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Research paper

A time-resolved fluorescence immunoassay for the detection of anti-*Neospora caninum* antibodies in sheep

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ABSTRACT

Neospora caninum is a protozoan parasite (Phylum Apicomplexa) that has been recently suggested as a relevant cause of reproductive disorders in small ruminants. The aim of the present study is to develop and validate a new serological test based on time resolved fluorescency using *N. caninum* GRA7 recombinant antigen (GRA7-TRFIA) for the detection of *N. caninum* antibodies in sheep. A total of 346 serum samples (208 from experimentally infected sheep, 117 from a dairy farm with a previous history of *Neospora*-associated abortion, and 21 negative sera) were used. The validation of the new assay was performed by the evaluation of assay precision, analytical sensitivity (Se), accuracy and cross reactivity. In the experimentally infected sheep, antibody kinetics was compared between GRA7-TRFIA and an in house *N. caninum* tachyzoite soluble extract-based ELISA (NcSALUVET ELISA) by Wilcoxon matched-pairs signed rank test. The cut-off and diagnostic Se and specificity (Sp) of GRA7-TRFIA was estimated by ROC analysis with field samples. In addition, concordance and correlation between GRA7-TRFIA and a commercial ELISA and NcSALUVET ELISA were assessed by *kappa* value and Spearman correlation coefficient, respectively.

Overall, GRA7-TRFIA showed an adequate precision, analytical Se and accuracy to detect anti-*N. caninum* antibodies in ovine serum, and no cross reactivity with the closely related protozoan *Toxoplasma gondii*. In naturally infected sheep, 100% Se and 95.35% Sp were obtained for a cut-off point of 62.68 Units of Fluorometry for *N. caninum* (UFN). Moreover, GRA7-TRFIA allowed earlier detection of *N. caninum* infection than NcSALUVET ELISA in experimentally infected sheep.

1. Introduction

Neospora caninum is a protozoan of the Subphylum Apicomplexa that causes neosporosis (Lindsay and Dubey, 1989). In cattle, this disease is characterized by mid-term abortions and congenitally infected calves with occasional malformations (Dubey and Schares, 2011; Dubey et al., 2017). Thus, it has a well-known negative economic impact in farms worldwide (Dubey and Schares, 2011; Reichel et al., 2013). Studies about the spread of the neosporosis and its consequences in small ruminants are relatively recent (West et al., 2006; Moreno et al., 2012; González-Warleta et al., 2014), even though abortions caused by *N. caninum* in sheep were described almost 30 years ago (Dubey and Lindsay, 1990; Hässig et al., 2003). Protozoan diseases causing abortions have been diagnosed for years by histopathological techniques from foetal tissues, *Toxoplasma gondii* being considered the main abortifacient parasite of sheep (Dubey, 2009). However, these techniques have limitations in distinguishing between *N. caninum* and *T. gondii* infections (Moreno et al., 2012; González-Warleta et al., 2014), so it can be assumed that neosporosis could have been underdiagnosed in sheep.

Nowadays, indirect fluorescence antibody test (IFAT) and several enzyme-linked immunosorbent assays (ELISA) are available for detecting anti-*N. caninum* antibodies in small ruminants (Pinheiro et al., 2015; Reichel et al., 2008; Andreotti et al., 2009; González-Warleta et al., 2014). Nevertheless, these methods have some limitations. For example, IFAT is not always suitable for large-scale analysis (Andreotti

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et al., 2009), is subjective, labor-intensive (Reichel et al., 2008) and, in bovine, it is less sensitive than other tests such as ELISA (Álvarez-García et al., 2003). As regards the ELISA techniques, several tests have been developed, but there is still a need to improve the reliability of results (Shaapan et al., 2008; Holec-Gasior, 2013).

