caninum*-positive sera were tested. Thus, additional cutoff value readjustment was suggested based on the epidemiological scenario.

The two in-house tests developed herein showed good diagnostic performance and can be indistinctly employed with ovine or caprine sera. For TgSALUVET WB, we established an exhaustive and restrictive criterion of seropositivity, and the remarkable cross-reactions found between *T. gondii* antigens and anti-*N. caninum* antibodies were considered. López-Ureña et al. (2023) recently defined a criterion of positivity for pig sera that consisted of the recognition of three out of eight IDAs (9–10, 19, 25, 28, 30, 33–35, 43–45, and 69 kDa). Despite the similarities found between both studies with commonly reported IDAs, the criterion established with pig sera was less restrictive, including additional IDAs, which could be explained by the fact that cross-reactions were not studied. However, considerations of cross-reactivity should not be discarded since most of such IDAs were also recognized prior to infection in pigs, except for the 9–10 and 69 kDa IDAs, and cross-reactions with other apicomplexan parasites relevant in this species were not investigated (e.g., *Cystoisospora suis*, *Sarcocystis* spp.). Hebbar et al. (2022) also studied cross-reactivity between anti-*N. caninum* IgGs and *T. gondii* antigens in goats by WB and reported minimal cross reactivity at 1/200 serum dilution. However, whether sensitivity could be compromised was not studied. Herein, the most problematic IDAs in terms of cross-reactivity were those corresponding to 30 and 37–40 kDa; thus, the unique recognition of these IDAs cannot be considered a criterion of seropositivity. Cross-reactivity between anti-*N. caninum* IgGs and *T. gondii* protein SAG1, which has a predictable molecular weight of 30 kDa, was previously described for other *T. gondii*-based serological tests, such as IDScreen (Sánchez-Sánchez et al., 2021b) and a SAG1-GRA8 chimeric antigen-based time-resolved fluorescence immunoassay (Huertas-López et al., 2021). The 30 kDa antigen has been described as an IDA together with others: 11–13, 18, 24, 34 and 42 kDa antigens in sheep sera (Wastling et al., 1994) and 28 and 34 kDa (Conde et al., 2001) or 12, 17, 23, 32, 55 and 75 kDa antigens in goats sera (Hebbar et al., 2022). Thus, it should be considered together with more specific IDAs in the criterion of seropositivity. In this regard, the 9–10 kDa IDA was the most specific antigen, as similarly reported by López-Ureña et al. (2023)).

TgSALUVET ELISA 2.0 also showed high initial diagnostic performance for both sheep and goats, as corroborated later in the comparative study. These results were expected, as lyophilized tachyzoites have been previously employed in ELISA tests that were highly sensitive and specific (García-Lunar et al., 2017; López-Ureña et al., 2023). This preliminary validation was carried out considering TgSALUVET WB as reference since this test mitigate the issue of false-positive reactors based on the specific criterium of seropositivity established herein. Although IFAT has been regarded as reference on several occasions, this assay may yield false-positive results and is further hampered by subjectivity in result interpretation as it is operator-dependent (Campero et al., 2018). Similarly, MAT was not deemed suitable as reference test due to its susceptibility to cross-reactivity issues with closely related Apicomplexan parasites (Mazuz et al., 2018).

We have compared a wide set of serological techniques, including in-house assays and commercial ELISA tests. This is the first time that the performance of Pigtype has been studied, whereas IDScreen, PrioCHECK and IDEXX had already been included in previous comparative studies. Herein, all tests showed good to excellent diagnostic performance and agreement regardless of the serum panel tested, with the exception of PrioCHECK, which presented a slight decrease in Sp and *K* values with respect to the other tests when analyzing serum samples from sheep naturally infected with *T. gondii*. However, a further improvement of all the ELISA tests was possible by readjusting their cutoff values, obtaining better performance and more harmonized results. Slight differences in terms of time of seroconversion were observed in the ELISA tests with sera from experimental infections, with time of seroconversion being earlier when considering a significant increase of IgG levels between

