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Experimental infection of sheep at mid-pregnancy with archetypal type II and type III *Toxoplasma gondii* isolates exhibited different phenotypic traits

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ABSTRACT

Toxoplasma gondii is a major cause of reproductive failure in small ruminants. Genotypic diversity of *T. gondii* strains has been associated with variations in phenotypic traits in *in vitro* and murine models. However, whether such diversity could influence the outcome of infection in small ruminants remains mostly unexplored. Here, we investigate the outcome of oral challenge in sheep at mid-pregnancy with 10 sporulated oocysts from three different *T. gondii* isolates belonging to archetypal II and III and selected according to their genetic and phenotypic variations shown in previous studies. Seventy-three pregnant sheep were divided in four groups: G1 infected with TgShSp1 isolate (type II, ToxoDB#3), G2 with TgShSp16 isolate (type II, ToxoDB#3), G3 with TgShSp24 isolate (type III, ToxoDB#2) and G4 of uninfected control sheep. Two different approaches were carried out within this study: (i) the outcome for the pregnancy after infection ($n = 33$) and (ii) the lesions and parasite tropism and burden at 14 and 28 days post infection (dpi) ($n = 40$). The onset of hyperthermia and seroconversion occurred one and two days later, respectively in G1 when compared to G2 and G3. However, sheep that suffered from reproductive failure, either by abortion, foetal dead at the time of euthanasia or stillbirth were similar among infected groups (50%, 40% and 47%, respectively). Histological lesions in placentomes and foetal tissues from euthanized animals from the second approach were only detected at 28 dpi and mainly in G1. At 14 dpi, *T. gondii*-DNA was only detected in G1 in the 11% of the placentomes. However, at 28 dpi the frequency of detection in placentomes was higher in G1 (96%) than in G2 and G3 (7% and 47%, respectively) besides in foetuses was lower in G2 (20%) than in G1 and G3 (100% and 87%, respectively). Regarding late abortions, stillbirths, and lambs of G1, G2 and G3, the frequency of microscopic lesions was similar between groups (79%, 78% and 67%, respectively) whereas *T. gondii*-DNA was evidenced in 100%, 55% and 100%, respectively. These recently obtained *T. gondii* isolates led to similar reproductive losses but intra- and inter-genotype variations in the rise of hyperthermia, dynamics of antibodies, frequency of lesions and parasite detection and distribution. Thus, the different phenotypic traits of the isolates could influence the outcome of the infection and mechanisms responsible for it, and further investigations are warranted.

1. Introduction

Toxoplasmosis, caused by *Toxoplasma gondii*, is a parasitic disease that may affect all warm-blooded animals and is responsible of important economic losses in sheep production worldwide (Stelzer et al., 2019). Transplacental transmission of *T. gondii* occurs mainly after primary infection of pregnant sheep with sporulated oocysts present in contaminated fodder or water (Dubey, 2009). Infections during the two first terms of gestation usually cause reabsorption or abortions while infections at late pregnancy usually produce stillbirths and/or birth of weak lambs (Innes et al., 2009). Two clinical presentations have been described in sheep according to the days post-infection (dpi) when abortions occur: early abortion (7–14 dpi) and late abortion (28 dpi onward) (Owen et al., 1998; Castaño et al., 2014). The former has been

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described in experimental infections and might be underdiagnosed in the field as, contrary to late abortions, neither parasite in the placenta or foetal tissues nor antibodies in the sheep are usually detected at the time of abortion (Benavides et al., 2017). Moreover, histological lesions in the placenta are different between both clinical presentations, while early abortions are characterised by infarcts at the placentomes, late abortions show multifocal foci of necrosis (Owen et al., 1998; Buxton et al., 2007; Castaño et al., 2014). Although there are still several unknown variables playing a role in the pathogenesis of ovine toxoplasmosis (Benavides et al., 2017), it has been stated that other factors apart from the period of gestation such as (i) parasite stage (oocysts or bradyzoites), (ii) immune response of the host, (iii) dose of infection, and (iv) parasite's genotype could also determine the outcome of infection (Castaño et al., 2016; Sánchez-Sánchez et al., 2019; Mukhopadhyay et al., 2020; Calero-Bernal et al., 2022).