Although recombinant antigens are not as sensitive and specific as those assays based on tachyzoite soluble extract for detecting Apicomplexa parasites (Aguado-Martínez et al., 2008), they have shown to be a good alternative providing accurate results with reduced costs (Holec-Gasior et al., 2014; Pinheiro et al., 2015; Abdelbaky et al., 2018). Concretely, recombinant antigens have been previously used for the diagnosis of *N. caninum* in small ruminants, such as NcSRS2 in sheep (Andreotti et al., 2009; Pinheiro et al., 2015), NcSAG1 in sheep and goats (Zhou et al., 2016) and NcMIC10 in goats (Yin et al., 2012). In bovines, among the different recombinant antigens employed, NcGRA7 has proven to be one of the most immunodominant proteins (Álvarez-García et al., 2007) with high diagnostic performance (Huang et al., 2007; Aguado-Martínez et al., 2008; Álvarez-García et al., 2013). Furthermore, NcGRA7 has shown high specificity (Sp) and no cross-reactivity with *T. gondii* in previous studies (Abdelbaky et al., 2018).

Time-resolved fluorescence immunoassays (TRFIA) have shown high sensitivity (Se), with a detection limit lower than ELISA techniques (Martínez-Subiela et al., 2011; Hu et al., 2015). These assays are based on the quantification of the fluorescence emitted by a lanthanide chelate label attached to antigens or antibodies (Martínez-Subiela et al., 2011). For example, these assays have been used successfully to detect progesterone in bovine milk samples (Oku et al., 2011), protein C reactive in cerebrospinal fluid of dogs (Martínez-Subiela et al., 2011) and antibodies anti-*Leishmania infantum* in blood serum and saliva of dogs (Cantos-Barreda et al., 2017a, 2017b). However, TRFIA has not been previously performed in ruminants for the diagnosis of *N. caninum* infection.

Therefore, the aim of the present study was to design and to validate a new serological TRFIA test based on recombinant NcGRA7 protein (GRA7-TRFIA) for the detection of anti-*N. caninum* IgG antibodies in sheep, in order to improve the Se of the current assays. For this purpose, (i) a GRA7-TRFIA was developed and validated, (ii) the ability of the technique to early detect antibodies compared to an in house *N. caninum* tachyzoite soluble extract-based ELISA (NcSALUVET ELISA, performed by Saluvet Innova S.L.) was evaluated with sera from sheep experimentally infected with *N. caninum*, and (iii) the optimal cut-off point was determined using sera from a flock naturally infected with *N. caninum*.

2. Material and methods

2.1. Serum samples

2.1.1. Serum samples from experimentally infected sheep

Serum samples previously used by Sánchez-Sánchez et al. (2018) were employed in this study. Briefly, 208 sera from 23 Rasa Aragonesa breed female sheep were parenterally infected with *N. caninum* tachyzoites at 90 days of pregnancy. Specifically, animals in groups 1–5 (Table 1) were sampled on 0, 3, 5, 7, 10 and 14 days post-infection (dpi), and then every week thereafter until abortion (between 32 and 44 dpi), delivery of weak lambs (between 42 and 51 dpi), or parturition (54 and 60 dpi). Sera were classified as positive and negative by an optimized NcSALUVET ELISA (Sánchez-Sánchez et al., 2018). Sera were stored at -80 °C until used in our trial. Serum samples from group 1 (G1) at 10 dpi and from sheep G1.1, G1.2, and G1.3 at 3 and 5 dpi were not available for its use in our trial.

Table 1
Sheep experimentally infected with *N. caninum* tachyzoites (Sánchez-Sánchez et al., 2018). IV = intravenous; SC = subcutaneous.

Group	G1	G2	G3	G4	G5
Number of infected sheep	6	5	5	4	3
Dose of *N. caninum* tachyzoites	10^5	10^4	10^3	10^2	10^4
Route of inoculation	IV	IV	IV	IV	SC

Analytical validation of the GRA7-TRFIA was carried out comparing the results obtained with TRFIA with those of NcSALUVET ELISA (Sánchez-Sánchez et al., 2018). Thus, it was possible to study the antibody kinetics, evaluating the ability of both tests to detect the presence of specific antibodies in the early stages of infection. For the cross-reactivity study, 22 *T. gondii*-positive sera by an in house *T. gondii* soluble extract antigen-based ELISA (Sánchez-Sánchez et al., 2019).