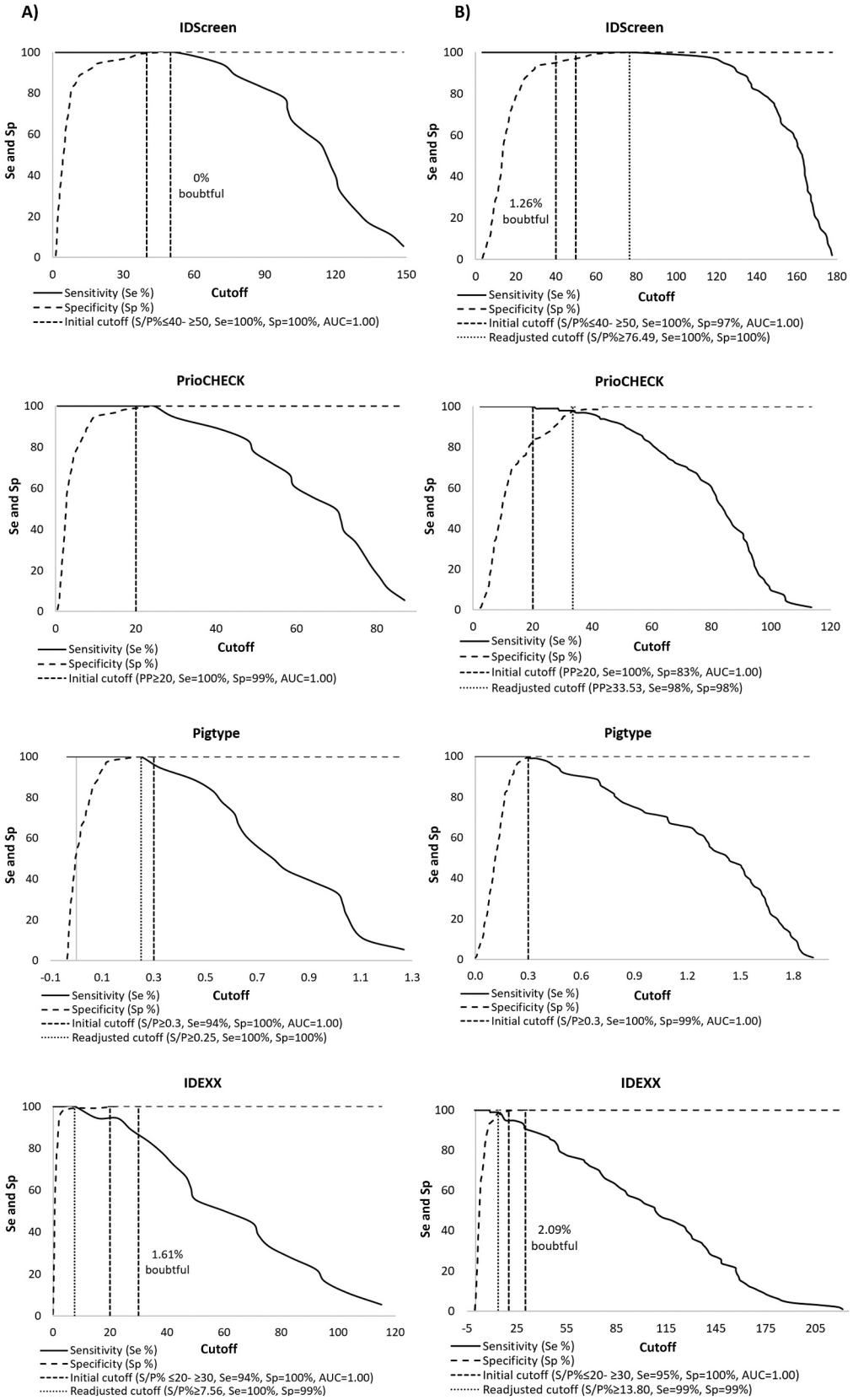

Fig. 3. TG-ROC graphs of four commercial and one in-house ELISA tests based on the reference criterion, using serum samples from sheep experimentally (Panel 1) **(A)** or naturally (Panel 4) **(B)** infected with *Toxoplasma gondii.* Cutoff, sensitivity (Se), specificity (Sp), and the area under the curve (AUC) values are shown for each ELISA test.

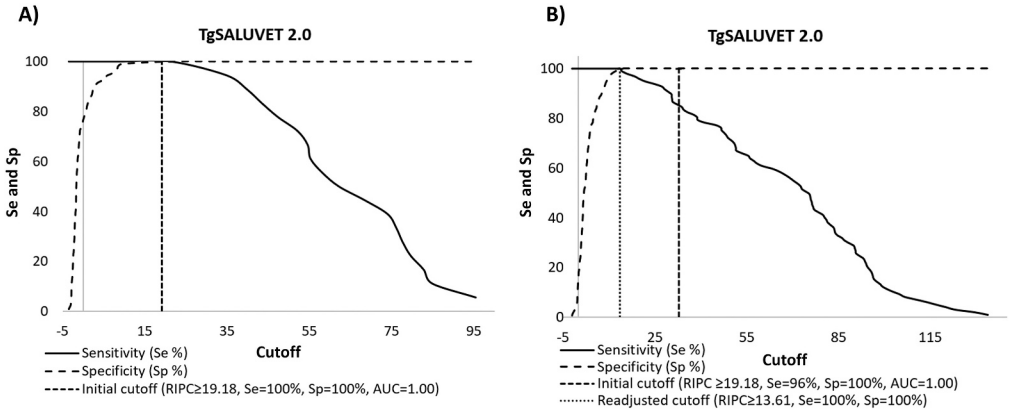

Fig. 3. (*continued*).

Table 3
Agreement between serological techniques using sera from *Toxoplasma gondii*-infected sheep before **(b)** and after **(a)** the TG-ROC analyses and cutoff value readjustment.

Experimental infections (Panel 1)	IDScreen		PrioCHECK		Pigtype		IDEXX		TgSALUVET ELISA 2.0		TgSALUVET WB	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	0.97	0.97	0.97	1.00	0.96	0.97	1.00	1.00	0.83	0.83
PrioCHECK	0.97	0.97	1.00	1.00	0.94	0.97	0.93	1.00	0.97	0.97	0.86	0.86
Pigtype	0.97	1.00	0.94	0.97	1.00	1.00	0.96	0.97	0.97	1.00	0.80	0.83
IDEXX	0.96	0.97	0.93	1.00	0.96	0.97	1.00	1.00	0.96	0.97	**0.78**	0.86
TgSALUVET ELISA 2.0	1.00	1.00	0.97	0.97	0.97	1.00	0.96	0.97	1.00	1.00	0.83	0.83
TgSALUVET WB	0.83	0.83	0.86	0.86	0.80	0.83	**0.78**	0.86	0.83	0.83	1.00	1.00

