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Optimization of the most widely used serological tests for a harmonized diagnosis of *Toxoplasma gondii* infection in domestic pigs

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ABSTRACT

The intake of *Toxoplasma gondii* tissue cysts through raw or undercooked pork meat is one of the main infection sources for humans. Thus, surveillance is recommended to control and prevent infection in domestic pigs. However, the lack of comparative studies hampers the updating of their performance and the comparison of seroprevalence data. Therefore, the aim of this study was to develop and validate three in-house tests and accomplish a comparative analysis of the most widely used serological tests employed in pigs. A panel of sera from pigs experimentally infected with either oocysts or tissue cysts from type II and III isolates (n = 158) was used to develop and validate a tachyzoite-based Western blot assay. Then, this technique was used as a reference to develop and preliminary validate a lyophilized tachyzoite-based enzyme-linked immunosorbent assay and an immunofluorescence antibody test. Next, a comparative study of the three in-house tests and three widely used commercial ELISAs (IDScreen®, PrioCHECK™ and Pigtype®) was accomplished with the abovementioned sera together with an additional serum panel of pigs experimentally infected with oocysts from the type II isolate (n = 44) and a panel of naturally infected pigs (n = 244). The results obtained by the majority of the tests were regarded as reference, and data analyses included TG-ROC calculations and agreement tests. Finally, the kinetics of anti-*T. gondii* IgGs from experimentally infected pigs was analyzed. Excellent sensitivity (Se) and specificity (Sp) values (≥ 93%) and moderate to near perfect agreement (k = 0.63–0.91) were observed using sera from experimental infections without requiring further readjustment, except for PrioCHECK (100% Se, 73% Sp). However, the Se of IDScreen® (87%) and TgSALUVET WB (71%) and the Sp of PrioCHECK (72%) were slightly or notably reduced when sera from naturally infected animals were analyzed, which also influenced the *kappa* values (k = 0.30–0.91). Cutoff readjustments increased the Se and Sp values to equal to or above 97% for all tests, except for TgSALUVET WB, which can be used as a reference for initial validation of tests, but it is not recommended for routine diagnosis. Seroconversion was recorded from two weeks post-infection by most of the tests, with significantly higher IgG levels in sera from pigs infected with the *T. gondii* type III *vs.* type II isolate. Again, differences regarding the test employed were observed. Differences in the diagnostic performance among tests evidenced the need to harmonize serological techniques to obtain comparable and reliable results.

1. Introduction

Toxoplasma gondii, the etiological agent of toxoplasmosis, is a worldwide distributed apicomplexan parasite that can infect any warm-blooded animal, including humans (Dubey, 2022). It has globally ranked as the third most important foodborne parasite (WHO and FAO, 2014). It is estimated that, at present, one-third of the global human population is chronically infected, with 90% of asymptomatic infections in immunocompetent individuals (McCall et al., 2022). However, clinical infections may include reproductive failures in pregnant women, especially during primary infections, such as abortion and ocular disease, as well as encephalitis, pneumonia, and death in immunocompromised patients

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(Koutsoumanis et al., 2018; Rostami et al., 2020; WHO and FAO, 2014). Thus, *T. gondii* is an opportunistic pathogen of concern for public health that should be approached through a One Health perspective.

There are three major routes of transmission for humans: the meat route through the ingestion of undercooked or raw meat containing tissue cysts; the environmental route through the consumption of sporulated oocysts that contaminate soil, water, vegetables, fruits and bivalves; and the maternal-fetal route by tachyzoites that pass from the mother to the fetus through the placenta (Almeria and Dubey, 2021; Attias et al., 2020; Dubey, 2009; López-Ureña et al., 2022). Up to 47.1% (16/34) of reported toxoplasmosis outbreaks from 1960 to March 2018 were associated with the consumption of contaminated meat and derivates (Pinto-Ferreira et al., 2019). In particular, pigs have been recognized as the principal food source of *T. gondii,* responsible for 41% and 50% of the cases in studies conducted in the United States and the Netherlands, respectively (Koutsoumanis et al., 2018).

In pigs, serology has been recommended as an epidemiological indicator for *T. gondii* infection with food safety purposes (EFSA, 2011). Indeed, a literature review indicated that *T. gondii* tissue cysts could be detected by bioassay or PCR methods in the meat of 58.8% (348/529) of *T. gondii*-seropositive pigs (Opsteegh et al., 2017). A wide variety of serological techniques have been used for the detection of anti-*T. gondii* antibodies in pigs, including enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFAT), Western blot (WB), indirect hemagglutination assay (IHA), modified agglutination test (MAT), Sabin-Fieldman dye test (DT), latex agglutination test (LAT) (Dubey et al., 2020) and protein microarray-based assay (Loreck et al., 2020). ELISA methods are the most widely used tests (Huertas-López et al., 2023; Liyanage et al., 2021), especially commercial ready-to-use kits, followed by MAT (Dubey et al., 2020) and IFAT (Huertas-López et al., 2023). In contrast, WB has been poorly used (Al-Adhami and Gajadhar, 2014; Basso et al., 2013; Garcia et al., 2008; Huertas-López et al., 2023) and has been recognized as a highly specific test. However, to date, there is no commonly adopted IFAT cutoff or WB positive pattern criterion. Moreover, the usefulness of WB for daily diagnosis purposes is unclear. In addition, despite the wide variety of serological tests used worldwide, the lack of comparative studies hampers the updating of their performance and the comparison of seroprevalence data. Only a restricted number of serological tests have been compared, and the main limitations addressed are as follows: i) commercial tests have been scarcely evaluated; ii) in the absence of a gold standard test, either in-house or commercial tests have been employed as a reference technique with unknown diagnostic performance data (Al-Adhami and Gajadhar, 2014; Garcia et al., 2006; Hill et al., 2006; Steinparzer et al., 2015); iii) sera from experimentally and naturally infected pigs were only used by Hill et al. (2006) *vs.* most studies that employed either sera from experimentally infected animals (Al-Adhami and Gajadhar, 2014; Garcia et al., 2006) or sera from natural infections (Basso et al., 2013; Cubas-Atienzar et al., 2019; Damriyasa et al., 2004; Kunic et al., 2022; Limon et al., 2017; Pablos-Tanarro et al., 2018; Papatsiros et al., 2016; Pardini et al., 2012; Steinparzer et al., 2015); and iv) the influence of *T. gondii* doses and isolates/strains remains unknown. In this context, the European Food Safety Authority (EFSA) pointed out the need for ring trials of the most widely used serological techniques to unify criteria and obtain comparable results since some of the tests and antigens currently accessible are not validated or standardized, and no standard sera samples are available (EFSA, 2011).

Accordingly, herein, a comparative study of the most widely used serological techniques for the diagnosis of *T. gondii* infections in domestic pigs following World Organization for Animal Health (WOAH) guidance (WOAH, 2023; 2021; 2018) was performed. First, the criterion of seropositivity was defined for a tachyzoite-based Western blot assay (TgSALUVET WB), which was later used as a reference test to develop and preliminary validate a lyophilized tachyzoite-based ELISA (TgSALUVET ELISA 2.0) and an immunofluorescence antibody test (TgSALUVET IFAT). Next, three commercial ELISA kits (IDScreen®, PrioCHECK™ and Pigtype®) and three in-house serological tests (TgSALUVET WB, TgSALUVET ELISA 2.0 and TgSALUVET IFAT) were compared and validated by analyzing a wide panel of sera from experimentally and naturally infected pigs. Finally, the influence of parasite stage and isolate on the kinetics of anti-*T. gondii* IgGs was studied for each test with sera from experimental infections.

2. Materials and methods

2.1. Panels of sera

Different serum panels obtained from pigs either experimentally or naturally infected with *T. gondii* were used in the different steps of this study **(**Fig. 1**)**. Serum samples were obtained from the jugular vein and kept at − 80 °C until use.

2.1.1. Sera from pigs experimentally infected with Toxoplasma gondii

Two panels of sera from pigs experimentally infected with *T. gondii* were used. These experiments were performed considering the European regulations, as well as the animal welfare and the good practices guidance for animals used for experimental research and other scientific practices, European Directive 2010/63/EU. The procedures were approved by the Ministry of Education, Youth and Sports from the Czech Republic (PP 55/2016) (Dámek, 2023a) and the Agency for Food, Environmental and Occupational Health & Safety (ANSES) from France (APAFIS N∘ 14,363–2018032908554996v3) (Dámek et al., 2023a, 2023b).

