Validation and field evaluation of a competitive inhibition ELISA based on the recombinant protein tSAG1 to detect anti-*Neospora caninum* antibodies in sheep and goats

María Belén Novoa (Conceptualization) (Methodology) (Formal analysis) (Investigation) (Validation) (Writing - original draft), Nerina Patricia Aguirre (Methodology) (Investigation), Nadia Ormaechea (Investigation), Sebastián Palmero (Investigation), Lisandro Rouzic (Investigation), Beatriz Susana Valentini (Investigation), Macarena Sarli (Investigation), Viviana Mercedes Orcellet (Investigation) (Resources), Rafael Marengo (Investigation) (Resources), Victor René Vanzini (Supervision), María Evangelina Primo (Conceptualization) (Methodology) (Formal analysis) (Validation) (Writing - review and editing)

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Running head: tSAG1-based ciELISA for anti-*N. caninum* antibodies detection in sheep and goats.

María Belén Novoa[a,b,*], Nerina Patricia Aguirre[a], Nadia Ormaechea[c], Sebastián Palmero[c], Lisandro Rouzic[c], Beatriz Susana Valentini[a], Macarena Sarli[a,b], Viviana Mercedes Orcellet[c], Rafael Marengo[c], Victor René Vanzini[a], María Evangelina Primo[a,b].

[a]Laboratory of Veterinary Immunology and Parasitology, National Institute of Agricultural Technology (INTA), Rafaela Agricultural Experimental Station, National Route 34, km 227 (2300), Rafaela, Santa Fe, Argentina.

[b]National Scientific and Technical Research Council (CONICET), Santa Fe, Argentina.

[c]Faculty of Veterinary Science, National University of the Littoral (UNL), Kreder 2805 (S3080HOF), Esperanza, Santa Fe, Argentina.

[*]Corresponding author: María Belén Novoa, Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, Ruta 34 km 227 (2300), Rafaela, Santa Fe, Argentina. novoa.maria@inta.gob.ar

Highlights

- ciELISA$_{tSAG1}$ can differentiate *N. caninum*-infected and uninfected sheep and goats.
- ciELISA$_{tSAG1}$ was highly sensitive and specific to detect anti-*N. caninum* antibodies.
- Cross-reactions were not observed between tSAG1 and anti-*Toxoplasma gondii* antibodies.
- At field evaluation the concordance between ciELISA$_{tSAG1}$ and IFAT was very good.
- The production of tSAG1 recombinant protein is simple and standardized.

Abstract

Neospora caninum is a protozoan parasite that causes abortion and reproductive failure in small ruminants. We validated and evaluated under field conditions a competitive inhibition ELISA based on the truncated SAG1 protein (tSAG1) from *N. caninum* for the detection of anti-*N. caninum* antibodies in sheep and goat flocks. The assay was validated using 80 positive and 142 negative serum samples from sheep and goats analyzed by IFAT and immunoblot (IB). ciELISA$_{tSAG1}$ was then used to evaluate the prevalence of anti-*N. caninum* antibodies in 1449 goats from 143 flocks and 385 sheep from 40 flocks and compared to IFAT. The prevalence of anti-*Toxoplasma gondii* antibodies was evaluated by IFAT. The ciELISA$_{tSAG1}$ cut-off was ≥ 36 percent inhibition, with a diagnostic sensitivity of 100.0% (95% CI = 95.4–100.0%) and a diagnostic specificity of 98.6% (95% CI = 95.0–99.8%) relative to the agreement

between IFAT and IB. The field evaluation revealed a concordance between ciELISA$_{tSAG1}$ and IFAT of 97.4%, with an agreement (κ) of 0.90 for sheep sera, and a concordance of 96.5% with κ = 0.85 for goat sera. The overall prevalence of anti-*N. caninum* antibodies in sheep was 14.3% by IFAT and 15.8% by ciELISA$_{tSAG1}$. In goats, prevalence was 12.9% by IFAT and 14.6% by ciELISA$_{tSAG1}$. The overall prevalence of anti-*T. gondii* antibodies was 28.8% in goats and 43.8% in sheep. The ciELISA$_{tSAG1}$ could be useful for large-scale detection of anti-*N. caninum* antibodies in sheep and goats, and for seroepidemiological investigations due to its appropriate sensitivity and specificity, and the simplicity of production.