Natural Infections (Panel 4)	IDScreen		PrioCHECK		Pigtype		IDEXX		TgSALUVET ELISA 2.0		TgSALUVET WB	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	**0.79**	0.95	0.96	0.99	0.89	0.97	0.91	1.00	0.84	0.88
PrioCHECK	**0.79**	0.95	1.00	1.00	0.80	0.94	**0.74**	0.95	**0.75**	0.95	**0.73**	0.84
Pigtype	0.96	0.99	0.80	0.94	1.00	1.00	0.92	0.96	0.93	0.99	0.87	0.87
IDEXX	0.89	0.97	**0.74**	0.95	0.92	0.96	1.00	1.00	0.98	0.97	0.88	0.89
TgSALUVET ELISA 2.0	0.91	1.00	**0.75**	0.95	0.93	0.99	0.98	0.97	1.00	1.00	0.89	0.88
TgSALUVET WB	0.84	0.88	**0.73**	0.84	0.87	0.87	0.88	0.89	0.89	0.88	1.00	1.00

The lowest *kappa* values (below 0.80) based on the cutoff values suggested by the manufacturers are marked in bold letters.

noninfected and infected sheep, from 12 to 21 dpi depending on the ELISA test, compared to seropositivity based on their initial cutoff values, from 21 dpi in all the ELISA tests, as similarly observed with PrioCHECK by Glor et al. (2013). Differences can be also explained by the different experimental designs followed (e.g., dose, strain) since seroconversion can vary between 12 dpi and 21 dpi even if the same test/antigen is employed as reported by other authors when using a *T. gondii* soluble antigen-based ELISA test (Castaño et al., 2014, 2019; Sánchez-Sánchez et al., 2019a).

Regarding the results obtained with sera from naturally infected sheep, IDScreen showed higher performance vs. PrioCHECK, which showed lower Sp compared with previous studies. Mangili et al. (2009) reported 83% Se for IDScreen when compared to an IFAT. In contrast, PrioCHECK showed 93–100% Se and Sp values when compared to an in-house IFAT and a commercial indirect hemagglutination test (ELI.H.A Toxo, ELITech Group, Switzerland) (Glor et al., 2013). These differences could be explained by the different sera and tests used as references. Furthermore, IDEXX showed similar results, as reported by other researchers, with 85–91% Se and 97–98% Sp (Mainar-Jaime and Barberán, 2007) or 91–92% Se and 97–99% Sp (Opsteegh et al., 2010), with both studies based on a Bayesian approach.

Finally, the cross-reactivity observed between anti-*N. caninum* antibodies and a high number of *T. gondii* antigens by TgSALUVET WB was reflected in the results obtained with all the ELISA tests that showed an elevated proportion of false-positive results (15–65%) regardless of the cutoff value employed. Accordingly, additional cutoff values were proposed to avoid false-positive results. The study of cross-reactions between anti-*N. caninum* antibodies and *T. gondii* antigens is highly recommended to obtain accurate results since *N. caninum* is an Apicomplexan parasite closely related to *T. gondii*, both with an orthologous cluster of proteins (Lorenzi et al., 2016). Cross-reactivity between *T. gondii* and *N. caninum* was observed as early as 1994 (Bjerkas et al., 1994) and has been continuously documented in different studies (Gondim et al., 2017; Huertas-López et al., 2021; Nishikawa et al., 2002; Sánchez-Sánchez et al., 2021b). Furthermore, *N. caninum* has also been identified as an important cause of reproductive failure in small ruminants (Moreno et al., 2012; Sánchez-Sánchez et al., 2018, 2021a), and *N. caninum* and *T. gondii* coinfections in small ruminant flocks have been noted (Moreno et al., 2012; Rossi et al., 2011; Sun et al., 2020; Villagra-Blanco et al., 2019). However, cross-reactivity with other closely related parasites, such as *Hammondia* spp. and *Sarcocystis* spp., cannot be ruled out since antibodies against such parasites were not tested prior to the infection in the sera panel employed. The existence of false-positive results with IDScreen has already been reported (Sánchez-Sánchez et al., 2021b), but this troubleshooting seems to be common to all the ELISA tests evaluated. Whether the nature or type of antigen might influence the results should be clarified since the antigens used in the tests evaluated were either a recombinant SAG1 protein, whole-tachyzoite