Panel 1 included sera from prepubertal Dan Hybrid-LY sows orally infected with 400 oocysts or 10 tissue cysts from either type II (CZ-Tiger isolate, ToxoDB#3, n = 6 and 7, respectively) or type III (CZ-Šimková isolate, ToxoDB#2; n = 6/group) isolates. Samplings were carried out prior to the infection and weekly from one to six weeks post-infection (wpi), with a few exceptions in which the samples were collected only in some weeks (n = 158) (Dámek, 2023a). These sera were used in step 1 to define the pattern of *T. gondii* tachyzoite antigen recognition by TgSALUVET WB, as well as to validate the TgSALUVET ELISA 2.0 and TgSALUVET IFAT, and in step 2 related to the comparative study (Fig. 1).

Panel 2 was composed of sera obtained from three 97-day-old Large White female pigs orally infected with 1000 oocysts of the clonal type II reference strain ME49 (ToxoDB #1). Sampling was carried out at 0, 2, 5, 9, 12, 14, 19, 26, 33, 40, 47, 54, and 58 days post-infection (dpi). One noninfected pig was used as a negative control, and blood samples were collected at 0, 26, 40, 47 and 54 dpi (n = 44) (Dámek et al., 2023a, 2023b). This panel of sera was used in step 1 for the initial validation of TgSALUVET ELISA 2.0 and TgSALUVET IFAT and in step 2 in the comparative study (Fig. 1).

2.1.2. Sera from pigs naturally exposed to Toxoplasma gondii

Panel 3 consisted of sera collected from slaughtered Black Iberian pigs (50% cross with Duroc-Jersey breed) during 2019–2020. These pigs had been reared in different systems ranging from extensive to semi-extensive regimes in farms allocated within the Dehesa ecosystem, composed mostly of acorn Mediterranean forest in the Extremadura region (southwestern Spain) (n = 244). Ten positive and 10 negative samples from this panel, based on TgSALUVET WB results, were used to test the precision of TgSALUVET ELISA 2.0 (inter- and intraplate variability) (step 1). The whole panel was included in the comparative study (step 2) (Fig. 1).

2.2. Parasites and antigen preparation

Parasite culture and antigen preparation were performed as previously described by García-Lunar et al. (2017) with slight modifications. Briefly, *T. gondii* tachyzoites from the type II ME49 strain were cultured in a monolayer of the VERO-81 cell line using DMEM (Dulbecco's

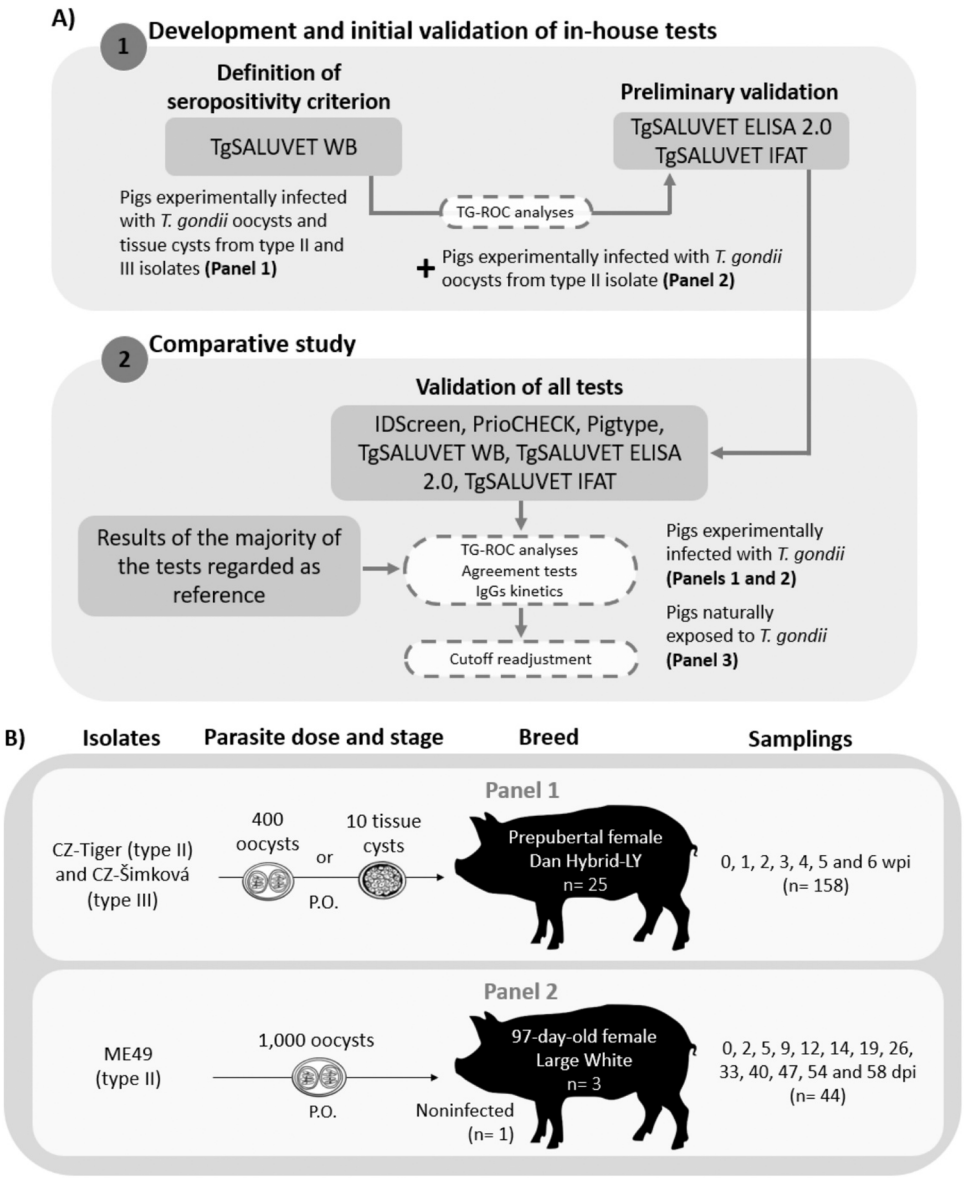

Fig. 1. A) Workflow followed in this study, including the serum panels used in each step. B) Experimental design of *Toxoplasma gondii* infections in pigs. P.O.: *Per os*. Wpi: weeks post-infection. Dpi: days post-infection. A checklist of the items included in the standards for reporting diagnostic accuracy studies (Kostoulas et al., 2017) has been provided as Supplementary Table 1.

Modified Eagle's Medium – high glucose, Sigma®, Ref. 6429) supplemented with 10% fetal bovine sera (FBS) and 1% antibiotics (Lonza, Ref. H317–745E). Fetal bovine serum was seronegative for *T. gondii* and other closely related parasites (*Besnoitia besnoiti* and *Neospora caninum*) to avoid cross-reactivity (García-Lunar et al., 2015). At 72 h post-infection (hpi), the culture was scraped off, and the parasites were purified using 3-μm Millipore filters (Millipore, Ref. TSTP02500) and counted in a Neubauer chamber. Parasites were pelleted by centrifugation at 1350 x *g* for 15 min (min) at 4 °C. For TgSALUVET WB, pellets of 1×10^8 tachyzoites were kept at − 80 °C until use. For TgSALUVET ELISA 2.0, glass vials, each with 1×10^8 tachyzoites in 4 mL of PBS, were stored at − 80 °C until being lyophilized (García-Lunar et al., 2017). For the TgSALUVET IFAT, pellets of 1×10^7 tachyzoites were resuspended in 1 mL of cold PBS, 5 μL of 37–38% formaldehyde (PanReac AppliChem, Ref. 131328.1211) was added, and the vials were stored at 4 °C until use.

2.3. Serological techniques

Three commercial ELISA kits (IDScreen, PrioCHECK and Pigtype) and three in-house tests (TgSALUVET WB, TgSALUVET ELISA 2.0, and TgSALUVET IFAT) were included in this comparative study (Fig. 1A, Table 1). Commercial tests were used as specified by the manufacturers' instructions.