2.1.2. Serum samples from a naturally N. caninum-infected sheep flock

A total of 117 blood serum samples collected from an Assaf breed dairy female sheep flock (Zamora, Spain) with a history of *N. caninum*-associated abortions were used for the evaluation of the diagnostic Se and Sp of our GRA7-TRFIA assay and, also, to compare these results with those obtained with a commercial ELISA and NcSALUVET ELISA. These sera were classified into 43 negative and 74 positive samples according to the results obtained by the commercial ELISA. They were stored at −80 °C until analysis by GRA7-TRFIA.

2.1.3. Negative serum samples

Twenty-one serum samples from two Rasa Aragonesa breed female sheep from those included as sentinel in Sánchez-Sánchez et al. (2019) study were used as negative control. They were also stored at −80 °C until analysis by GRA7-TRFIA.

2.2. Experimental design

2.2.1. Assay precision

Intra- and inter-assay precision were performed in order to evaluate the assay precision. The intra-assay precision was calculated by analysis of a serum which contains a high amount of anti-*N. caninum* antibodies and another one with low amount, each of them being analyzed five times in a single analytical run. For calculating the inter-assay precision, the same samples were analyzed in duplicate by five analytical runs on five different days within two weeks. The samples were divided into aliquots and frozen, so vials were only defrosted as required for each analytical run in order to prevent any possible variation as a result of repeated freeze-thaw cycles. Both intra- and inter-assay precision were expressed by the coefficient of variation (CV) (Andreasson et al., 2015).

2.2.2. Assay sensitivity

The assay Se was calculated by estimating the analytical detection limit and the lower limit of quantification. The detection limit was defined as the lowest amount of anti-*N. caninum* antibodies that could be distinguished from a sample of zero value, and it was calculated as the mean value of twelve replicate determinations of the zero standard (assay buffer) plus three standard deviations.

The lower limit of quantification was calculated based on the lowest UFN that could be measured in the linear part of the calibration curve with a CV below 20% and above the limit of detection. This parameter was estimated using a serum with medium amount of anti-*N. caninum* antibodies; this sample was serially diluted in assay buffer and each dilution was analyzed five times in the same run.

2.2.3. Assay accuracy

Accuracy was directly calculated by linearity under dilution, recovery procedure and comparison with the results of the in house ELISA.

To study the linearity under dilution, determinations of anti-*N. caninum* antibodies were made by serial dilutions with assay buffer of two serum samples with high and low amount of these antibodies,

respectively. The amount of anti-*N. caninum* antibodies in diluted samples were measured with the GRA7-TRFIA and, then, curves which represent UFN measured versus UFN expected were created, and the coefficient of determination (R^2) were calculated.

Recovery procedure was assessed using a serum containing high amount of anti-*N. caninum* antibodies and another one with low quantity. The first sample was diluted two-fold (50%), four-fold (25%) and ten-fold (10%) with the low antibody amount sample (50%, 75% and 90%, respectively). Secondly, the low antibody amount sample was diluted four-fold (25%) and ten-fold (10%) with the high antibody amount sample (75% and 90%, respectively). Detected and expected antibody amounts for each diluted serum sample were compared, and then, the percentages of recovery were estimated.

2.2.4. Cross-reactivity

Cross-reactivity study was carried out by GRA7-TRFIA analysis of 22 sera from *N. caninum* negative sheep which had been diagnosed *T. gondii*-positive by in house ELISA (RIPC > 10).

2.2.5. Antibody kinetics

For this purpose, 229 samples from sheep experimentally infected with *N. caninum* and two negative control sheep obtained in different dpi were analyzed by GRA7-TRFIA and NcSALUVET ELISA, and results were statistically compared by Wilcoxon matched-pairs signed rank test. UFN and RIPC group means were graphically compared.