Key words: ciELISA; SAG1; *N. caninum*; diagnosis; sheep; goat; *T. gondii;* prevalence.

1. Introduction

Neospora caninum is an obligate intracellular apicomplexan protozoan that causes neosporosis (Dubey et al., 1988a). In cattle, this disease causes abortions and neurological signs in newborn calves (Barr et al., 1991; Dubey et al., 1992), producing severe economic losses to the cattle industry (Reichel et al., 2013). The ability of *N. caninum* to produce abortion in sheep and goats has been demonstrated experimentally (Arranz-solís et al., 2015; Barr et al., 1992; Buxton et al., 1997; Porto et al., 2016), and the occurrence of abortions and reproductive failure in flocks due to *N. caninum* infection has been confirmed in both species (González-Warleta et al., 2014; Hecker et al., 2019; Moreno et al., 2012; West et al., 2006).

Ruminants are intermediate hosts that become infected through the ingestion of pastures, food or water contaminated with *N. caninum* oocysts shed in feces of canids,

the definitive hosts. *N. caninum* can also be transmitted transplacentally, from the dam to the fetus (Dubey et al., 2007). The parasites remain quiescent in tissue cysts, and reactivation occurs under circumstances of immunosuppression such as pregnancy (Guido et al., 2016). In this case, parasite reactivation can cause abortion or congenital infection (González-Warleta et al., 2018). There is no effective chemotherapeutic treatment or vaccine for the prevention of *N. caninum* infection; it can be partially controlled using management strategies based on efficient detection tests (Reichel et al., 2014).

The seroepidemiology of *N. caninum* infection in small ruminants is partially known worldwide. The indirect immunofluorescent antibody test (IFAT) is still used to detect anti-*N. caninum* antibodies in cattle and small ruminants, although it is a laborious technique and interpretation of results is subjective. Various enzyme linked immunosorbent assays (ELISAs) are available and are replacing IFAT in large-scale seroepidemiological studies in cattle. Some of these assays were adapted for their use in small ruminants (González-Warleta et al., 2014; Pinheiro et al., 2015; Reichel et al., 2008). Commercial kits that use the required anti-species antibodies are available to detect antibodies in cattle and small ruminants (Alvarez-García et al., 2013). Commercial ELISAs available for small ruminants, LSIVet Ruminant (Laboratoire Service International, Lyon, France) and IDEXX Neospora Ab (IDEXX Laboratories, Westbrook, ME), are indirect ELISAs (iELISA) based on tachyzoite lysates that can be used to detect anti-*N. caninum* antibodies in sheep and goats. The cytoplasmic antigens of the parasite included in these lysates might detect antibodies against *Toxoplasma gondii*, a closely related parasite with high prevalence in sheep and goat flocks worldwide (Lindsay and Dubey, 2020; Tenter et al., 2000). Recombinant antigens may increase test specificity by reducing possible cross-reactions with antibodies against

apicomplexan common antigens. SAG1 protein is immunodominant and conserved among *N. caninum* isolates (Hemphill et al., 1999); therefore, it is a promising candidate antigen to detect anti-*N. caninum* antibodies in different animal species. Competitive ELISA (cELISA) has the benefits of generating less nonspecific reactivity than iELISAs and not requiring species-specific antibodies.

Our group has developed and validated a competitive inhibition ELISA (ciELISA) based on a truncated variant of the major surface antigen (tSAG1) from *N. caninum* tachyzoites produced as a recombinant protein and the RafNeo5 monoclonal antibody (MAb), both produced in our laboratory. This ciELISA$_{tSAG1}$ has been validated to detect anti-*N. caninum* antibodies in cattle, with a diagnostic sensitivity of 98.7% (95% CI = 96.8–99.7%) and a diagnostic specificity of 97.9% (95% CI = 96.4–99.0%) using a cut-off of ≥ 29 percent inhibition (%I) (Novoa et al., 2020). The aim of this study was to validate and evaluate under field conditions the ciELISA$_{tSAG1}$ to detect anti-*N. caninum* antibodies in sheep and goats.