2.3.1. Western blot (TgSALUVET WB)

Aliquots of 2×10^7 tachyzoites were resuspended in loading buffer (final concentration: 10% glycerol, 50 mM TRIS at pH 6.8, 2% SDS, 0.05% bromophenol blue and 100 mM DTT). The samples were subjected to a cold wet ultrasonic bath for 15 min, followed by a wet bath at 100 °C for 5 min. Then, the samples were loaded in a one-well comb in a 15% polyacrylamide gel for electrophoresis at 150 V (constant) for approximately 1.5 h in a Mini Protean® Tetra System (Bio-Rad Laboratories). Prestained protein standards (Precision Plus Protein™ Kaleidoscope™, Bio-Rad Laboratories, Ref. 1310375) were added to one side of each gel to estimate the molecular weight of the antigens. After that,

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

Technique	Antigen	Target species	Sample type	Sample dilution: type	Cutoff	Diagnostic performance*
ID Screen® Toxoplasmosis Indirect Multi species (IDScreen)	P30	Ruminants, swine, dogs, cats	Serum, plasma and meat juice	1/10: serum and plasma 1/2: meat juice	S/P% ≤ 40, ≥ 50	Se= 100%, Se= 100%[a]
PrioCHECK® Porcine Toxoplasma Ab Kit (PrioCHECK)	Tachyzoite**	Pigs	Serum, plasma and meat juice	1/50: serum and plasma 1/10: meat juice	PP ≥ 20	Not specified[b]
Pigtype® Toxoplasma Ab Indical Bioscience/ Qiagen (Pigtype)	Tachyzoite**	Cattle, sheep, goats, cats, dogs, foxes, pigs and wild boars	Serum, plasma (meat juice only from pigs and wild boars)	1/100: serum and plasma 1/10: meat juice	S/P ≥ 0.3 ***	Not specified[c]
TgSALUVET WB (reduced conditions)	Tachyzoites	Pigs	Serum	1/20	To be determined in this study	To be determined in this study
TgSALUVET ELISA 2.0	Lyophilized tachyzoites	Pigs	Serum	1/100	To be determined in this study	To be determined in this study
TgSALUVET IFAT	Tachyzoites	Pigs	Serum	1/100 and 1/200	To be determined in this study	To be determined in this study

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 in the user's manual within the kit. **No more details were provided. *** Short protocol suggested in the manual was followed. [a]Based on 33 positive samples from France and 300 negative samples tested (animal species not specified). Intra-plate repeatability= 3–4%, inter-plate reproducibility= 4–6%. [b]No data available in the kit manual, only available in an online flyer from ThermoFisher (https://www.thermofisher.com/order/catalog/product/es/es/7610230); sera: Se= 98% and Sp= 100% (based on 50 positive and 270 negative porcine samples), meat juice: Se= 97%, Sp= 100% (based on 33 positive and 116 negative samples), using as reference IFAT, WB ad ELISA. [c]Microtiter plate coefficient of variation= 6.8%.

the antigens were transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad, Ref. 1620112) in a Mini Protean II™ (Bio-Rad Laboratories) at 400 mA (constant) for 1 h. The membranes were stained with 0.1% Red Ponceau (Sigma Ref. P3504) to visualize the electrotransferred protein bands. Next, the membranes were washed with 0.05% TBS-Tween 20 (TBS-T) and blocked with 5% powdered skim milk TBS-T for 2 h at room temperature. Then, they were washed three times for 5 min each with TBS-T, carefully dried with filter paper, and stored in dry filter paper and plastic bags (Ziploc) at − 20 °C until further use.

For immunoblots, the membranes were soaked in TBS-T and cut into strips of approximately 1–2 mm. The strips were placed on stands with individual rails. Serum samples were diluted at 1/20 in blocking solution, added to separate rails with a strip and incubated in a rocker for 1.5 h at room temperature. After that, three washes with TBS-T for 5 min each were performed. Then, protein G conjugated with peroxidase (Sigma Ref. P8170) was added at a dilution of 1/600 in TBS-T and incubated under the same conditions and protected from light. Finally, two washes of 5 min each were performed with TBS-T followed by an additional wash with TBS. The reaction was revealed using 4-chloro-1-naphthol solution (Thermo Scientific, Ref. 34010) and stopped with Milli-Q water using the colorimetric reaction of the positive and negative controls as a reference, which were initially selected from pigs naturally exposed to *T. gondii* (Panel 3) based on this technique; these results were later confirmed by all included serological tests in the comparative study. The strips were placed in a template and scanned with the GS-800 Calibrated Densitometer (Bio-Rad) for analysis.

2.3.2. Lyophilized tachyzoite-based ELISA (TgSALUVET ELISA 2.0)

A lyophilized *T. gondii* tachyzoite-based ELISA was developed and validated for diagnostic purposes and previously used for analyzing the kinetics of anti-*T. gondii* IgGs in experimentally infected piglets (Largo-de la Torre et al., 2022). The detailed protocol is as follows. Initially, 1×10^5 *T. gondii* tachyzoites diluted in 100 μL of 0.1 M carbonate-bicarbonate, pH 9.6, were added per well in 96-well plates (Thermo Scientific MaxiSorp, Ref. 442404) and kept at 4 °C overnight. After three washes with 0.05% PBS-Tween 20 (PBS-T), the plates were blocked with 300 μL per well of 5% powdered skim milk PBS-T and incubated at room temperature for 2 h. Then, they were washed, and 100 μL of diluted sera at 1/100 in blocking solution was added per well. After 1 h of incubation at 37 °C and three washes, 100 μL of protein G with peroxidase (Sigma—Aldrich, Ref. P8170), diluted at 1/6000 in PBS-T, was added per well and incubated for 1 h under the same conditions. Next, the plates were washed, and 100 μL of TMB Ultra was added per well (Thermo Fisher, Ref. 34028). The reaction was stopped by adding 100 μL/well of 2 N sulfuric acid after 10 min. The final optical density (OD) was read at 450 nm using a microplate reader (Multiscan RC 6.0, Labsystems). The results were interpreted as the relative index percent (RIPC): ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100. The same sera samples used as positive and negative controls in TgSALUVET WB were used for the TgSALUVET ELISA 2.0 test.

2.3.3. Immunofluorescence antibody test (TgSALUVET IFAT)

A total of 8×10^4 formalin-fixed tachyzoites were added per 4-mm well (Thermo Scientific Diagnostic Microscope Slides, Ref. ER-311B-CE24), dried at room temperature and then postfixed with acetone (PanReac AppliChem, Ref. 211007.1211) at − 20 °C for 10 min. Next, the slides were washed with Milli-Q water for 10 min under movement (100 rpm) and dried at room temperature. Positive and negative controls were diluted at 1/100, samples were diluted at 1/100 and 1/200 in PBS, and 8 μL of each was placed in the wells. The slides were incubated in wet, dark chambers for 30 min at 37 °C. After that, a quick wash with PBS was performed, followed by two additional washes of 10 min each in a rocker. Rabbit anti-pig IgG (whole molecule) FITC antibody (Sigma, Ref. F1638) was diluted at 1/64 in 0.2% Evans Blue Dye PBS, and 8 μL was added per well. The samples were incubated and washed under the same conditions, with an additional final wash of 10 min with Milli-Q water. The slides were dried at room temperature in the dark, and the cover slides were fixed with Fluoroshield™ (Sigma, Ref. F6182). The same serum controls used in the TgSALUVET WB were included in each slide for TgSALUVET IFAT. Samples were classified as positive if more than 50% of the tachyzoites showed complete peripheral intense fluorescence, and the results were based on the consensus of two experienced operators, with the samples whose results were discrepant being classified as doubtful.

2.4. Data analysis

The molecular weight of the detected proteins by TgSALUVET WB was estimated using Quantity One 4.5.1 software (Bio-Rad). Immunodominant antigens or fractions (IDAs) were identified by their frequency and intensity of recognition, and this was achieved by two operators to avoid bias. A chi-square test was performed to identify differences between experimental groups in the frequency of recognition of each identified antigen. Sera collected at 6 wpi (Panel 1) were used for this purpose. The intensity was scaled as weak (+), medium (++) or high (+++). A two-way analysis of variance (ANOVA) with repeated measures, followed by a *post hoc* Tukey or Dunnett test if applicable (GaphPad Prism), was performed to confirm significant differences in the total antigens and IDAs recognized after the infection with respect to prior to the infection, as well as between experimental groups (Panel 1). Differences were considered significant when the *P* values were lower than 0.05. When the criterion of positivity was defined, if a mismatch result was observed between both operators, the sample was regarded as doubtful by TgSALUVET WB.