2.2.6. Diagnostic performance of GRA7-TRFIA and comparison with commercial ELISA and NcSALUVET ELISA

Results of GRA7-TRFIA performed with 117 samples from a naturally *N. caninum* infected sheep flock (74 positives, and 43 negatives according to commercial ELISA) were analyzed by a ROC curve. The area under the curve (AUC) was calculated and cut-off for GRA7-TRFIA was established in order to obtain the highest Se and Sp values.

Comparison between GRA7-TRFIA and commercial ELISA methods was assessed by *kappa* value, and between GRA7-TRFIA and NcSALUVET ELISA by Spearman correlation coefficient, using the same 117 naturally infected serum samples.

2.3. Serological tests

2.3.1. GRA7-TRFIA

NcGRA7 antigen used in GRA7-TRFIA was produced by Rekom laboratories® (Granada, Spain) according to the sequence proposed by (Álvarez-García et al., 2007) without the signal peptide. Its N-terminus was fused to a his-tag. Then, it was also biotinylated in its C-terminus by Rekom laboratories®.

Streptavidin-coated microtitration wells (DELFIA streptavidin microtitration strips, PerkinElmer Life and Analytical Sciences®, Turku, Finland) were filled with 100 ng of biotinylated recombinant antigen NcGRA7 (Rekom laboratories®, Granada, Spain) diluted in 200 µL of assay buffer (DELFIA assay buffer, PerkinElmer Life and Analytical Sciences®, Turku, Finland) and incubated for 1 h at room temperature with continuous shaking.

The strips were then washed three times with 200 µL/well of wash buffer (DELFIA wash concentrate, PerkinElmer Life and Analytical Sciences®, Turku, Finland). 200 µL/well of 1:500 diluted serum samples were added hereunder. For the second time, the strips were incubated for 1 h at room temperature.

A second wash was done and then, 100 ng of europium-labelled antibody anti-IgG (Rabbit anti-sheep IgG-heavy and light chain Antibody Affinity purified, Bethyl®, bioNova científica SL®, Madrid, Spain) diluted in 200 µL of assay buffer were added to each well, followed by 1 h of incubation at room temperature.

After a third wash, 200 µL/well of enhancement solution (DELFIA enhancement solution, PerkinElmer Life and Analytical Sciences®, Turku, Finland) were added and the strips were shaken for 5 min. The emitted fluorescence was measured as CPS (counts per second) using a multilabel counter (VICTOR2 1420, PerkinElmer Life and Analytical Sciences®, Turku, Finland). The CPS values were proportional to the quantity of anti-*N. caninum* antibodies in the serum sample.

Results were expressed as Units of Fluorometry for *N. caninum* (UFN), considering that 1 UFN is equivalent to 5000 CPS. To convert CPS to UFN by WorkOut Plus program (WorkOut Plus 2.5, Dazdaq Limited®, Brighton, England), a standard curve was used. A serum sample with high amount of anti-*N. caninum* antibodies was used to create the standard curve in every analytical run (Cantos-Barreda et al., 2017b).

2.3.2. NcSALUVET ELISA

The 117 sera from a naturally *N. caninum* infected sheep flock, the 208 sera obtained from sheep experimentally infected with *N. caninum*, and the 21 negative control sera were analyzed by NcSALUVET ELISA performed following the protocol described by Álvarez-García et al. (2003) to detect anti-*N. caninum* IgG antibodies. Results were expressed in RIPC (relative index percent) according to this formula (Sánchez-Sánchez et al., 2018):

RIPC = (OD_{405} sample - OD_{405} negative control) / (OD_{405} positive control - OD_{405} negative control) × 100

Serum samples from experimentally infected sheep were considered positive when RIPC value was equivalent to RIPC > 10. Negative and positive controls were the same used in Sánchez-Sánchez et al., 2018 study.