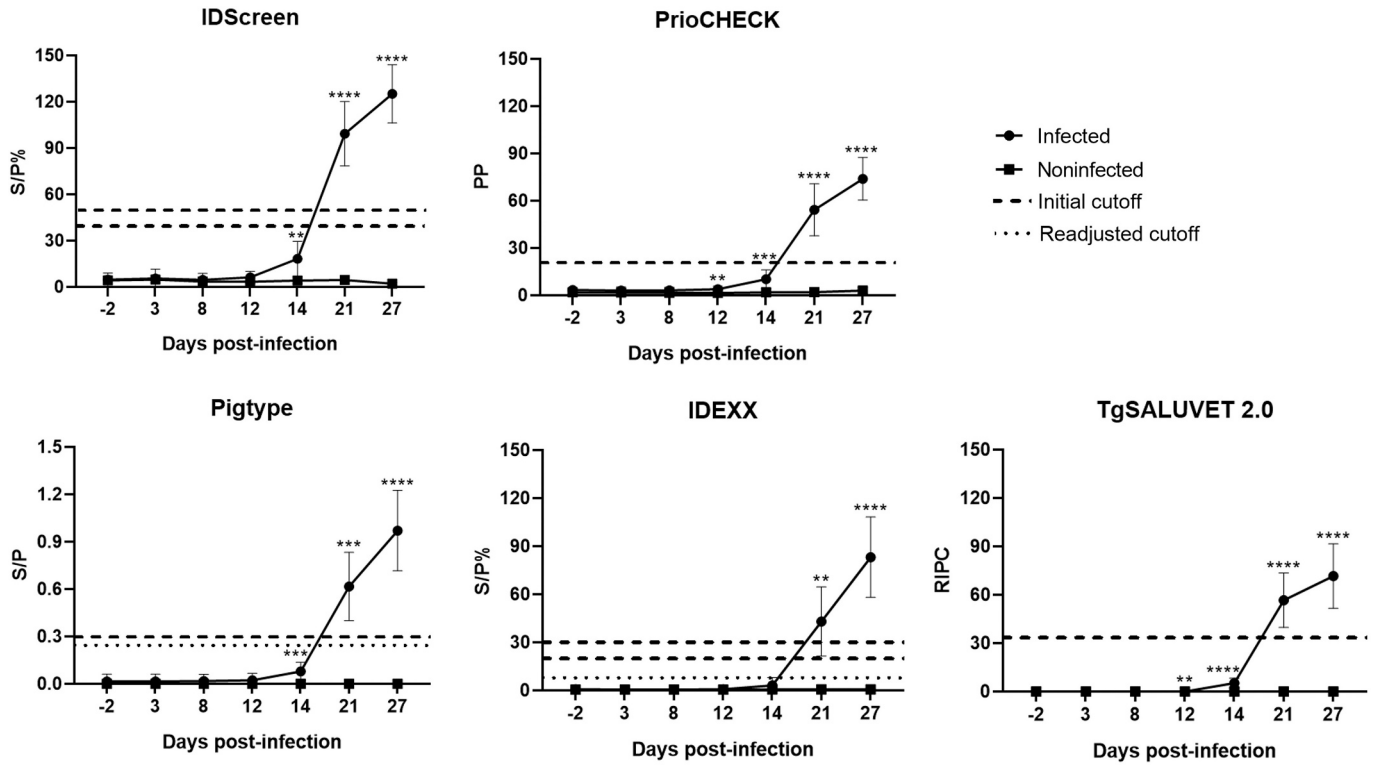

Fig. 4. Kinetics of anti-*Toxoplasma gondii* IgGs by all the ELISA tests using serum samples from sheep experimentally infected with *Toxoplasma gondii* oocysts (Panel 1). Significant differences were analyzed within sampling days between noninfected and infected groups for each ELISA test and were identified as follows (Sidak's multiple comparisons tests): * = $P < 0.05$, **: $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$.

Table 4
Cross-reactivity between anti-*Neospora caninum* IgG and *Toxoplasma gondii* antigens in *Toxoplasma gondii*-based ELISA tests according to the different cutoff values employed.

ELISA tests	Positive-*N. caninum* serum samples (Panel 3, n = 26) that tested positive by *T. gondii*-based ELISA tests							
	Initial cutoff values			First readjusted cutoff values			Second readjusted cutoff values	
	Cutoff value*	n	*P*	Cutoff value*	n	*P*	Cutoff**	n
IDScreen (S/P%)	≤ 40, ≥ 50	14	<0.0001	≥ 76.49	4	0.1213	≥ 99.23	0
PrioCHECK (PP)	≥ 20	13	0.0001	≥ 33.53	5	0.0593	≥ 53.91	0
Pigtype (S/P)	≥ 0.3	11	0.0010	N/A	11	0.0010	≥ 0.48	0
IDEXX (S/P%)	<20, ≥ 30	6	0.0287	≥ 13.80	17	<0.0001	≥ 69.08	0
TgSALUVET 2.0 (RIPC)	19.18	6	0.0287	≥ 13.61	10	0.0024	≥ 32.21	0

N/A: do not apply. S/P%, PP and RIPC = ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100, S/P = ([sample OD- negative control OD]/[positive control OD- negative control OD]).

* The corresponding diagnostic performance are specified in Fig. 3.

** The diagnostic performance parameters were as follows: IDScreen= 99% Se, 100% Sp; PrioCHECK= 88% Se, 100% Sp; Pigtype= 94% Se, 100% Sp; IDEXX= 72% Se, 100% Sp; TgSALUVET ELISA 2.0= 86% Se, 100% Sp. The negative serum samples used as control in this analysis (Panel 1 prior to the infection, n= 20) tested negative in all ELISA tests when applying the initial or both readjusted cutoff values.

extract or lyophilized tachyzoites. It has been claimed that recombinant or chimeric antigens might be more specific (Liyanage et al., 2021). For example, Holec-Gąsior et al. (2014) reported the absence of false-positive results with an ELISA based on *T. gondii* GRA1, P22 and ROP1 recombinant proteins or on four tetravalent chimeric proteins (AMA1N-SAG2-GRA1-ROP1, AMA1C-SAG2-GRA1-ROP1, AMA1-SAG2-GRA1-ROP1, and SAG2-GRA1-ROP1-GRA2) when testing *N. caninum*-positive sheep sera. However, the characteristics of these sera were not mentioned (e.g., experimental or natural infection origin and IgG levels). These results contrast with the results presented herein since IDScreen, which initially showed the highest number of false-positive results, was the only ELISA based on a recombinant protein, P30 (SAG1).

In summary, all the serological tests compared herein are accurate enough for serological diagnosis of *T. gondii* infection in small ruminants. However, ideally, readjusted cutoff values are recommended for a higher diagnostic performance. Moreover, cross-reactions between anti-*N. caninum* antibodies and *T. gondii* antigens harm the analytical specificity more than initially thought and should be considered when defining a criterion of seropositivity by WB and when using such serological tests for diagnostic purposes. To discard false-positive reactors, a practical recommendation for diagnostic laboratories could be the use of both readjusted cutoff values estimated with sera from naturally infected sheep, and those samples with results in between (doubtful results), should be further analyzed by a specific and confirmatory WB test. The employment of well-characterized sera, including false-positive reactors, should be an essential requirement for future method developments or validation studies carried out in small ruminants.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.105052.