TgSALUVET ELISA 2.0 precision was determined by analyzing each sample in triplicate in three different ELISA plates. The intraplate coefficient of variation (CV) was determined as follows: 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). Intra- and interplate CVs below 20% were assumed to have acceptable repeatability (Jacobson, 1998). A preliminary TgSALUVET ELISA 2.0 cutoff selection was performed with a nonparametric two-graph receiver operating characteristic (TG-ROC) analysis with SigmaPlot 12.0 software. Serum panels from experimental infections and TgSALUVET WB as a reference test in the absence of a gold standard method were used to obtain a proof-of-concept Se and Sp results.

The TgSALUVET IFAT cutoff was defined by performing an agreement test between the IFAT results with respect to the results obtained with TgSALUVET WB with sera from experimental infections. Cohen's Kappa coefficients were analyzed by the WinEpi platform (Thrusfield et al., 2001) (http://www.winepi.net/) using a confidence level of 95%. The results were expressed as *kappa* (*k*) values, and the serum dilution that presented the highest agreement was selected for the comparative study. Then, the initial diagnostic performance of TgSALUVET IFAT was defined using TgSALUVET WB as reference test in the WinEpi platform, with a confidence level of 95%.

For the comparative study, TG-ROC analyses were performed using SigmaPlot 12.0 Software, with a confidence level of 95%, independently for samples from experimentally (Panels 1 and 2) and naturally (Panel 3) infected pigs. For this, each sample was classified as positive, negative, or doubtful based on the result of most of the tests (at least by four out of six techniques). Samples with positive results by three techniques and negative results by the other three techniques were considered doubtful and were excluded from the analysis. For qualitative assays such as TgSALUVET WB and TgSALUVET IFAT, the diagnostic performance was determined using WinEpi as described before but using the results from the majority of the tests as a reference test. The agreement among tests was assessed before and after the TG-ROC analysis by analyzing Cohen's Kappa coefficients as specified above.

Anti-*T. gondii* IgG levels for each experimental group and each week/day of sampling were compared (Panels 1 and 2). For Panel 1, a mixed-effects analysis with repeated measures and multiple comparisons was performed to confirm differences within and between experimental groups based on parasite stage and isolate, followed by a Tukey test if applicable. For the results obtained with Panel 2, a one-way ANOVA was used to compare the differences in infected pigs among sampling days, followed by a Dunnett test. These tests were performed using GraphPad Prism, version 8.0.1, not assuming sphericity and applying a Geisser-Greenhouse correction if recommended. Differences were considered significant when the *P* values were lower than 0.05.

3. Results

3.1. Pattern of antigen recognition and establishment of the seropositive criterion for TgSALUVET WB

A serum sample from an experimentally infected pig (Panel 1) at 6 wpi with the highest number of antigens recognized was regarded as a reference to determine the mean molecular weight (MW) value of the detected antigens (Fig. 2A). Up to 17 antigens were recognized by seropositive animals with molecular weights that ranged between 9 and 82 kDa (Fig. 2A). The frequency and intensity of antigen recognition are shown in Table 2. Eight IDAs were identified (9–10, 19, 25, 28, 30, 33–35, 43–45, and 69 kDa). These IDAs were detected by all infected pigs from at least one of the experimental groups at 6 wpi with a medium or high intensity (Fig. 2A). Nevertheless, 64% of the pig sera recognized the 19 kDa antigen with medium intensity prior to the infection, as well as the 25, 28, 30, 33–35, 39, and 43–45 kDa antigens, but with lower frequency and/or intensity rates. Only two IDAs, the 9–10 and 69 kDa antigens, were not recognized prior to the infection, and the 9–10 kDa antigen was recognized with a higher frequency and intensity (Table 2). A given sample was classified as positive if at least three IDAs were recognized with medium or high intensity since the recognition of three IDAs with medium or high intensity was observed only in seropositive samples (Fig. 2B, Table 2). Furthermore, the presence of these IDAs was not dependent on the experimental group (stage and isolate) ($P \geq 0.05$), except for the 28 kDa antigen, which was recognized by up to 100% of the pigs infected with the type III isolate *vs.* 17–50% of the pigs infected with the type II isolate (Table 2).

Moreover, statistically significant differences in the total number of recognized antigens and IDAs were recorded from 2 wpi onward compared with samples prior to the infection (Fig. 3). After 2 wpi, the number of recognized antigens increased weekly, with significant differences in all cases at any sampling week *vs.* 6 wpi. When considering *T. gondii* stages and isolates, significant differences between experimental groups were detected from 3 or 4 wpi in the number of IDAs and total recognized antigens, respectively. In detail, a higher number of recognized antigens was observed in pigs infected with the type III isolate than in those infected with the type II isolate as follows: any stage of type III isolate *vs.* oocysts type II isolate at 4–5 wpi, tissue cysts type III isolate *vs.* oocysts type II isolate at 6 wpi, and tissue cysts type III isolate *vs.* tissue cysts type II isolate at 5–6 wpi ($P < 0.05$) (Fig. 3A). Similarly, a higher number of recognized IDAs was recorded in pigs infected with the type III isolate as follows: tissue cysts from the type III isolate *vs.* oocysts from the type II isolate at 3 wpi, any stage from the type III isolate *vs.* oocysts from the type II isolate at 4–5 wpi, and tissue cysts from the type III isolate *vs.* tissue cysts from the type II isolate at 3 and 5 wpi ($P < 0.05$) (Fig. 3B).

Finally, based on the established criterion of positivity, seropositive pigs were recorded from 2 wpi onward in Panels 1 and 2.

3.2. Optimization and diagnostic performance of TgSALUVET ELISA 2.0

The coefficients of variation for the intra- and interplate repeatability were 3.6% and 4.8%, respectively. Six out of 202 samples belonging to serum Panels 1 and 2 were excluded from the TG-ROC analysis due to doubtful results obtained with the TgSALUVET WB reference test (No. of sera samples included in the TG-ROC analysis based on TgSALUVET WB: negative= 81, positive= 115). The area under the curve (AUC) was 0.97 ($CI_{95\%}$: 0.95–0.99), and 95% Se ($CI_{95\%}$: 89–98) and 95% Sp ($CI_{95\%}$: 88–99) were obtained for the selected cutoff, RIPC $\leq$ 15.27- $\geq$ 31.09. Using this cutoff, seropositivity was recorded from 2 wpi or 19 dpi onward in Panels 1 and 2, respectively, with 7.4% doubtful results.