2. Materials and Methods

2.1. Study area

A cross-sectional study was conducted involving 143 goat flocks and 40 sheep flocks in the provinces of Santa Fe and Entre Ríos in Argentina. These flocks were destined for meat production, the most prevalent production type for local consumption in this area. Most of the farms involved in the study had small flocks with fewer than 20 animals and a low level of replacement of breeding animals.

2.2. Serum samples

All animals used in this study were handled in strict accordance with good animal practice and the conditions defined by the Animal Ethics Committee of the Veterinary

Science Faculty of the Littoral National University, Esperanza, Santa Fe, Argentina (license 226/15, file 20,390).

2.2.1. Test Validation

In order to validate the ciELISA$_{tSAG1}$ for detection of anti-*N. caninum* antibodies in small ruminants, 80 positive (40 sheep and 40 goat) and 142 negative (74 sheep and 68 goat) serum samples were used. Negative sera belonged to negative flocks and positive sera to flocks with positive animals due to natural infection. The sera were analyzed using IFAT and immunoblot (IB), and the animals were categorized as infected or uninfected when there was agreement between the assays.

2.2.2. Control Sera

Control sheep serum negative to *N. caninum* and *T. gondii* (sNcTgC-) was obtained from a sheep confirmed as uninfected after 3 serum samplings taken at 30-d intervals, analyzed by IFAT and IB for both parasites. This sheep was then inoculated intravenously with 1 dose of 10^6 live tachyzoites of *N. caninum* (Buxton et al., 2001). The sheep weak positive control (sNcC+) and the sheep strong positive control (sNcC++) sera were obtained after bleeding the sheep 21 and 42 d after inoculation, respectively, and stored in aliquots at -20°C until use.

Goat positive control to *N. caninum* and *T. gondii* (gNcC+, gTgC+) and goat negative control (gNcTgC-) to *N. caninum*, consisted of a pool of positive goat sera from naturally infected animals and a pool of negative goat sera of animals from *N. caninum* and *T. gondii* infection-free flocks, respectively, tested by IFAT and IB.

Sera from a sheep experimentally infected with *T. gondii* was used to evaluate cross-reactivity of antibodies against *T. gondii* with tSAG1 protein. In order to obtain this serum, a sheep negative to anti-*N. caninum* antibodies by IFAT and IB was infected with 10^6 tachyzoites of *T. gondii* (Lopes et al., 2011). Infection was confirmed by IFAT

and sera obtained 42 d after experimental inoculation was used as a sheep C+ to *T. gondii* (sTgC+). The RafNeo5 MAb that specifically recognizes the SAG1 protein from *N. caninum* was also used as a positive control in the evaluation of tSAG1 recombinant protein. This is a mouse IgG1 kappa isotype MAb obtained previously by immunization of mice with a soluble fraction of sonicated tachyzoites (Valentini et al., 2016).

2.2.3. Field Evaluation

For the field evaluation of the ciELISA$_{tSAG1}$, serum samples of 1449 goats from 143 flocks and 385 sheep from 40 flocks were analyzed by IFAT and ciELISA$_{tSAG1}$. Additionally, an IB was performed on 50 serum samples of the total of 61 that showed disagreement between IFAT and ciELISA$_{tSAG1}$. IFAT to detect anti-*T. gondii* antibodies was also performed in these serum samples.

2.3. Parasites

Tachyzoites of NC-1 strain of *N. caninum* (Dubey et al., 1988b) were maintained in vitro, harvested and purified as described previously (Novoa et al., 2020).

2.4. Immunoblot

Specific anti-*N. caninum* antibodies were detected by IB as described previously (Campero et al., 2015; Schares et al., 1999). Control sera and sheep and goat serum samples were diluted 1:100 in tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.05% V/V of Tween-20 and 5% W/V of skimmed milk (Svelty; Nestlé, Buenos Aires, Argentina) (TBS-T-M). Anti-sheep or anti-goat IgG peroxidase conjugates produced in rabbit (1:1,000 in TBS-T-M) (Sigma-Aldrich) were used. The antigen-antibody reaction was revealed with the colorimetric substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) and 0.1% V/V of hydrogen peroxide (H_2O_2). The reaction against immunodominant antigens (IDAs) with relative molecular masses of 17, 21, 29, 30, 33, 37, 43, 55 and about 70-80 kDa was

recorded (Schares et al., 1999; Staubli et al., 2006). A serum sample was considered positive when two or more IDAs were recognized (Staubli et al., 2006).