In this sense, genotype-phenotype association in *T. gondii* is still blur, therefore an extra effort dealing with molecular (*e.g.*, genotyping) and phenotypic characterization using harmonized methods, and the search for highly informative and discriminatory phenotypic markers will be of major interest (Calero-Bernal et al., 2022). *Toxoplasma gondii* isolates have been traditionally classified into three different clonal lineages according to their cumulative mortality in murine models [highly virulent (type I) (haplogroup 1), intermediate virulent (type II) (haplogroup 2) and non-virulent (type III) (haplogroup 3)] despite the existence of other virulent haplogroups (Howe and Sibley, 1995; Behnke et al., 2016; Fernández-Escobar et al., 2022). In this sense, type II *T. gondii* genotypes are the most predominant in European sheep, together with a lesser extent of type III and recombinant genotypes (Fernández-Escobar et al., 2022). However, recent studies have shown not only inter-genotype differences but also intra-genotype variations in several phenotypic markers evaluated *in vitro* (AH1 cell line and OvMOs) and *in vivo* (murine and porcine models) (Taniguchi et al., 2018, Fernández-Escobar et al., 2020a, 2020b; 2021; Largo-de la Torre et al., 2022; Vallejo et al., submitted). Whether such phenotypic diversity is also found when infecting sheep remains unclear, as inferences from the murine model should be taken with caution (Sánchez-Sánchez et al., 2019).

Thus, the aim of present study was to investigate the influence on the experimental infection of sheep at mid-pregnancy with *T. gondii* oocysts of different genotypes belonging to the two predominant archetypal types in Europe [TgShSp1 (type II, genotype#3), TgShSp16 (type II, genotype#3) and TgShSp24 (type III, genotype#2)]. This study defines the outcome of three recently obtained isolates with different genotype that have already shown variation in their phenotypic traits in *in vitro* studies and considered as non-virulent (<30% cumulative mortality) in murine models.

2. Materials and methods

2.1. Ethic statements

Animal handling, blood sampling and euthanasia were carried out in accordance with Spanish Law 6/2013, concerning animals, their exploitation, transportation, experimentation, and sacrifice; Royal Decree 118/2021 for the protection of animals employed in research and teaching, and EU regulations, Directive 2010/63/UE, related to the protection of animals used for scientific goals. Animal procedures for *T. gondii* infection of mice and cats (PROEX 062/19) for oocyst production were approved by the Animal Welfare Committee of the Community of Madrid, Spain. Animal procedures for *T. gondii* infection of sheep were approved by the CSIC Animal Ethic Committee (ref. 1063/2021). The experimental procedure was authorised by the regional government, Junta de Castilla y León. All animals used in this study were handled by veterinarians in strict accordance with good clinical practices, and all efforts were made to minimise suffering. Animals were monitored for clinical signs of disease throughout the study.

2.2. Selection of T. gondii isolates

Three *T. gondii* isolates were selected from a large panel of 30 recently isolated Spanish strains, obtained from ovine tissue samples (Fernández-Escobar et al., 2020a, 2020b). Isolates were selected according to genotypic and phenotypic parameters (clonal type, genotype, host of origin, mouse virulence and parasite invasion rates in ovine trophoblasts and macrophages) summarized in Table 1 (Fernández-Escobar, et al., 2021; Vallejo et al., submitted).

2.3. Generation of sporulated T. gondii oocysts

Sporulated *T. gondii* oocysts of each isolate were obtained at SALUVET facilities (Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid) through oral infection of cats as previously mentioned (Muller et al., 2017). Concisely, 8-weeks-old female CD1 mice (Javier-Labs, Laval, France) were intraperitoneally inoculated with 10^3 tachyzoites of each isolate that had been kept at low cell culture passage ($n < 10$). After 2 months, asymptomatic mice were euthanized and 3 months-old specific pathogen free kittens (Isoquimen S.L., Barcelona, Spain) were fed with a pool of brains from mice infected with each isolate. Faeces were collected daily from kittens and unsporulated oocysts were sporulated in 2% H_2SO_4 during 4 days at room temperature. The oocysts used for infections were maintained at 4 °C one year from production until inoculum preparation. To prepare the inoculum, sporulated oocysts were quantified using a single-use Neubauer chamber (DHCN01 Neubauer Improved CYTO, Gentaur, UK) and subsequently diluted in PBS. Sheep were orally challenged with 7 mL PBS containing 10 sporulated oocysts of each isolate.

2.4. Experimental design

A total of 73, 14–24 months-old, primiparous pregnant Rasa Aragonesa sheep, obtained from a commercial flock without previous history of toxoplasmosis, and seronegative against abortifacient agents (*T. gondii*, *Neospora caninum,* border disease virus, Schmallenberg virus, *Coxiella burnetii*, and *Chlamydia abortus*) were randomly distributed into four experimental groups and housed in the facilities of Instituto de Ganadería de Montaña (IGM) (CSIC-University of León), Grulleros, León, Spain. These sheep had been oestrus-synchronised and mated with pure-breed Rasa Aragonesa tups for two days. Pregnancy and foetal viability were confirmed by ultrasound scanning on day 40 post-mating.