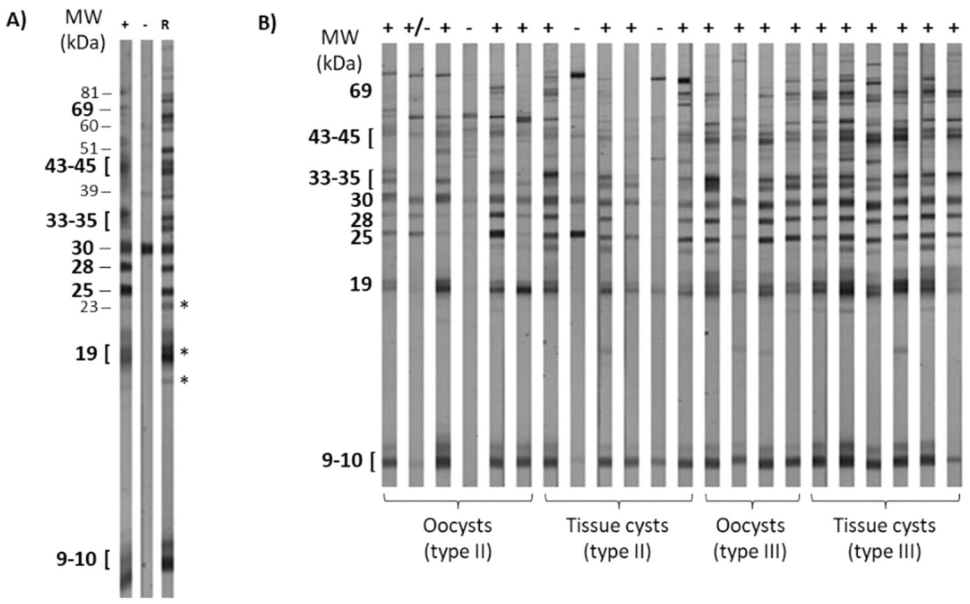

Fig. 2. *Toxoplasma gondii* tachyzoite antigens recognized by experimentally infected pigs at six weeks post-infection. A) + : positive control serum, -: negative control serum, R: positive control serum used as reference where "* " shows a high recognition (+++) of 19 kDa antigen, medium recognition (++) of 23 kDa antigen and a weak recognition (+) of 19 kDa antigen. B) Positive (+), negative (-) and doubtful (+/-) sera from experimentally infected pigs with either oocysts or tissue cysts from type II or III isolates at six weeks post-infection based on the defined positivity criteria: recognition of at least three immunodominant antigens (IDAs) with medium or high intensity. MW: molecular weight. IDAs are shown in bold letters.

Table 2
Frequency and intensity of recognition of *Toxoplasma gondii* tachyzoite antigen by sera from pigs experimentally infected with either oocysts or tissue cysts from type II and III isolates at six weeks post-infection.

Antigens (kDa)[a]	Oocysts type II isolate	Tissue cysts type II isolate	Oocysts type III isolate	Tissue cysts type III isolate	X^2	Prior to infection
81	50/++	50/ +++	25/+	50/++	*	36/+
69	17/++	33/++	75/++	100/ +++	Ns	0
60	0	17/++	0	17/++	Ns	0
51	83/++	17/++	50/++	67/++	****	41/++
43–45	33/++	33/+	100/++	100/ +++	Ns	50/+
39	17/+	33/+	0	33/++	***	23/++
33–35	50/+	67/++	75/+++	100/ +++	Ns	23/+
30	83/++	83/++	100/ +++	100/ +++	Ns	23/++
28	17/+++	50/++	75/++	100/ +++	*	14/+
25	50/++	83/++	75/+++	100/ +++	Ns	4/+
23	0	33/+	0	33/+	Ns	0
19	67/++	67/++	75/++	100/++	Ns	64/++
9–10	67/++	83/++	100/ +++	100/ +++	Ns	0

[a] An antigenic fraction comprised more than one antigenic band. X^2: Chi-square test. Immunodominant antigens are represented in bold letters. Frequency: percentage. Intensity: +++ (high), ++ (medium), + (weak). The intensity was the mean within each experimental group. Ns: non-significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.3. Validation of TgSALUVET IFAT

A better agreement between TgSALUVET IFAT and TgSALUVET WB was obtained for 1/200 ($k = 0.80$) *vs.* 1/100 ($k = 0.74$) cutoff. Accordingly, a 1/200 cutoff was selected for the comparative study (Supplementary Table 2), with an initial diagnostic performance of 99% Se ($CI_{95\%}$: 97–101) and 78% Sp ($CI_{95\%}$: 69–87) when using TgSALUVET WB as a reference test. Based on this cutoff, seropositivity was also recorded from 2 wpi onward in pigs from Panels 1 and 2.

3.4. Comparative study

All ELISA tests showed high Se and Sp values (97–100%) for the cutoffs suggested by the manufacturers with sera from experimentally infected pigs, except for PrioCHECK, which showed 73% Sp (Fig. 4A). Moreover, TgSALUVET WB and TgSALUVET IFAT also showed good diagnostic performance (Se= 95%, Sp= 100% and Se= 99%, Sp= 93%, respectively). Since doubtful results were obtained with IDScreen (0.99%; 2/202) and TgSALUVET ELISA 2.0 (7.43%; 15/202), the cutoffs were readjusted to avoid doubtful results and maintain good diagnostic performance (Se= 99%, Sp= 100% and Se= 97%, Sp= 100%, respectively). On the other hand, the PrioCHECK cutoff was also readjusted to increase Se and Sp up to 97% (Fig. 4A). After cutoff value readjustments, the diagnostic performance of TgSALUVET WB and TgSALUVET IFAT was hardly modified (Se= 91%, Sp= 100% and Se= 100%, Sp= 91%, respectively). The cutoffs, Se and Sp, as well as the AUC values for each ELISA, are shown in Fig. 4A. Summarized information regarding confidence intervals for each parameter is available in Supplementary Table 3. All tests also showed good to near perfect agreement before the TG-ROC analysis ($k = 0.80–0.91$) with sera from experimental infections, except for PrioCHECK with moderate agreement in all pairwise comparisons ($k = 0.63–0.75$) that improved after the cutoff readjustment ($k = 0.79–0.92$) (Table 3).

When TG-ROC analyses were carried out with sera from naturally infected pigs, several tests showed lower Se and/or Sp values (TgSALUVET WB: Se= 71%, Sp= 99%; PrioCHECK: Se= 100%, Sp= 72%; IDScreen: Se= 87%, Sp= 100%; TgSALUVET ELISA 2.0: Se= 100%, Sp= 92%). Pigtype and TgSALUVET IFAT maintained good diagnostic performance (97% Se and Sp; and 97% Se and 100% Sp, respectively). Cutoff readjustments improved the diagnostic performance of all techniques, with Se and Sp values equal to or higher than 97%, except for TgSALUVET WB, whose Se value increased up to 79%. The cutoffs, Se and Sp, as well as the AUC values for each ELISA, are shown in Fig. 4B. Summarized information regarding confidence intervals is for each parameter available as Supplementary Table 3. Low to moderate agreement between techniques was observed, and PrioCHECK showed the lowest agreement ($k = 0.30–0.46$), while TgSALUVET IFAT showed the highest *kappa* values ($k = 0.70–0.91$). After the cutoff readjustment, only IDScreen and TgSALUVET IFAT reached moderate to good agreement in some pairwise comparisons (Table 3).

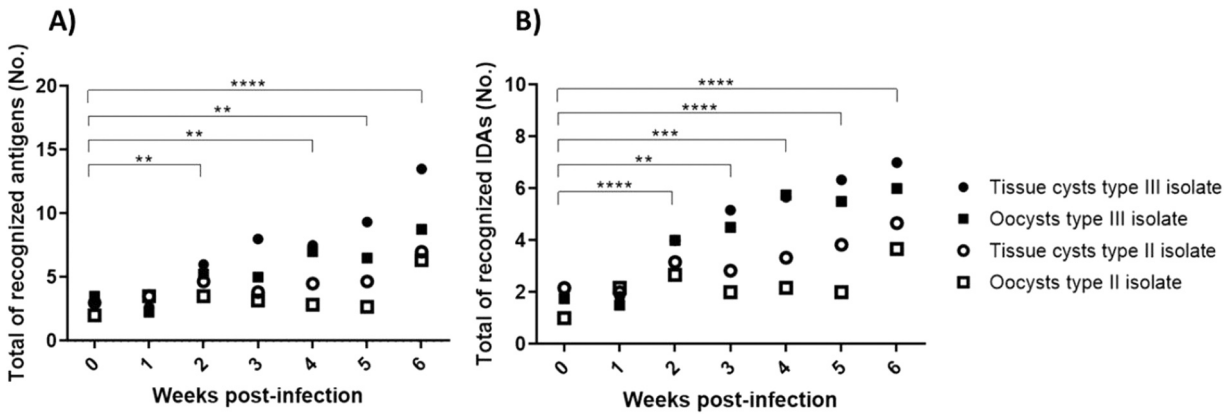

Fig. 3. Kinetics of total (A) or immunodominant (B) antigen recognition by sera from experimentally infected pigs by TgSALUVET WB. IDAs: immunodominant antigens. Significant differences between each sampling week with respect to prior to the infection are identified as follows (Dunnett's multiple comparison test): * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$.