Cross-reactivity of antibodies from *T. gondii* experimentally infected sheep to tSAG1 was analyzed by IB using purified tSAG1 as antigen. tSAG1 was separated by electrophoresis in an SDS-polyacrylamide gel under non-reducing conditions and transferred to an adsorbent nitrocellulose membrane. Different strips of the nitrocellulose membrane were incubated with control sera sNcTgC-, sNcC+ and sTgC+, diluted 1:100 in TBS-T-M, and with the MAb RafNeo5 that specifically recognizes SAG1 *N. caninum* protein in a 1:1,000 dilution. After 3 washes, anti-sheep IgG peroxidase conjugate (Sigma-Aldrich) diluted 1:1,000 in TBS-T-M was added to the nitrocellulose membrane previously incubated with the serum samples. Anti-mouse IgG peroxidase conjugate (Jackson Immunoresearch Laboratories) diluted 1:1,000 in TBS-T-M was added to the nitrocellulose membrane previously incubated with the MAb. The reaction was revealed as described above.

2.5. Indirect fluorescent antibody test

For the indirect fluorescent antibody test (IFAT) to detect anti-*N. caninum* or anti-*T. gondii* antibodies, purified whole tachyzoites were used according to the technique described previously, with modifications (Novoa et al., 2020; Pare et al., 1995). Test sera diluted 1:50 was considered the cut-off (Hecker et al., 2013; Jolley et al., 1999), and the mouse anti-sheep/goat IgG fluorescein isothiocyanate-conjugate (Sigma-Aldrich) was diluted 1:400 in phosphate-buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Slides were observed under an epi-fluorescence microscope (Eclipse 80i; Nikon, Melville, NY). Fluorescence over the whole tachyzoite surface was considered positive, and on the apical nonspecific part was considered negative (Björkman and Uggla, 1999).

2.6. Competitive-inhibition ELISA based on tSAG1 and RafNeo5 MAb

ciELISA$_{tSAG1}$ was performed as described for cattle (Novoa et al., 2020). Each ELISA plate included control serum (sNcC++, sNcC+, sNcTgC-, gNcC+, gNcTgC-) and RafNeo5 MAb without serum (Cc) to determine its maximal $OD_{405\ nm}$. The color of the reaction was measured at 405 nm in an ELISA plate reader (Labsystems Multiskan FC; Microlat, Buenos Aires, Argentina). Results were expressed as %I, according to the formula %I = 100 – [(sample OD/Cc OD) x 100].

2.7. Data analyses

A receiver operating characteristic (ROC) analysis (MedCalc 13.0) was performed with the serum samples used for the test validation to determine the optimal cut-off value, diagnostic sensitivity (D-Se), and diagnostic specificity (D-Sp), with 95% CI for ciELISA$_{tSAG1}$.

The prevalence of antibodies against *N. caninum* and *T. gondii* in sheep and goat flocks was analyzed. Concordance (%) and kappa values (κ) between IFAT and ciELISA$_{tSAG1}$ tests, used for determination of anti-*N. caninum* antibodies, with 95% CI, were evaluated using the software MedCalc. Strength of agreement was considered poor ($\kappa \leq 0.20$), fair ($\kappa = 0.21$-0.40), moderate ($\kappa = 0.41$-0.60), good ($\kappa = 0.61$-0.80), or very good ($\kappa = 0.81$-1.00) (Altman, 1990).

3. Results

3.1. Immunoblot

The IB performed with tSAG1 antigen revealed specific antigen-antibody reactions between sNcC++ serum and tSAG1, showing a thick band between 25 and 37 kDa markers, and a weak band between 50 and 75 kDa markers. The molecular weights obtained for the bands correspond to the expected monomeric and dimeric forms of tSAG1. The same pattern was revealed in the nitrocellulose membrane incubated with

RafNeo5 MAb. sTgC+ and sNcTgC- sera did not reveal antigen-antibody reactions (Fig. 1).

3.2. Validation of ciELISA$_{tSAG1}$

The ciELISA$_{tSAG1}$ cut-off determined with the 222 serum samples used for test validation was ≥ 36%I, with a D-Se of 100.0% (95% CI = 95.4–100.0%) and a D-Sp of 98.6% (95% CI = 95.0–99.8%). The mean %I and standard deviation (SD) were 86% (± 17%) for positive and 11% (± 16%) for negative sera (Fig. 2).