The four groups were as follows: group 1 (G1, $n = 20$) infected with TgShSp1 isolate, group 2 (G2, $n = 20$) infected with TgShSp16 isolate, group 3 (G3, $n = 19$) infected with TgShSp24 isolate and group 4 (G4, $n = 14$) as non-infected control sheep. On day 90 of gestation the sheep from G1, G2 and G3 were orally infected with 10 sporulated oocysts of each isolate as previously described (Sánchez-Sánchez et al., 2019). Two different approaches were conducted within the same study aiming at studying the outcome of the infection as well as characterising the

Table 1
Toxoplasma gondii isolates used in the present study.

	TgShSp1	TgShSp16	TgShSp24
Clonal type	Type II	Type II	Type III
Genotype (ToxoDB#)	#3	#3	#2
Host of origin	Ovine foetal brain	Adult ovine myocardium	Adult ovine myocardium
Mouse virulence (cumulative mortality, %)	0	20.8	18.2
Parasite invasion rate in ovine trophoblasts (AH-1 cell line)	Low	Medium	High
Parasite invasion rate in ovine macrophages	Low	Medium	High
Ovine macrophages differentation	M2	M2	M1

lesions and the parasite tissue distribution and burden in maternal and foetal tissues. For this latter, the experimental design involved a serial euthanasia of five pregnant sheep at 14 and 28 dpi in each of the four experimental groups ($n = 40$). Additionally, and with the purpose of analysing the clinical outcome of infection, the remaining 33 sheep were left until early or late abortions or delivery occurred. One day after lambing, all sheep and lambs were euthanized.

2.5. Clinical monitoring and collection of samples

Sheep were observed daily throughout the experimental infection period. Rectal temperatures were measured daily, from two days before infection until 28 dpi; and, regarding physiological range values, rectal temperatures above 40 ºC were considered hyperthermic (Diffay et al., 2002). Blood samples were collected two days before challenge and 3, 5, 8, 12, 14, 19, 22 and 28 dpi. After serial euthanasia, until abortion or birth took place, sampling was performed weekly and any event during the study was annotated. Briefly, blood samples were collected from the jugular vein into collection tubes without anticoagulant (Becton Dickinson and Company, UK) and serum was obtained by centrifugation and stored at − 20 ºC until analysis. Abortions were classified into two categories: early abortions (between 8 and 14 dpi) and late abortions (from 28 to 50 dpi).

Sheep and lambs were euthanized by intravenous administration of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain) with a previous sedation by intramuscular administration of xylazine (Bayer, Mannheim, Germany). A regulated, orderly, and complete necropsy was performed immediately after euthanasia and gross lesions, when present, were annotated. During serial euthanasia, foetuses were separated from the placenta, and 9 placentomes (3 cranial, 3 medial and 3 caudal) were randomly retrieved from each placenta. Placentomes showing whitish discolouration and generalised autolysis adjacent to mummified foetuses, were not considered. In addition, iliofemoral and jejunal mesenteric lymph nodes were also collected. Placentomes and lymph nodes were stored in 10% formalin for histopathological examinations and frozen at − 20 ºC for *T. gondii* DNA detection (Castaño et al., 2016).

Foetal tissues collected from early abortions, serial euthanasia, late abortions, stillbirths and lambs, included the brain, lung (left dorsal caudal lobe), skeletal muscle from the left hind limb (semitendinous muscle), liver (right lobe) and heart (apex). Samples were fixed in 10% formalin for histopathological processing (all the samples) or maintained at − 20 ºC for *T. gondii* DNA detection (brain, liver, and in the case of serial euthanasia, the heart). Foetuses were considered as infected if at least one out of the three analysed organs (brain, liver and heart) tested positive by PCR. Foetal brains were first fixed in 90% ethanol and 10% formaldehyde, that gave them a firmer consistency, and after 48 h in 10% neutral buffered formalin solution for another 48 h (Gutiérrez-Expósito et al., 2020). In those foetuses obtained during the serial euthanasia (at 14 and 28 dpi), blood from cardiac lumen or thoracic fluid was also collected and serum was stored at − 20 ºC until serological analysis. In lambs born alive, no serum was obtained as they had suckled colostrum before euthanasia. Due to the advanced degree of autolysis, neither serum nor thoracic fluid were taken from aborted foetuses either early or late abortions.

2.6. Histological processing and βAPP immunohistochemistry

After formalin fixation, placentomes and foetal tissues were trimmed and conventionally processed for embedding in paraffin wax (Castaño et al., 2014; Sánchez-Sánchez et al., 2019). Specifically, from each foetal brain, 4 different sections were processed (frontal lobe, corpus callosum, midbrain and cerebellum) as described previously (Gutiérrez-Expósito et al., 2020). Histological sections were stained with haematoxylin and eosin (H-E) and studied under an optical microscope by 2 veterinary pathologists (RV and JB).