3.5. Kinetics of antibody levels in experimentally infected pigs by ELISA tests

In general terms, a significant increase in anti-*T. gondii* IgGs was detected in both panels of sera from 2 to 3 wpi with respect to samples prior to the infection, except in Panel 2 for PrioCHECK, which showed an earlier increase in IgG levels (from 9 dpi), and Pigtype and TgSALUVET ELISA 2.0, which showed a delayed increase in IgG levels (from 26 dpi). Similar seroconversion results at approximately 2 wpi were obtained regardless of the cutoffs employed (initial *vs.* readjusted cutoffs). However, when the cutoff suggested by PrioCHECK was used, three animals from Panel 1 were seropositive prior to infection, and seroconversion was recorded earlier, from 1 wpi.

When the kinetics of IgGs were studied between experimental groups (sera from Panel 1), a significantly higher level of antibodies was detected in pigs infected with the type III isolate by all ELISA tests as follows: pigs infected with any stage of the type III isolate *vs.* oocysts from the type II isolate at 2–4 and 6 wpi for IDScreen, 2–6 wpi for Pigtype, and 3–6 wpi for TgSALUVET ELISA 2.0; tissue cysts from the type III isolate *vs.* oocysts from the type II isolate at 5 wpi for IDscreen, 5–6 wpi for PrioCHECK and 2 wpi for TgSALUVET ELISA 2.0; and tissue cysts from the type III isolate *vs.* tissue cysts from the type II isolate at 4 wpi for IDScreen and 3–5 wpi for Pigtype (Fig. 5A). Nevertheless, no significant differences were observed between groups infected with oocysts and tissue cysts from the same genotype.

4. Discussion

Herein, a comparative study of a wide set of serological techniques used for the detection of anti-*T. gondii* IgGs in domestic pigs has been performed, including routinely used commercial ELISA kits (IDScreen, PrioCHECK and Pigtype), as well as three in-house methods (WB, ELISA and IFAT) that were previously validated. Updated diagnostic performance data are offered, and new cutoffs are suggested to obtain harmonized results. For this study, sera from pigs experimentally infected with different stages and isolates of *T. gondii,* as well as from pigs naturally exposed to *T. gondii,* were analyzed by all the above-mentioned serological tests. Moreover, differences in the levels of anti-*T. gondii* IgGs regarding the *T. gondii* stage and isolate were investigated for all tests.

The developed in-house WB and ELISA tests showed good to excellent proof-of-concept diagnostic performance in their initial validation. Moreover, TgSALUVET IFAT was a highly specific test. *Toxoplasma gondii*-based WBs have been previously developed for pig sera. However, they have been performed under different conditions, and to date, there is no consensus on the criterion of positivity. Some authors considered the recognition of the 30 kDa antigen in addition to the 22 kDa or 42 kDa antigens under nonreducing conditions to be positive (Basso et al., 2013; Pardini et al., 2012). In contrast, Al-Adhami and Gajadhar (2014) classified a sample as positive if the pattern of antigen recognition was similar to that shown by the positive control, where the 150, 100, 79, 45, 39, 35, 30 and 24 kDa antigens were recognized. Recently, a commercial WB kit validated for humans was adapted to pig sera, and the recognition of the 30 kDa antigen together with either the 31 kDa or 33 kDa antigen was considered a positive result (Olsen et al., 2022). In this scenario, herein, an exhaustive analysis with a wide panel of sera from experimentally infected pigs using different parasite stages and isolates has been used to define a criterion of positivity based on the frequency and intensity of antigen recognition. The 9–10, 19, 25, 28, 30, 33–35, 43–45 and 69 kDa bands were identified as IDAs, and only the 9–10 and 69 kDa IDAs were not recognized prior to infection. Therefore, we established a restrictive criterion of positivity that consisted of the recognition of at least three of the IDAs (9–10, 19, 25, 28, 30, 33–35, 43–45 and 69 kDa) with medium or high intensity to avoid false-positive results. The recognition of antigens prior to infection can be due to cross-reactivity with closely related parasites since some proteins are conserved among Apicomplexan parasites (Lorenzi et al., 2016). For example, anti-*N. caninum* antibodies present in small ruminant sera cross-reacted with the 30 kDa *T. gondii* surface antigen (known as TgSAG1 or SRS29B) (Huertas-López et al., 2021; Sánchez-Sánchez et al., 2021). In fact, TgSAG1 is highly immunogenic (Velmurugan et al., 2008) and is used in serological tests (*e.g.*, IDScreen). Thus, WB results based solely on recognition of the 30 kDa antigen should be interpreted with caution.

The establishment of a restrictive criterion of positivity for TgSALUVET WB allowed the definition of a cutoff value for two additional in-house tests, namely TgSALUVET ELISA 2.0 and TgSALUVET IFAT. TgSALUVET ELISA 2.0 was previously used to analyze the kinetics of anti-*T gondii* IgGs in experimentally infected piglets with oocysts from type II and type III isolates (Largo-de la Torre et al., 2022), but there was a lack of validation for positive and negative discrimination. To date, this is the first ELISA that employs *T. gondii* lyophilized tachyzoites for diagnostic purposes in swine. Traditionally, whole or soluble tachyzoite extracts have been used as antigens in ELISA tests, and uniquely in the case of the closely related parasite *B. besnoiti,* a lyophilized tachyzoite-based ELISA was developed (García-Lunar et al., 2017). In that case, an increased Sp was reported compared to a soluble extract-based ELISA, likely due to an enrichment of membrane *vs.* cytosolic antigens (García-Lunar et al., 2017). The TgSALUVET IFAT initially showed excellent Se, with lower Sp. Since a limitation claimed for IFAT is the subjective results, consensus from two or more experienced operators is strongly recommended to avoid subjectivity and