2.3.3. ID Screen® Neospora caninum indirect multi-species

The ELISA commercial kit ID Screen® *Neospora caninum* Indirect Multi-species (IDvet®, Grabels, France) was used to analyze the sera from naturally *N. caninum* infected sheep flock, according to the protocol described by the manufacturer. The cut off value was ≥50/41 according to this formula (Álvarez-García et al., 2013):

Sample/Positive = (OD sample – OD negative control) / (OD positive control – OD negative control)

2.4. Data analysis

Statistical analysis for analytical validation was performed to calculate mean and median values, intra- and inter-assay CVs, detection limit and graphical comparison of UFN and RIPC group means using routine descriptive statistical procedures and computer software (Microsoft Excel®, Version 2016). Wilcoxon matched-pairs signed rank test to compare antibody kinetics between GRA7-TRFIA and NcSALUVET ELISA in sheep experimentally infected with *N. caninum*, and ROC analysis for optimal cut-off determination and comparison between GRA7-TRFIA and both ELISA methods in sera from a naturally *N. caninum*-infected sheep flock were carried out by the GraphPad software (Graph Pad Prism, Version 6, San Diego, CA, USA). Statistical significance was considered when $P < 0.05$.

3. Results

3.1. Assay precision

Results of intra- and inter-assay precision are showed in Table 2. Coefficient of variation of intra-assay precision of the samples with high and low antibodies amount ranged from 5.8% to 9.1%. For inter-assay precision, CV ranged between 8.5% and 15.7%.

3.2. Assay sensitivity

The analytical detection limit was 4.2 UFN. For estimating the lowest limit of quantification, all the CVs were below 15% when diluting. Therefore, the limit of quantification is equal to the limit of detection (4.2 UFN).

Table 2
Intra-assay and inter-assay precision obtained for the measurement of anti-*N. caninum* IgGs by GRA7-TRFIA. X = mean; UFN = Units of Fluorometry for *N. caninum*; SD = standard deviation; CV = coefficient of variation.

	Serum sample	Intra-assay			Inter-assay		
		X (UFN)	SD	CV (%)	X (UFN)	SD	CV (%)
IgG	Low	39.36	2.29	5.82	48.28	7.59	15.72
	High	2250.95	205.44	9.12	2307.17	195.13	8.45

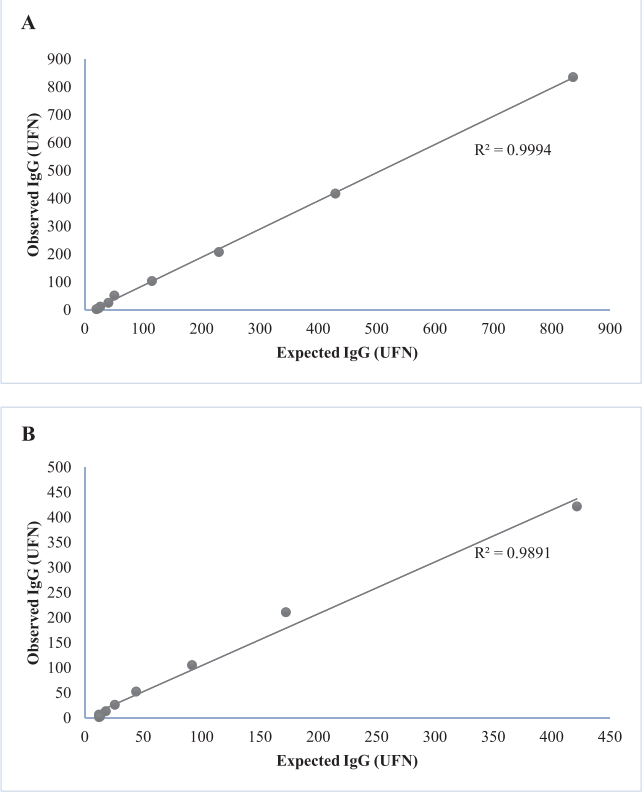

Fig. 1. Analysis of linearity under dilution of two serum samples containing high (A) and low (B) amounts of anti-N. caninum IgGs. R^2 = coefficient of determination. UFN = Units of Fluorometry for N. caninum.

3.3. Assay accuracy

Regarding to linearity under dilution, GRA7-TRFIA results of the serial dilutions of each sample were graphically represented in "y" axis as "observed UFN", while "expected UFN" were confronted in "x" axis, resulting in linear regression equations (Fig. 1). R^2 was 0.99 for the high antibody amount sample and 0.98 for the low amount one.