3.3. Field evaluation

3.3.1. Goat Flocks

Concordance between ciELISA$_{tSAG1}$ and IFAT in field sera was 96.5% with a κ value of 0.85 (95% CI = 0.81–0.89). The overall prevalence of anti-*N. caninum* antibodies in goats was 12.9% by IFAT and 14.6% by ciELISA$_{tSAG1}$. Anti-*N. caninum* antibodies were detected in 55.3% (*n* = 79) and 63.6% (*n* = 91) of the 143 goat flocks analyzed by IFAT and ciELISA$_{tSAG1}$, respectively. The prevalence of anti-*N. caninum* antibodies in flocks ranged from 0 to 90% (Fig. 3). Using the established ≥ 36%I cut-off, the mean %I (± SD) at ciELISA$_{tSAG1}$ was 84% (± 23%) and 7% (± 15%) for positive and negative goat sera, respectively (Fig. 4). The overall prevalence of anti-*T. gondii* antibodies in goats was 28.8%. Anti-*T. gondii* antibodies were detected in 79.9% (*n* = 114) of the 143 goat flocks. The prevalence in flocks ranged from 0 to 100% (Fig. 3). Sixty and 62 of the serum samples from the 417 goats that were positive to *T. gondii* were also positive to *N. caninum* by IFAT or ciELISA$_{tSAG1}$, respectively.

3.3.2. Sheep flocks

In sera from sheep flocks, the concordance between ciELISA$_{tSAG1}$ and IFAT was 97.4% with a κ value of 0.90 (95% CI = 0.84–0.96). The overall prevalence of anti-*N. caninum* antibodies in sheep was 14.3% by IFAT and 15.8% by ciELISA$_{tSAG1}$. Anti-*N. caninum*

antibodies were detected in 65.0% (*n* = 26) and 67.5% (*n* = 27) of the 40 sheep flocks analyzed by IFAT and ciELISA$_{tSAG1}$, respectively. The prevalence of anti-*N. caninum* antibodies in sheep flocks ranged from 0 to 60% (Fig. 3). Using the established ≥ 36%I cut-off, the mean %I (± SD) at ciELISA$_{tSAG1}$ was 84% (± 23%) and 9% (± 16%) for positive and negative sheep sera, respectively (Fig. 4). The overall prevalence of anti-*T.gondii* antibodies in sheep was 43.9%. Anti-*T.gondii* antibodies were detected in 95% (*n* = 38) of the 40 sheep flocks. The prevalence in flocks ranged from 0 to 100% (Fig. 3). Thirty-three and 36 of the serum samples from the 169 sheep that were positive to *T. gondii* were also positive to *N. caninum* by IFAT or ciELISA$_{tSAG1}$, respectively.

3.3.3. Disagreement between IFAT and ciELISA$_{tSAG1}$

There was no agreement between IFAT and ciELISA$_{tSAG1}$ in 61 (3.5% of the goat serum samples and 2.6% of the sheep serum samples) of the 1834 evaluated sera. IB was performed on 50 of those serum samples (42 from goat and 8 from sheep). Of these, 37 serum samples were positive by ciELISA$_{tSAG1}$ and 13 were positive by IFAT. The results of the IB in 25 samples (17 positives and 8 negatives) agreed with those of the ciELISA$_{tSAG1}$, and in the other 25 samples (5 positives and 20 negatives), they agreed with those of the IFAT. From the total serum samples that were positive to *T. gondii* (n = 586), 17 showed disagreement between IFAT and ciELISA$_{tSAG1}$ in the detection of anti-*N. caninum* antibodies. In those serum samples, the IB results showed agreement with the ciELISA$_{tSAG1}$ results in 10 serum samples and with IFAT in 6 serum samples. One serum sample was unavailable to perform the IB.