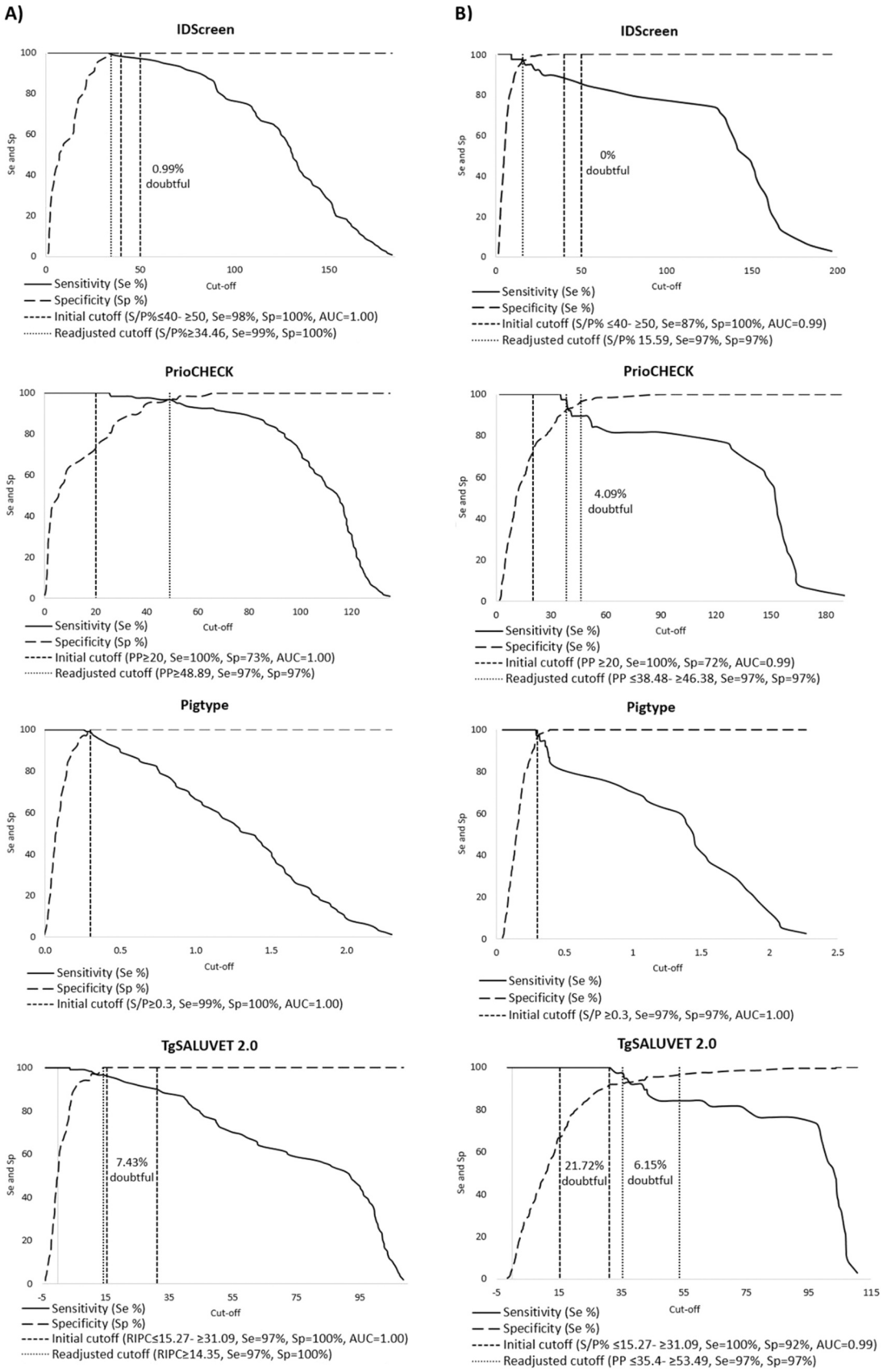

Fig. 4. Two-graph receiver operating characteristic (TG-ROC) analyses based on the reference criterion. A) Sera from experimentally infected pigs; B) sera from naturally infected pigs. Cutoff, sensitivity (Se), specificity (Sp), and area under the curve (AUC) values are shown for each ELISA.

Table 3
Agreement (*kappa* value) among techniques using sera from *Toxoplasma gondii* experimentally or naturally infected pigs before (b) and after (a) the TG-ROC analysis.

Experimental infections	IDScreen		PrioCHECK		Pigtype		TgSALUVET ELISA 2.0		TgSALUVET WB		TgSALUVET IFAT	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	**0.71**	0.90	0.90	0.90	0.91	0.90	0.83	0.84	0.85	0.90
PrioCHECK	**0.71**	0.90	1.00	1.00	**0.72**	0.86	**0.67**	0.92	**0.63**	**0.79**	**0.75**	0.90
Pigtype	0.90	0.90	**0.72**	0.86	1.00	1.00	0.91	0.91	0.82	0.82	0.86	0.86
TgSALUVET ELISA 2.0	0.91	0.90	**0.67**	0.92	0.91	0.91	1.00	1.00	0.89	0.81	0.85	0.86
TgSALUVET WB	0.83	0.84	**0.63**	**0.79**	0.82	0.82	0.89	0.81	1.00	1.00	0.80	0.80
TgSALUVET IFAT	0.85	0.90	**0.75**	0.90	0.86	0.86	0.85	0.86	0.80	0.80	1.00	1.00
Natural infections	**IDScreen**		**PrioCHECK**		**Pigtype**		**TgSALUVET ELISA 2.0**		**TgSALUVET WB**		**TgSALUVET IFAT**	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	0.36	**0.82**	0.74	0.77	0.65	0.79	0.73	0.63	**0.91**	**0.80**
PrioCHECK	0.36	**0.82**	1.00	1.00	0.43	0.71	0.46	0.78	0.30	0.71	0.39	**0.82**
Pigtype	0.74	0.77	0.43	0.71	1.00	1.00	0.72	0.75	0.64	0.64	0.77	0.77
TgSALUVET ELISA 2.0	0.65	0.79	0.46	0.78	0.72	0.75	1.00	1.00	0.55	0.73	0.70	**0.86**
TgSALUVET WB	0.73	0.63	0.30	0.71	0.64	0.64	0.55	0.73	1.00	1.00	0.73	0.73
TgSALUVET IFAT	**0.91**	**0.80**	0.39	**0.82**	0.77	0.77	0.70	**0.86**	0.73	0.73	1.00	1.00

The lowest kappa values ($k \leq 0.80$) are marked in bold letters.
The highest kappa values ($k \geq 0.80$) are marked in bold letters.

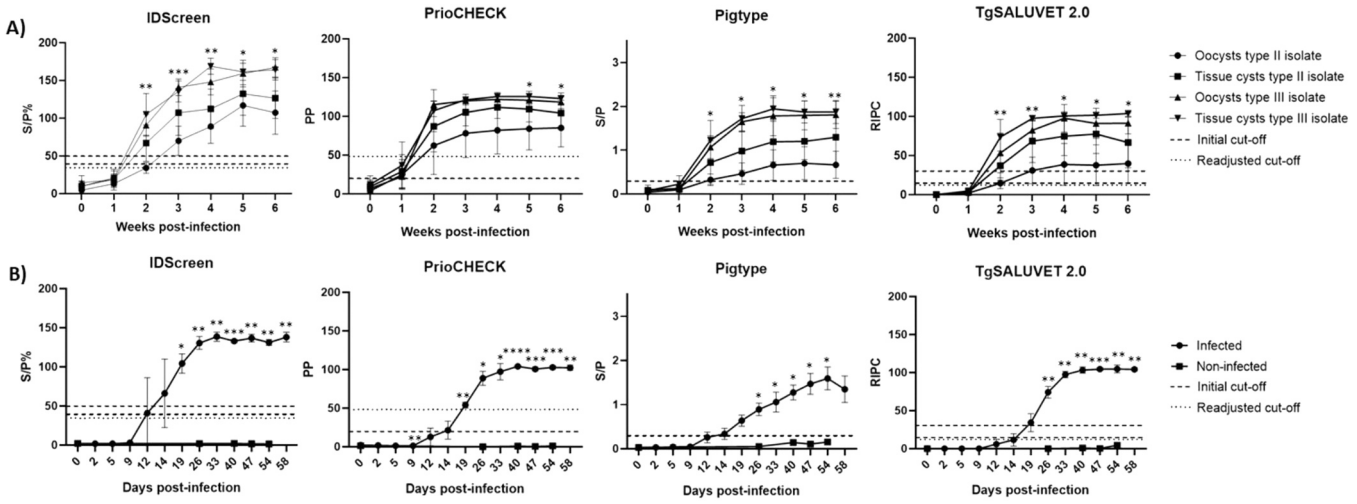

Fig. 5. Anti-*Toxoplasma gondii* IgG kinetics as assessed by different ELISAs in pigs experimentally infected with *T. gondii*. Data are presented as the mean in S/P%, PP, S/P or RIPC (as specified by manufacturers) by experimental group ± standard deviation (SD). Placed horizontally: A) Panel 1: significant differences between experimental groups within sampling weeks (Tukey's multiple comparisons test, representing the highest *P* value within sampling week; *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$). B) Panel 2: significant differences between the IgG mean levels at 0 dpi *vs.* 2–58 dpi (Dunnett's multiple comparisons test; *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$ and ****= $P < 0.0001$). No comparisons were carried out between infected and noninfected pigs since there were missing samples on some sampling days.

obtain reliable and comparable results by IFAT tests, as done herein. Indeed, a previous interlaboratory study of serological tests employed in the diagnosis of bovine neosporosis evidenced high discrepancies among IFAT tests (Campero et al., 2018).

These results, together with the results obtained with commercial ELISAs in the comparative study, showed the usefulness of sera from experimentally infected animals for the validation of serological tests. In general terms, all tests showed excellent diagnostic performance when analyzing sera from experimental infections; thus, the cutoffs recommended by the manufacturers can be employed, except for PrioCHECK (Se= 100%, Sp= 73%, $k = 0.63$–0.75). Good diagnostic performance with sera from experimental infections is expected since these sera are from controlled infections and homogeneous experimental groups. Accordingly, the diagnostic performance of these serological tests could be overestimated. A previous comparative study of serological tests used for the diagnosis of bovine neosporosis, which also included sera samples from experimental infections and used the results obtained by the majority of the tests as a reference, also showed good to excellent test performance (Álvarez-García et al., 2013; von Blumröder et al., 2004). However, regarding the serodiagnosis of *T. gondii* infection, in contrast with these outcomes, lower Se values have been reported in previous comparative studies of different tests when using sera from experimental infections, probably due to the different techniques used as references, for which initial validation and performance were not clearly stated in all cases. Among them, a MAT and a rhoptry ELISA showed Se values of 87% and 94%, respectively, but 100% Sp in both cases when an IFAT was used as a reference (Garcia et al., 2006). Al-Adhami and Gajadhar (2014) also reported 85–89% Se and 94–97% Sp for an in-house ELISA when SafePath ELISA, MAT and WB were used as references.