Recovery results, which ranged from 82.5% to 120%, are represented in Table 3. The quantity of anti-*N. caninum* antibodies in the diluted samples decreased in proportion to the addition of the sample with low antibody amount. Likewise, an increase in antibodies was detected when serum with high antibody amount was added to the sample containing low quantity of antibodies.

3.4. Cross-reactivity

None of the 22 sera from cross-reactivity study tested positive to anti-*N. caninum* GRA7-TRFIA. All results were < 28.18 UFN.

Table 3
Recovery of anti-*N. caninum* IgG antibodies. *High = high amount of IgG antibodies in serum samples; *Low = low amount of IgG antibodies in serum samples; UFN = Units of Fluorometry for *N. caninum*.

	% Sample (high)*	% Sample (low)*	Expected (UFN)*	Detected (UFN)*	Recovery (%)
IgG	100	0		3139.9	
	90	10	2826.3	3395.3	120
	75	25	2355.8	1943.9	82.5
	50	50	1571.7	1481.2	94.2
	25	75	787.6	836.9	106.3
	10	90	317.1	351	110.7
	0	100		3.4	

3.5. Antibody kinetics

Comparative results for the detection of anti-*N. caninum* antibodies by NcSALUVET ELISA and GRA7-TRFIA are presented in supplementary files. Fig. 2 show the timeline of UFN or RIPC group means analyzed by GRA7-TRFIA and NcSALUVET ELISA. Comparison of the day of seroconversion using GRA7-TRFIA and NcSALUVET ELISA in every single animal is shown in Table 4. In general, similar results from both techniques were obtained except from those on days close to seroconversion. In the majority of cases, seroconversion was detected around 13–14 dpi with GRA7-TRFIA, while it was detected around 21 dpi with NcSALUVET ELISA. Concretely, GRA7-TRFIA was able to find seropositivity earlier than NcSALUVET ELISA in all animals except in sheep G1.1, G1.3, G1.6, G5.1 and G4.2, in which seroconversion was detected at the same time by both methods.

The time of seroconversion was significantly different between both tests (Table 5, Wilcoxon matched-pairs signed rank test; $P < 0.0001$). Median of differences between both groups was 7.00.

3.6. Diagnostic performance of GRA7-TRFIA and comparison with commercial ELISA and NcSALUVET ELISA

ROC curve for GRA7-TRFIA showed an area under the curve (AUC) of 0.9749, with a standard error of 0.01178 (95% CI, Fig. 3). Using a cut-off point of 62.68 UFN, 100% Se and 95.35% Sp were obtained. GRA7-TRFIA and commercial ELISA showed high agreement, with a *kappa* value of 0.963 (95% CI: 0.912–1.000). Correlation between GRA7-TRFIA and NcSALUVET ELISA was positive and statistically significant (Spearman correlation coefficient 0.814, $P < 0.0001$).

4. Discussion

A new TRFIA technique based on the recombinant NcGRA7 antigen has been analytically validated for the detection and measurement of specific anti-*N. caninum* IgG antibodies in sheep sera. As far as author's knowledge, this is the first time that a TRFIA technique has been used for the detection and quantification of specific anti-*N. caninum* IgG antibodies in sheep. Moreover, this is also the first time that recombinant NcGRA7 antigen is used for TRFIA, and specifically for *N. caninum* antibodies detection in sheep.

For analytical validation, intra- and inter-assay precision were calculated and they were below 20%, which is the accepted limit for immunoassays (Jacobson, 1998; U.S. Department of Health and Human Services, 2013). Particularly, intra-assay precision was below 10%, which makes duplication of samples unnecessary and reduces the time and amount of sample used in every assay. This accuracy of the results is similar to that obtained in other studies which analyze TRFIA (Parra et al., 2005; Martínez-Subiela et al., 2011; Cantos-Barreda et al., 2017b). The low limit of detection and quantification showed a high analytical Se for this technique, which allows to detect very small amounts of antibodies. Moreover, GRA7-TRFIA can be performed using