4. Discussion

ciELISA$_{tSAG1}$, which was validated for use in cattle, can be used to differentiate *N. caninum*-infected sheep and goats from uninfected ones. The Se and Sp results obtained at validation demonstrated great potential of the studied test, with 100% and 98.6%,

respectively. These results were supported by the field study with a large number of sera, in which the concordance between IFAT and ciELISA$_{tSAG1}$ was 96.5% and 97.4% for goat and sheep sera, respectively. Strength of agreement between assays was very good, as demonstrated by a κ value of 0.85 for goat sera and of 0.90 for sheep sera. These concordance results obtained with a single cut-off point are a benefit of the ciELISA$_{tSAG1}$, since it can be used to detect anti-*N. caninum* antibodies in mixed flocks without the need to process goat and sheep sera separately. In the analyzed field samples with disagreement between IFAT and ciELISA$_{tSAG1}$, the concordance between ELISA and IB or IFAT and IB was the same (50%). The IFAT is the most widely used technique nowadays, despite its disadvantages: it is laborious, subjective and a small amount of sera can be processed per slide. The very good κ value obtained between IFAT and the ciELISA$_{tSAG1}$ allows the use of the ciELISA$_{tSAG1}$ for anti-*N. caninum* antibody detection in sheep and goats. Commercial iELISA for small ruminants, LSIVet Ruminant and IDEXX Neospora Ab are based on tachyzoite lysates (Alvarez-García 2013; Reichel et al., 2008). ciELISA$_{tSAG1}$ has the benefit that its antigen is a recombinant protein. tSAG1 recombinant production does not depend on the maintenance of cell culture; rather, it can be produced easily in large quantities and is homogeneous in different production batches. These characteristics improve the standardization of antigen production for test kit development. Furthermore, in the IB performed with tSAG1 as antigen, we demonstrated that tSAG1 recombinant protein did not react with sera from animals infected with *T. gondii*, an important requirement when working with species that have a high prevalence of toxoplasmosis (Dubey, 2009). In addition, the commercial vaccine for toxoplasmosis is a live attenuated vaccine (Buxton, 1993) and generates antibodies against *T. gondii* proteins that could be homologous to *N. caninum* proteins (Liao et al., 2005). The IFAT performed to detect

anti-*T. gondii* antibodies at the field study strengthen this statement, given that of the 586 serum samples that were positive for the detection of anti-*T. gondii* antibodies, 488 tested negative to the ciELISA$_{tSAG1}$. Moreover, when serum samples that tested positive for antibodies against *T. gondii* by IFAT were used to detect anti-*N. caninum* antibodies by ciELISA$_{tSAG1}$ or IFAT the agreement of the IB results was better with the ciELISA$_{tSAG1}$ results than with the IFAT results.

An applied printing immunoassay (APIA) and an iELISA based on recombinant tSAG1 protein were previously evaluated for use in cattle (Chahan et al., 2003; Wilkowsky et al., 2011). A Se of 85% and a Sp of 96% relative to the commercial iELISA IDEXX Neospora Ab were reported for APIA (Wilkowsky et al., 2011). The iELISA reported a Se of 100 % and a Sp of 95,8% relative to IB (Chahan et al., 2003). These two test were not evaluated with small ruminant sera. Some iELISAs based on recombinant proteins previously validated for cattle were adapted for their use in small ruminants. In sheep samples, an iELISA based on SRS2 recombinant protein showed a Se of 100% and a Sp of 94.5% (Pinheiro et al., 2015) and an iELISA that associates SAG1 and SRS2 recombinant proteins showed a Se of 100% and a Sp of 97,2% relative to IFAT results (Sinnot et al., 2020). A Se of 100% and a Sp of 98.6% were obtained by ciELISA$_{tSAG1}$ using 222 serum samples from sheep and goats categorized as infected or uninfected when there was agreement between IFAT and IB. The comparison of ciELISA$_{tSAG1}$ with a combination of assays instead of a unique assay, reduces the rate of false positivity or false negativity of the standard of comparison (Jacobson, 1998). The sensitivity of the test is favored by the fact that sera can be diluted 1:2 given that the ciELISA$_{tSAG1}$ has less nonspecific reactivity than the iELISAs. The very good agreement results obtained between IFAT and ciELISA$_{tSAG1}$ when using 1834 samples from a field study, supports the usefulness of the test.