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Declaration of Competing Interest

Authors declare no conflict of interest.

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References

Ahaduzzaman, M., Hasan, T., 2022. Seroprevalence of *Toxoplasma gondii* infection in sheep and goats from different geographical regions of the world: systematic review and meta-analysis. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.14753.

Basso, W., Holenweger, F., Schares, G., Müller, N., Campero, L.M., Ardüser, F., Moore-Jones, G., Frey, C.F., Zanolari, P., 2022. *Toxoplasma gondii* and *Neospora caninum* infections in sheep and goats in Switzerland: Seroprevalence and occurrence in aborted foetuses. Food Waterborn. Parasitol. 28 https://doi.org/10.1016/J.FAWPAR.2022.E00176.

Belluco, S., Simonato, G., Mancin, M., Pietrobelli, M., Ricci, A., 2017. *Toxoplasma gondii* infection and food consumption: a systematic review and meta-analysis of case-controlled studies. Crit. Rev. Food Sci. Nutr. 58 (18), 3085–3096. https://doi.org/10.1080/10408398.2017.1352563.

Bjerkas, I., Jenkins, M.C., Dubey, J.P., 1994. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. Clin. Diagn. Lab. Immunol. 1 (2), 214–221. https://doi.org/10.1128/cdli.1.2.214-221.1994.

Campero, L.M., Moreno-Gonzalo, J., Venturini, M.C., Moré, G., Dellarupe, A., Rambeaud, M., Echaide, I.E., Valentini, B., Campero, C.M., Moore, D.P., Cano, D.B., Fort, M., Mota, R.A., Serrano-Martínez, M.E., Cruz-Vázquez, C., Ortega-Mora, L.M., Álvarez-García, G., 2018. An Ibero-American inter-laboratory trial to evaluate serological tests for the detection of anti-*Neospora caninum* antibodies in cattle. Trop. Anim. Health Prod. 50 (1), 75–84. https://doi.org/10.1007/S11250-017-1401-X.

Castaño, P., Fuertes, M., Ferre, I., Fernández, M., del Ferreras, M.C., Moreno-Gonzalo, J., González-Lanza, C., Katzer, F., Regidor-Cerrillo, J., Ortega-Mora, L.M., Pérez, V., Benavides, J., 2014. Placental thrombosis in acute phase abortions during experimental *Toxoplasma gondii* infection in sheep. Vet. Res. 45 (1), 9. https://doi.org/10.1186/1297-9716-45-9.

Castaño, P., Fernández, M., Regidor-Cerrillo, J., Fuertes, M., Horcajo, P., Ferre, I., Ferreras, M.C., Ortega-Mora, L.M., Pérez, V., Benavides, J., 2019. Peripheral and placental immune responses in sheep after experimental infection with *Toxoplasma gondii* at the three terms of gestation. Vet. Res. 50 (1), 66. https://doi.org/10.1186/s13567-019-0681-8.

Conde, M., Molina Caballero, J.M., Rodríguez-Ponce, E., Ruiz, A., González, J., 2001. Analysis of IgG response to experimental infection with RH *Toxoplasma gondii* in goats. Comp. Immunol. Microbiol. Infect. Dis. 24 (3), 197–206. https://doi.org/10.1016/S0147-9571(00)00027-8.

Djurković-Djaković, O., Dupouy-Camet, J., van der Giessen, J., Dubey, J.P., 2019. Toxoplasmosis: overview from a one health perspective. Food Waterborn. Parasitol. 15, e00054 https://doi.org/10.1016/j.fawpar.2019.e00054.

Dubey, J.P., 2021. Outbreaks of clinical toxoplasmosis in humans: five decades of personal experience, perspectives and lessons learned. Parasit. Vectors 14, 263. https://doi.org/10.1186/s13071-021-04769-4.

Dubey, J.P., Murata, F.H.A., Cerqueira-Cézar, C.K., Kwok, O.C.H., 2020a. Public health and economic importance of *Toxoplasma gondii* infections in goats: the last decade. Res. Vet. Sci. 132, 292–307. https://doi.org/10.1016/j.rvsc.2020.06.014.

Dubey, J.P., Murata, F.H.A., Cerqueira-Cézar, C.K., Kwok, O.C.H., Su, C., 2020b. Economic and public health importance of *Toxoplasma gondii* infections in sheep: 2009–2020. Vet. Parasitol. 286, 109195 https://doi.org/10.1016/j.vetpar.2020.109195.

EFSA, 2013a. Scientific opinion on the public health hazards to be covered by inspection of meat from sheep and goats. EFSA J. 11 (6) https://doi.org/10.2903/J.EFSA.2013.3265.

EFSA, 2013b. Technical specifications on harmonised epidemiological indicators for biological hazards to be covered by meat inspection of domestic sheep and goats. EFSA J. 11 (6), 3277. https://doi.org/10.2903/j.efsa.2013.3277.

Fernández-Escobar, M., Calero-Bernal, R., Benavides, J., Regidor-Cerrillo, J., Guerrero-Molina, M.C., Gutiérrez-Expósito, D., Collantes-Fernández, E., Ortega-Mora, L.M., 2020. Isolation and genetic characterization of *Toxoplasma gondii* in Spanish sheep flocks. Parasit. Vectors 13 (1), 1–14. https://doi.org/10.1186/S13071-020-04275-Z/FIGURES/2.

Friesema, I.H.M., Hofhuis, A., Hoek-van Deursen, D., Jansz, A.R., Ott, A., van Hellemond, J.J., van der Giessen, J., Kortbeek, L.M., Opsteegh, M., 2023. Risk factors for acute toxoplasmosis in the Netherlands. Epidemiol. Infect. 151, e95 https://doi.org/10.1017/S0950268823000808.