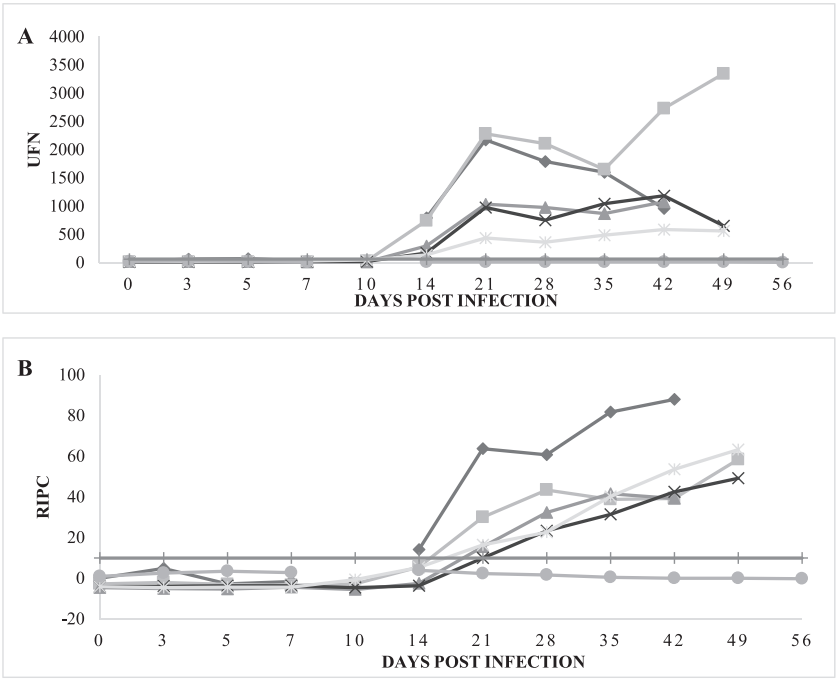

Fig. 2. Timeline of Units of Fluorometry for *N. caninum* (UFN) (2A) and Relative Index Percent (RIPC) (2B) group means analyzed with GRA7-TRFIA and NcSALUVET ELISA, respectively. ◆ = Group 1; ■ = Group 2; ▲ = Group 3; X = Group 4, * = Group 5; ● = Negative control; + = Cut off (UFN = 62.68; RIPC = 10).

Table 4
Comparison of the day of seroconversion using GRA7-TRFIA and NcSALUVET ELISA.

Sheep	GRA7-TRFIA (day)	NcSALUVET ELISA (day)
1.4	3	21
5.2	7	21
2.3	10	14
1.1	13	13
1.2	13	20
1.3	13	13
1.5	14	21
1.6	14	14
2.1	14	21
2.2	14	21
2.5	14	21
5.4	14	28
3.1	14	28
3.2	14	28
3.3	14	21
3.4	14	28
3.5	14	21
4.1	14	28
4.3	14	21
2.4	21	28
5.1	21	21
4.2	21	21
4.4	21	34

Table 5
Statistical study of time of seroconversion detected by GRA7-TRFIA and NcSALUVET ELISA.

Statistic	GRA7-TRFIA	NcSALUVET ELISA
Minimum	3.00	13.00
10% Percentile	8.20	13.40
25% percentile	13.00	21.00
Median	14.00	21.00
75% Percentile	14.00	28.00
90% Percentile	21.00	28.00
Maximum	21.00	34.00
Median of differences	7.00	
Wilcoxon matched-pairs signed rank test (*P*-value)	< 0.0001	

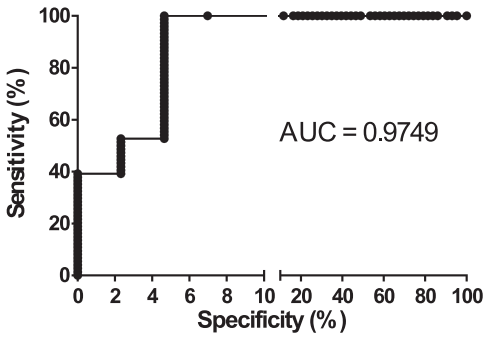

Fig. 3. ROC curve for anti-*N. caninum* IgG GRA7-TRFIA.