The prevalence of anti-*N. caninum* antibodies (15.8% for sheep and 14.6% for goats) detected by ciELISA$_{tSAG1}$ was higher than values estimated in other regions of Argentina (Gos et al., 2017; Hecker et al., 2013). Our study detected a prevalence of anti-*N. caninum* antibodies similar to values detected in the southeast of Brazil, north of the analyzed region (Andrade et al., 2013; Figliuolo et al., 2004). The prevalence of anti-*T. gondii* antibodies detected was similar to those detected in other studies, with a large number of infected flocks (Gos et al., 2017, Hecker et al., 2013; Fligliuolo et al., 2004). The difference in the prevalence of neosporosis between the littoral region and other regions in Argentina and its similarity with southern brazil region could be associated with climatic factors, type of production or management system, since this difference was previously described in cattle (Novoa et al., 2020). The risk factors involved in the seroprevalence of neoporosis in the littoral region of Argentina will be analyzed in ongoing studies.

5. Conclusion

The ciELISA$_{tSAG1}$ is suitable for seroepidemiological studies in sheep and goats, since it is simpler and more objective than IFAT, and a very good agreement between both techniques was observed. The polarized distribution of the positive and negative results in ciELISA$_{tSAG1}$ allows an increase or decrease of the cut-off under different epidemiological situations, without significantly affecting the diagnostic sensitivity and specificity (Wright et al., 1993). Investigations at a larger scale are required to provide detailed information of the seroprevalence of anti-*N. caninum* antibodies in small ruminants and to establish the role of *N. caninum* in reproductive losses in sheep and goat flocks worldwide and the ciELISA$_{tSAG1}$ is a useful tool to achieve this purpose.

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Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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CRediT authorship contribution statement

María Belén Novoa: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Writing - Original Draft

Nerina Patricia Aguirre: Methodology, Investigation

Nadia Ormaechea: Investigation

Sebastián Palmero: Investigation

Lisandro Rouzic: Investigation

Beatriz Susana Valentini: Investigation

Macarena Sarli: Investigation

Viviana Mercedes Orcellet: Investigation, Resources

Rafael Marengo: Investigation, Resources

Victor René Vanzini: Supervision

María Evangelina Primo: Conceptualization, Methodology, Formal analysis, Validation, Writing - Review & Editing

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Figure 1. Immunoblot analysis of recombinant tSAG1 antigen. Lane 1: Sheep serum positive to *N. caninum* ; Lane 2: Sheep serum positive to *Toxoplasma gondii*; Lane 3: Sheep serum negative to *N. caninum* and *T. gondii*. Sera belong to sheep experimentally infected with *N. caninum*, *T. gondii*, or an uninfected sheep. Lane 4: RafNeo5 MAb. MW = molecular weight marker Precision Plus Protein All Blue Pre-stained Protein Standards (Bio-Rad Laboratories).

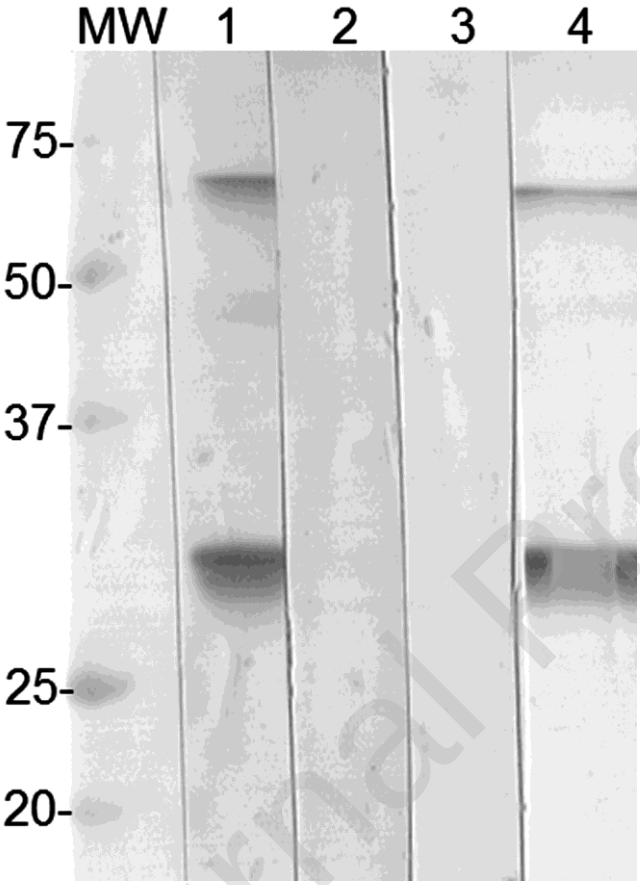

Figure 2. ROC analyses of the ciELISA$_{tSAG1}$ for diagnosis of neosporosis in small ruminants. Determination of the cut-off using 80 positive and 142 negative serum samples from sheep and goats. Results are expressed as percentage of inhibition (%I).