Nevertheless, as expected, the scenario changed when sera from naturally infected pigs were employed following the WOAH recommendations (WOAH, 2023), which better mimics what may occurs in the field (unknown stage, doses and route, presence of co-infections, among other variables). The results evidenced the need for cutoff readjustments, especially for PrioCHECK and IDScreen, since a decrease in both the diagnostic performance and agreement was recorded. There are a

few comparative studies of commercial tests that analyzed sera from naturally infected pigs. IDScreen was used more frequently, but with variable results, probably due to the different tests employed as references. For example, in contrast to our results, IDScreen showed lower Se when compared to a commercial MAT (Toxo-Screen DA Biomérieux) (Se= 57%, Sp= 99%) (Steinparzer et al., 2015), but high agreement ($k = 0.96$) when compared to an IFAT kit (Fuller Laboratories, Fullerton CA) (Papatsiros et al., 2016), or low to moderate agreement ($k = 0.39–0.68$) when compared to the same commercial MAT kit (Cubas-Atienzar et al., 2019; Pablos-Tanarro et al., 2018; Steinparzer et al., 2015). Other commercial tests previously evaluated were PrioCHECK and Pigtype. In contrast to our results, PrioCHECK obtained lower Se and higher Sp values compared to a commercial MAT (Se= 65%, Sp= 97%, $k = 0.62$), while Pigtype showed the worst performance compared to the same test (Sp= 57%, Sp= 99%, $k = 0.64$) (Steinparzer et al., 2015). However, better performance was reported for PrioCHECK in a comparative study with an in-house TgSAG-1-ELISA, WB and IFAT that used a Bayesian latent class approach, with 99% Se and 93% Sp (Basso et al., 2013). In the present study, PrioCHECK was the test that showed the lowest Sp (72–73%), as well as the lowest agreement ($k = 0.30–0.75$) regardless of the serum panel employed. Based on the comparative study, Pigtype and TgSALUVET IFAT were initially well validated since they maintained high Se and Sp values and did not require cutoff readjustment with any serum panel. Finally, despite the low Se value obtained with TgSALUVET WB in natural infections even after the ELISA cutoff readjustments (Se= 79%), the criterion of positivity could not be changed since a lower intensity and frequency of IDAs recognition was observed with this serum panel. For example, it was found that the 9–10 kDa antigen, which was frequently detected with medium-high intensity in experimentally infected pigs (Table 2), was present in less than 50% of seropositive pigs from Panel 3 based on the defined criterion of positivity. Thus, our results demonstrate that TgSALUVET WB could be used as a reference test in an initial validation of serological techniques, but it is not recommended for routine serodiagnosis.

All in-house and commercial tests detected seroconversion at 2–3 wpi on average, except for PrioCHECK, which was earlier and could be directly related to its high Se but low Sp. Comparable anti-*T. gondii* IgG kinetics have been reported in other studies based on some of the ELISAs tested here and performed with pigs experimentally infected with *T. gondii* oocysts. One of them documented seroconversion from 1.5 to 2 wpi until the end of the experiment (8–30 wpi) based on PrioCHECK or IDScreen (Kauter et al., 2022), whereas the other reported a significant IgG increase from 14 dpi based on TgSALUVET ELISA 2.0 prior to its validation for diagnostic purposes (Largo-de la Torre et al., 2022). Interestingly, all ELISA tests, as well as TgSALUVET WB, showed significantly higher levels of anti-*T. gondii* IgGs in pigs infected with the type III isolate with respect to the type II isolate, with an earlier IgG level increase detected with IDScreen, Pigtype and TgSALUVET ELISA 2.0, from 2 wpi onward, but a more delayed increase with TgSALUVET WB and PrioCHECK, from 3 and 5 wpi, respectively. The higher antibody levels associated with the type III isolate could be explained by the higher virulence and earlier dissemination of this isolate. This was demonstrated previously in a mouse model where higher rates of mortality and morbidity of type III were shown compared to those with a set of type II isolates (Fernández-Escobar et al., 2021, 2020). Higher virulence and earlier dissemination have also been documented in type III *vs.* type II isolates in a piglet model of toxoplasmosis, with higher temperatures for a longer period in pigs infected with the type III isolate, as well as a higher frequency of detection and parasite burden quantified by qPCR in mice inoculated with tissue originating from pigs infected with the type III isolate (Largo-de la Torre et al., 2022). In this experiment, pigs infected with the type III isolate also showed higher IgG levels with respect to the other group based on TgSALUVET ELISA 2.0, while no significant differences were recorded between groups when using PrioCHECK (Largo-de la Torre et al., 2022), which agrees with the present results.

Multiple variables, such as the reference sera, the methodology and the antigen used, influence the performance of a test and therefore are key points to be considered when validating a serological technique (WOAH, 2021). Unfortunately, there is no sera panel that can represent all target infected populations and environmental conditions (WOAH, 2009) or commonly accepted reference tests. Consequently, in this comparative study, we used different panels of sera and employed the results obtained by most of the included tests as reference criteria, an approach that seems to be appropriate for further studies. The usefulness of the different serum panels has been discussed above. However, other more sophisticated statistical approaches, such as the Bayesian Latent Class Models, are highly recommended in the absence of a gold standard test (Johnson et al., 2019; Kostoulas et al., 2017). On the other hand, limited conclusions could be drawn with respect to the influence of the antigen employed since the different tests that showed good diagnostic performance employed different antigens (Table 1). Nevertheless, it was reported for small ruminants that false-positive reactions can be found with anti-*N. caninum* antibodies in TgSAG1-based tests (WB and IDScreen) due to cross reactions (Huertas-López et al., 2021; Sánchez-Sánchez et al., 2021). To date, there are only a few studies about *N. caninum* seroprevalence in domestic pigs (Damriyasa et al., 2004; Lopes et al., 2021; Villa et al., 2022), probably because it has low relevance in this species since its contribution to inducing transplacental infections and reproductive failures in natural infections is still unclear (Snak et al., 2019). However, the presence of anti-*N. caninum* antibodies in naturally exposed domestic pigs has been documented (Damriyasa et al., 2004; Lopes et al., 2021; Villa et al., 2022), so cross-reactivity with this parasite, as well as with other closely related ones, should be considered in future studies.

It must be taken into consideration that serum, plasma, or meat juice can be used in some of the commercial ELISA kits, and different performances have been observed in the same test based on the type of sample (Garcia et al., 2008). Thus, similar approaches should be followed with any other sample types (*e.g.*, meat juice, whole blood) and with sera from other host species considering the multispecies specifications of some of the commercial ELISA kits evaluated here.

5. Summary and conclusions

We offer updated performance information of widely employed serological tests, including commercially available methods that are accessible for research and diagnostic laboratories. In this comparative study, all tests proved to be well validated when analyzing sera from experimental infections, except for PrioCHECK, but several ELISAs required further readjustments for sera originating from natural infections to obtain harmonized data. TgSALUVET WB, which turned out to be useful as a reference test for an initial validation of serological techniques, is not recommended for routine diagnosis. These results must be taken into consideration for epidemiological surveys and studies that aim to evaluate IgG kinetics based on different stages or isolates since some tests were more sensitive than others in detecting variations among experimental groups. The establishment of an international network for creating a common well-characterized serum biobank would favor future test validations.

CRediT authorship contribution statement

Blaga Radu: Writing – review & editing, Investigation. **López-Ureña Nadia María:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Calero-Bernal Rafael:** Writing – review & editing, Visualization, Supervision, Resources, Methodology. **González-Fernández Nuria:** Methodology, Investigation, Formal analysis, Data curation. **Álvarez-García Gema:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis,

Conceptualization. **Koudela Bretislav:** Writing – review & editing, Investigation. **Ortega-Mora Luis Miguel:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nadia Maria Lopez-Urena, Rafael Calero-Bernal, Radu Blaga, Bretislav Koudela, Luis Miguel Ortega-Mora, Gema Alvarez-Garcia reports financial support was provided by Horizon Europe.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.110024.

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