García-Lunar, P., Moré, G., Campero, L., Ortega-Mora, L.M., Álvarez-García, G., 2015. Anti-*Neospora caninum* and anti-*Sarcocystis* spp. specific antibodies cross-react with *Besnoitia besnoiti* and influence the serological diagnosis of bovine besnoitiosis. Vet. Parasitol. 214 (1–2), 49–54. https://doi.org/10.1016/J.VETPAR.2015.09.011.

García-Lunar, P., Ortega-Mora, L.M., Schares, G., Diezma-Díaz, C., Álvarez-García, G., 2017. A new lyophilized tachyzoite based ELISA to diagnose *Besnoitia* spp. infection in bovids and wild ruminants improves specificity. Vet. Parasitol. 244, 176–182. https://doi.org/10.1016/J.VETPAR.2017.07.029.

Gazzonis, A.L., Zanzani, S.A., Villa, L., Manfredi, M.T., 2020. *Toxoplasma gondii* infection in meat-producing small ruminants: meat juice serology and genotyping. Parasitol. Int. 76, 102060 https://doi.org/10.1016/J.PARINT.2020.102060.

Gharekhani, J., Yakhchali, M., Esmaeilnejad, B., Mardani, K., Majidi, G., Sohrabei, A., Berahmat, R., Hazhir Alaei, M., 2018. Seroprevalence and risk factors of *Neospora caninum* and *Toxoplasma gondii* in small ruminants in southwest of Iran. Arch. Razi Instit. 73 (4), 305–310. https://doi.org/10.22092/ARI.2017.109958.1119.

Glor, S.B., Edelhofer, R., Grimm, F., Deplazes, P., Basso, W., 2013. Evaluation of a commercial ELISA kit for detection of antibodies against *Toxoplasma gondii* in serum, plasma and meat juice from experimentally and naturally infected sheep. Parasit. Vectors 6 (1), 85. https://doi.org/10.1186/1756-3305-6-85.

Gondim, L.F.P., Mineo, J.R., Schares, G., 2017. Importance of serological cross-reactivity among *Toxoplasma gondii*, *Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *Besnoitia besnoiti*. Parasitology 144 (7), 851–868. https://doi.org/10.1017/S0031182017000063.

González-Warleta, M., Castro-Hermida, J.A., Regidor-Cerrillo, J., Benavides, J., Álvarez-García, G., Fuertes, M., Ortega-Mora, L.M., Mezo, M., 2014. *Neospora caninum* infection as a cause of reproductive failure in a sheep flock. Vet. Res. 45 (1) https://doi.org/10.1186/s13567-014-0088-5.

Hebbar, B.K., Roy, M., Mitra, P., Chavhan, K., Chaudhari, S., Shinde, S., Deshmukh, A.S., 2022. Seroprevalence, risk factors, and serological cross-reactivity for diagnosis of *Toxoplasma gondii* and *Neospora caninum* infections in goats in India. Microb. Pathog. 173, 105780 https://doi.org/10.1016/J.MICPATH.2022.105780.

Holec-Gąsior, L., Ferra, B., Hiszczyńska-Sawicka, E., Kur, J., 2014. The optimal mixture of *Toxoplasma gondii* recombinant antigens (GRA1, P22, ROP1) for diagnosis of ovine toxoplasmosis. Vet. Parasitol. 206 (3–4), 146–152. https://doi.org/10.1016/j.vetpar.2014.09.018.

Huertas-López, A., Martínez-Subiela, S., Cerón, J.J., Vázquez-Calvo, Á., Pazmiño-Bonilla, E.D., López-Ureña, N.M., Martínez-Carrasco, C., Álvarez-García, G., 2021. Development and validation of a time-resolved fluorescence immunoassay for the detection of anti-*Toxoplasma gondii* antibodies in goats. Vet. Parasitol. 293, 109432 https://doi.org/10.1016/J.VETPAR.2021.109432.

Innes, E.A., Bartley, P.M., Buxton, D., Katzer, F., 2009. Ovine toxoplasmosis. Parasitology 136 (14), 1887–1894. https://doi.org/10.1017/S0031182009991636.

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. Rev. Sci. Techn. (Int. Off. Epizootics) 17 (2), 469–486. https://doi.org/10.20506/RST.17.2.1119.

Kostoulas, P., Nielsen, S.S., Branscum, A.J., Johnson, W.O., Dendukuri, N., Dhand, N.K., Toft, N., Gardner, I.A., 2017. STARD-BLCM: standards for the reporting of diagnostic accuracy studies that use Bayesian latent class models. Prev. Vet. Med. 138, 37–47. https://doi.org/10.1016/j.prevetmed.2017.01.006.

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., de Cesare, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cacciò, S., Chalmers, R., Deplazes, P., Devleesschauwer, B., Innes, E., Romig, T., van der Giessen, J., Hempen, M., Van der Stede, Y., Robertson, L., 2018. Public health risks associated with food-borne parasites. EFSA J. 16 (12) https://doi.org/10.2903/J.EFSA.2018.5495.

Lindsay, D.S., Dubey, J.P., 2020. Neosporosis, toxoplasmosis, and Sarcocystosis in ruminants: an update. Vet. Clin. N. Am. Food Anim. Pract. 36 (1), 205–222. https://doi.org/10.1016/j.cvfa.2019.11.004.

Liyanage, K.L.D.T.D., Wiethoelter, A., Hufschmid, J., Jabbar, A., 2021. Descriptive comparison of ELISAs for the detection of *Toxoplasma gondii* antibodies in animals: a systematic review. Pathogens 10 (5), 605. https://doi.org/10.3390/pathogens10050605.