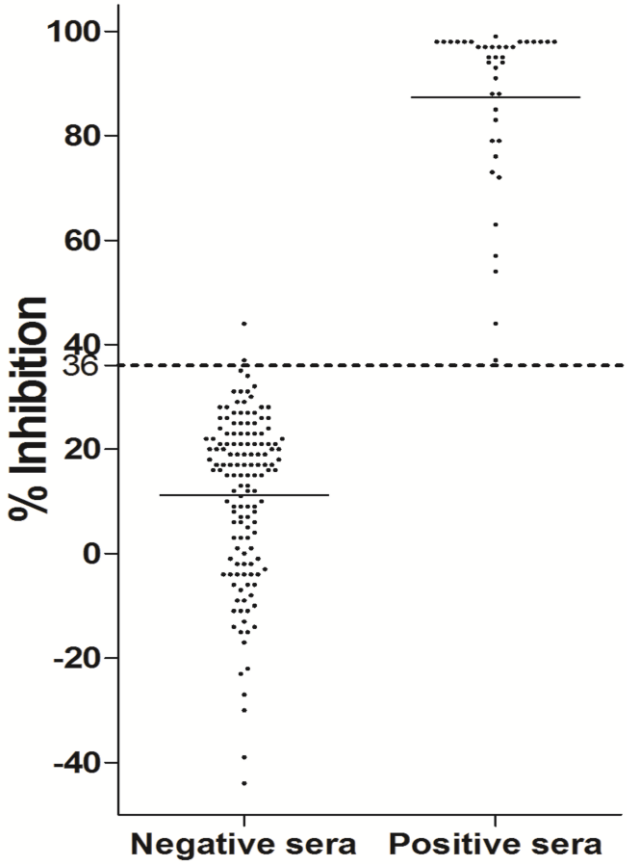

Figure 3. Frequency distribution of *T. gondii* infection prevalence analyzed using IFAT (black bars) and *N. caninum* infection prevalence analyzed using IFAT (grey bars) and ciELISA$_{tSAG1}$ (dotted white bars) in 143 goat flocks and 40 sheep flocks. The amount of flocks is shown as a percentage of the total of flock for each species.

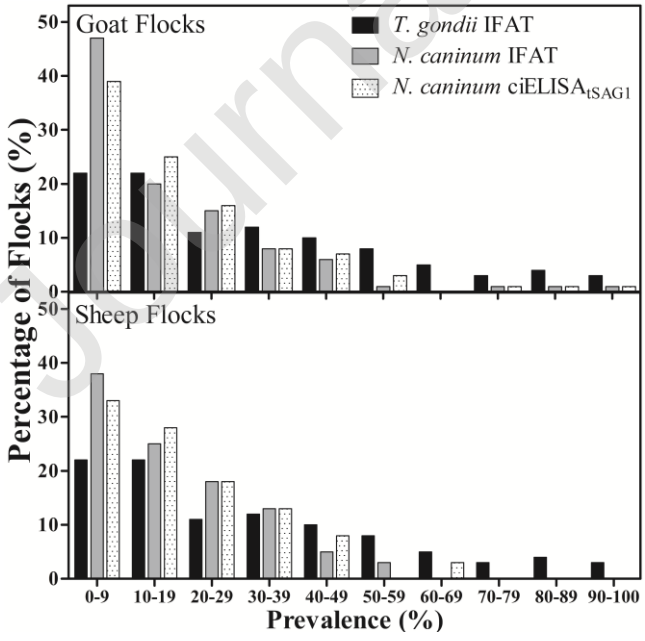

Figure 4. Field study of agreement between IFAT and ciELISA$_{tSAG1}$ in 1449 goat serum samples from 143 flocks and 385 sheep serum samples from 40 flocks. The cut-off ≥ 36% determined at validation was used. ciELISA$_{tSAG1}$ results are expressed as percentage of inhibition (%I).