1:500 diluted sera, while the maximum dilutions required to analyze sera with IFAT are 1:50 (Andreotti et al., 2009; Pinheiro et al., 2015), and 1:100 with commercial ELISA and NcSALUVET ELISA (Sánchez-Sánchez et al., 2018). Therefore, our results indicate that GRA7-TRFIA is the most recommended technique when small amounts of serum are available. It should be noted that ELISA test based on NcSRS2 antigen described by Andreotti et al. (2009) uses 1:1000 diluted sera, although the Se provided for its optimal cut-off (98.6%) is lower than that of GRA7-TRFIA (100%).

Accuracy of GRA7-TRFIA has been proven by the high correlation coefficients in linearity under dilution and recovery results between 82.5 and 120%, which are within acceptance range for the recovery (Andreasson et al., 2015). Furthermore, no cross-reaction with *T. gondii* was observed. This lack of cross-reactivity indicates that GRA7-TRFIA is very specific. However, in other studies, cross-reactivity between *N. caninum* and *Sarcocystis cruzi* has been source of concern (Paulo et al., 2005). Therefore, cross-reactivity with other Apicomplexan parasites (such as *Sarcocystis* spp. and *Besnoitia* spp.) should be assessed in the future, particularly if this new TRFIA is going to be employed with cattle sera (García-Lunar et al., 2015).

GRA7-TRFIA is able to detect *N. caninum* infection earlier than NcSALUVET ELISA. Specifically, seroconversion of all infected animals was detected at the latest on 21 dpi by GRA7-TRFIA, while with

NcSALUVET ELISA it occurred on 35 dpi,. So, GRA7-TRFIA makes it possible to detect *N. caninum* infection at earlier stages compared to NcSALUVET ELISA.

On the other hand, in naturally infected sheep, GRA7-TRFIA showed good agreement with NcSALUVET ELISA and commercial ELISA, with 100% of Se and 95.35% of Sp for an optimal cut-off point of 62.68 UFN. These results indicate that this technique is highly sensitive and specific in line with other ELISA assays previously used in sheep (Andreotti et al., 2009; Pinheiro et al., 2015) and bovines (Aguado-Martínez et al., 2008). Other studies which compare TRFIA and ELISA techniques found scarce differences (Fransson et al., 2007; Hu et al., 2015) or similar results (Parra et al., 2005; Cantos-Barreda et al., 2017b) between both techniques.

This study has been focused in serum samples, as do several other available tests with accurate performance. However, current techniques have some limitations that could be improved by using the GRA7-TRFIA developed in our study. Firstly, some techniques fail to detect chronic *N. caninum* infections in cattle (Dubey and Schares, 2011; Guido et al., 2016); in these cases, a higher sensitive technique would be useful to detect anti-*N. caninum* antibodies. Secondly, current ELISA tests has been developed to detect *N. caninum* antibodies from bulk-milk samples (Dubey and Schares, 2006). But, as far as the authors' knowledge, there are no studies about the application of TRFIA for the detection of anti-*N. caninum* antibodies on milk or bulk milk samples from sheep. This technique could be highly useful due to its high Se and also knowing that the TRFIA usually have less interferences with lipemia compared to ELISA assays (Parra and Cerón, 2007).

5. Conclusions

All the obtained results provide the first evidence of the usefulness of GRA7-TRFIA for quantification of anti-*N. caninum* IgG antibodies in ovine sera. This assay shows a high Se and allows an earlier diagnosis of the infection than a tachyzoite soluble extract-based ELISA. This new diagnostic technique is easy, fast and usable for large-scale screening, and thus, it could be used for *N. caninum* control programs.

Ethics approval

All procedures involving animals were approved by the Murcia University Ethical Committee, in compliance with laws RD32/2007 and RD1201/2005 relating to animal experimentation in Spain.

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Declaration of Competing Interest

The authors declare that there are no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.108994.

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