López-Ureña, N.M., Calero-Bernal, R., González-Fernández, N., Blaga, R., Koudela, B., Ortega-Mora, L.M., Álvarez-García, G., 2023. Optimization of the most widely used serological tests for a harmonized diagnosis of *Toxoplasma gondii* infection in domestic pigs. Vet. Parasitol. 322, 110024 https://doi.org/10.1016/j.vetpar.2023.110024.

Lorenzi, H., Khan, A., Behnke, M.S., Namasivayam, S., Swapna, L.S., Hadjithomas, M., Karamycheva, S., Pinney, D., Brunk, B.P., Ajioka, J.W., Ajzenberg, D., Boothroyd, J. C., Boyle, J.P., Dardé, M.L., Diaz-Miranda, M.A., Dubey, J.P., Fritz, H.M., Gennari, S. M., Gregory, B.D., Kim, K., Saeij, J.P.J., Su, C., White, M.W., Zhu, X.Q., Howe, D.K., Rosenthal, B.M., Grigg, M.E., Parkinson, J., Liu, L., Kissinger, J.C., Roos, D.S., Sibley, L.D., 2016. Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. Nat. Commun. 7 https://doi.org/10.1038/NCOMMS10147.

Mainar-Jaime, R.C., Barberán, M., 2007. Evaluation of the diagnostic accuracy of the modified agglutination test (MAT) and an indirect ELISA for the detection of serum

antibodies against *Toxoplasma gondii* in sheep through Bayesian approaches. Vet. Parasitol. 148 (2), 122–129. https://doi.org/10.1016/j.vetpar.2007.05.018.

Mangili, P.M., Vesco, G., Feliziani, F., Paoloni, A., Menichelli, M., Cagiola, M., Marini, C., Pourquier, P., Papa, P., 2009. Development and Evaluation of the Performance of an in-House ELISA to be Used for the Indirect Diagnosis of Toxoplasmosis in Sheep [PDF File]. Retrieved from. https://www.innovative-diagnostics.com/pdfs/pdfs/TOXOS-MS/doc085.pdf.

Martínez-Rodriguez, L.C., Tafur-Gómez, G.A., Guzman-Barragan, B.L., 2020. *Toxoplasma gondii* in small ruminants in northeastern areas of Colombia: Seroprevalence and risk factors. Parasit. Epidemiol. Control 10, e00147. https://doi.org/10.1016/J.PAREPI.2020.E00147.

Mazuz, M.L., Alvarez-García, G., King, R., Savisky, I., Shkap, V., Ortega-Mora, L.M., Gutiérrez-Expósito, D., 2018. Exposure to *Neospora* spp. and *Besnoitia* spp. in wildlife from Israel. Int. J. Parasitol.: Parasit. Wildlife 7 (3), 317–321. https://doi.org/10.1016/j.ijppaw.2018.08.002.

Moreno, B., Collantes-Fernández, E., Villa, A., Navarro, A., Regidor-Cerrillo, J., Ortega-Mora, L.M., 2012. Occurrence of *Neospora caninum* and *Toxoplasma gondii* infections in ovine and caprine abortions. Vet. Parasitol. 187 (1–2), 312–318. https://doi.org/10.1016/J.VETPAR.2011.12.034.

Nishikawa, Y., Claveria, F.G., Fujisaki, K., Nagasawa, H., 2002. Studies on serological cross-reaction of *Neospora caninum* with *Toxoplasma gondii* and *Hammondia heydorni*. J. Vet. Med. Sci. 64 (2), 161–164. https://doi.org/10.1292/JVMS.64.161.

Opsteegh, M., Teunis, P., Mensink, M., Züchner, L., Titilincu, A., Langelaar, M., van der Giessen, J., 2010. Evaluation of ELISA test characteristics and estimation of *Toxoplasma gondii* seroprevalence in Dutch sheep using mixture models. Prev. Vet. Med. 96 (3–4), 232–240. https://doi.org/10.1016/J.PREVETMED.2010.06.009.

Pinto-Ferreira, F., Caldart, E.T., Pasquali, A.K.S., Mitsuka-Breganó, R., Freire, R.L., Navarro, I.T., 2019. Patterns of transmission and sources of infection in outbreaks of human toxoplasmosis. Emerg. Infect. Dis. 25, 2177–2182. https://doi.org/10.3201/EID2512.181565.

Rossi, G.F., Cabral, D.D., Ribeiro, D.P., Pajuaba, A.C.A.M., Corrêa, R.R., Moreira, R.Q., Mineo, T.W.P., Mineo, J.R., Silva, D.A.O., 2011. Evaluation of *Toxoplasma gondii* and *Neospora caninum* infections in sheep from Uberlândia, Minas Gerais state, Brazil, by different serological methods. Vet. Parasitol. 175 (3–4), 252–259. https://doi.org/10.1016/J.VETPAR.2010.10.017.

Sánchez-Sánchez, R., Ferre, I., Re, M., Vázquez, P., Ferrer, L.M., Blanco-Murcia, J., Regidor-Cerrillo, J., Pizarro Díaz, M., González-Huecas, M., Tabanera, E., García-Lunar, P., Benavides, J., Castaño, P., Hemphill, A., Hulverson, M.A., Whitman, G.R., Rivas, K.L., Choi, R., Ojo, K.K., Barrettf, L.K., Van Voorhis, W.C., Ortega-Mora, L.M., 2018. Safety and efficacy of the bumped kinase inhibitor BKI-1553 in pregnant sheep experimentally infected with *Neospora caninum* tachyzoites. Int. J. Parasitol. Drugs Drug Resist. 8 (1), 112–124. https://doi.org/10.1016/J.IJPDDR.2018.02.003.

Sánchez-Sánchez, R., Ferre, I., Regidor-Cerrillo, J., Gutiérrez-Expósito, D., Ferrer, L.M., Arteche-Villasol, N., Moreno-Gonzalo, J., Müller, J., Aguado-Martínez, A., Pérez, V., Hemphill, A., Ortega-Mora, L.M., Benavides, J., 2019a. Virulence in mice of a *Toxoplasma gondii* type ii isolate does not correlate with the outcome of experimental infection in pregnant sheep. Front. Cell. Infect. Microbiol. 8 https://doi.org/10.3389/fcimb.2018.00436.

Sánchez-Sánchez, R., Ferre, I., Re, M., Ramos, J.J., Regidor-Cerrillo, J., Díaz, M.P., González-Huecas, M., Tabanera, E., Benavides, J., Hemphill, A., Hulverson, M.A., Barrett, L.K., Choi, R., Whitman, G.R., Ojo, K.K., Van Voorhis, W.C., Ortega-Mora, L.M., 2019b. Treatment with bumped kinase inhibitor 1294 is safe and leads to significant protection against abortion and vertical transmission in sheep experimentally infected with *Toxoplasma gondii* during pregnancy. Antimicrob. Agents Chemother. 63 (7) https://doi.org/10.1128/AAC.02527-18.

Sánchez-Sánchez, R., Vázquez-Calvo, Á., Fernández-Escobar, M., Regidor-Cerrillo, J., Benavides, J., Gutiérrez, J., Gutiérrez-Expósito, D., Crespo-Ramos, F.J., Ortega-Mora, L.M., Álvarez-García, G., 2021a. Dynamics of *Neospora caninum*-associated abortions in a dairy sheep flock and results of a test-and-cull control programme. Pathogens 10 (11), 1518. https://doi.org/10.3390/pathogens10111518.

Sánchez-Sánchez, R., Ferre, I., Re, M., Pérez-Arroyo, B., Cleofé-Resta, D., García, V.H., Díaz, M.P., Ferrer, L.M., Ruiz, H., Vallejo-García, R., Benavides, J., Hulverson, M.A., Choi, R., Whitman, G.R., Hemphill, A., van Voorhis, W.C., Ortega-Mora, L.M., 2021b. A short-term treatment with BKI-1294 does not protect foetuses from sheep experimentally infected with *Neospora caninum* tachyzoites during pregnancy. *International journal for parasitology*. Drugs and Drug Resistan. 17, 176–185. https://doi.org/10.1016/J.IJPDDR.2021.10.001.

Stelzer, S., Basso, W., Benavides Silván, J., Ortega-Mora, L.M., Maksimov, P., Gethmann, J., Conraths, F.J., Schares, G., 2019. *Toxoplasma gondii* infection and toxoplasmosis in farm animals: risk factors and economic impact. Food Waterborn. Parasitol. 15, e00037 https://doi.org/10.1016/j.fawpar.2019.e00037.

Sun, L.X., Liang, Q.L., Nie, L.B., Hu, X.H., Li, Z., Yang, J.F., Zou, F.C., Zhu, X.Q., 2020. Serological evidence of *Toxoplasma gondii* and *Neospora caninum* infection in black-boned sheep and goats in Southwest China. Parasitol. Int. 75, 102041 https://doi.org/10.1016/J.PARINT.2019.102041.

Thrusfield, M., Ortega, C., de Blas, I., Noordhuizen, J.P., Frankena, K., 2001. WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Vet. Rec. 148, 567–572. https://doi.org/10.1136/VR.148.18.567.

Vallejo-Blanco, R., Benavides, J., Arteche-Villasol, N., Sánchez-Sánchez, R., Calero-Bernal, R., Ferreras, M.C., Criado, M., Pérez, V., Ortega-Mora, L.M., Gutiérrez-Expósito, D., 2023. Experimental infection of sheep at mid-pregnancy with archetypal type II and type III *Toxoplasma gondii* isolates exhibited different phenotypic traits. Vet. Parasitol. 315, 109889 https://doi.org/10.1016/J.VETPAR.2023.109889.

Villagra-Blanco, R., Esquivel-Suárez, A., Wagner, H., Romero-Zúñiga, J.J., Taubert, A., Wehrend, A., Hermosilla, C., Dolz, G., 2018. Seroprevalence and factors associated with *Toxoplasma gondii*-, *Neospora caninum*-and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica. Vet. Parasitol.: Reg. Stud. Report. 14, 79–84. https://doi.org/10.1016/j.vprsr.2018.09.006.

Villagra-Blanco, R., Barrantes-Granados, O., Montero-Caballero, D., Romero-Zúñiga, J.J., Dolz, G., 2019. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* infections and associated factors in sheep from Costa Rica. Parasit. Epidemiol. Control 4. https://doi.org/10.1016/j.parepi.2019.e00085.

Wastling, J.M., Harkins, D., Buxton, D., 1994. Western blot analysis of the IgG response of sheep vaccinated with S48 *Toxoplasma gondii* (Toxovax). Res. Vet. Sci. 57 (3), 384–386. https://doi.org/10.1016/0034-5288(94)90135-X.

WHO, & FAO, 2014. Multicriteria-Based Ranking for Risk Management of Food-Borne Parasites: Report of a Joint FAO/WHO Expert Meeting, 3–7 September 2012. FAO Headquarters, Rome, Italy. https://apps.who.int/iris/handle/10665/112672.