Additionally, in the cases of early abortions, sections from the same four areas of the brain were cut and mounted on poly-L-lysine coated slides for immunohistochemical labelling. Beta amyloid precursor protein (βAPP) monoclonal antibody (clon 2cc11; Millipore) was used for labelling necrotic areas in the white matter (*i.e.*, leucomalacia) as previously described (Gutiérrez-Expósito et al., 2020). Briefly, deparaffinization, rehydration and epitope retrieval were accomplished in Tris-based solution (PT-Link System, Agilent Technologies) at pH= 6. In addition, slides were immersed in a 3% H_2O_2 in methanol solution for 30 min in dark at room temperature and incubated overnight at 4 ºC with the primary antibody diluted (1:35000) in a phosphate-buffered saline in a humidified chamber. Next day, after washing, incubation with a secondary antibody for 40 min and with 3,3-diaminobenzidine (DAB; Agilent Technologies) for 5 min was performed, producing brown signal. Finally, slides were rinsed in tap water and counterstained with Mayer's haematoxylin for 10 s

2.7. DNA extraction and PCR procedures for parasite detection and quantification

T. gondii detection was studied in 9 placentomes of each sheep, lymph nodes and foetal (brain, liver and heart) and lamb tissues (brain and liver). DNA extraction and PCR procedures were carried out as previously described (Sánchez-Sánchez et al., 2019). Briefly, one gram of each tissue originating from three different areas of the collected tissue was homogenized and around 50 mg of that mix was destined to DNA extraction using the Maxwell® 16 Tissue DNA Purification Kit with the Maxwell 16 Instrument (AS1030 Promega, WI, USA) following manufacturer's protocol. QuantiFluor™ ONEdsDNA System kit and Quantus™ Fluorometer (Promega, WI, USA) were used for DNA quantification. The concentration of DNA for all samples was adjusted to 50 ng/µl. Parasite DNA detection was carried out by a nested PCR adapted to a single tube for the internal transcribed spacer (ITS1) region of *T. gondii* as previously described (Hurtado et al., 2001). Each reaction was performed in a final volume of 25 µl with five µl of DNA. Control animals were included in each round of DNA extraction and PCR as negative controls. Appropriate positive controls were also included in each round. Twelve µl of the PCR products were visualised under UV light in 1.5% agarose gel/Gel Red to detect the *T. gondii*-specific 227 base pairs (bp) amplification product (Hurtado et al., 2001).

Afterwards, positive DNA-*T. gondii* samples by nested-PCR were subjected to parasite burden quantification by real-time PCR targeting the 529RE fragment as previously described (Castaño et al., 2016). Standard curves for *T. gondii* and ovine DNA (β-actin) showed a slope of − 3.61 and − 3.45, respectively, and an $R^2 > 0.99$. Parasite burden was expressed as the number of tachyzoites/mg DNA.

2.8. Serology

Specific IgG antibodies against *T. gondii* in sheep serum were detected by a commercial ELISA (ID Screen® Toxoplasmosis Indirect Multi-species) following manufacturer's instructions. Indirect fluorescent antibody test (IFAT) was performed to detect IgG against *T. gondii* infection in foetal serum and thoracic fluids, adapting previously described IFAT in *Neospora caninum* infected animals (Álvarez-García et al., 2003), using purified *T. gondii* tachyzoites (ME49 strain) and anti-sheep IgG FITC conjugate (F5137, Sigma-Aldrich, Madrid, Spain) at 1:200 dilution in Evans blue (E2129, Sigma-Aldrich, Madrid, Spain) 0.2% in PBS (Castaño et al., 2016). Serum was diluted at 2-fold serial dilutions up to endpoint titer. Continuous tachyzoite membrane fluorescence at a titer ≥ 8 for sera or foetal fluids was considered a positive reaction.

2.9. Statistical analysis

Rectal temperature and dynamics of antibodies were studied using a

non-parametric ANOVA followed by a *post hoc* Dunn's multiple comparison. The number of death foetuses, percentage of cases showing lesions as well as differences in frequency of parasite detection by PCR were evaluated using the χ^2-test or Fisher's exact *F*-test. Differences in parasite burden were analysed using the non-parametric Kruskal-Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann-Whitney test for pairwise comparisons. All these analyses were performed with GraphPad Prism 8.0 software. Statistical significance for analyses was established at $p < 0.05$.

3. Results

3.1. Clinical observations

3.1.1. Rectal temperature

All infected groups (G1, G2 and G3) experienced a transitory hyperthermia above 40 ºC between 6 and 10 dpi whereas rectal temperatures of G4 remained always under 39.5 ºC. Specifically, G2 and G3 had fever between 6 and 9 dpi and G1 between 7 and 10 dpi (Fig. A). Infected G2 and G3 showed statistically significant differences with non-infected G4 between 5 and 10 dpi ($p < 0.0001$) whilst group G1 and G4 showed differences between 7 and 10 dpi ($p < 0.0001$), therefore the rise of temperature in G1 occurred two days later than in G2 and G3 ($p < 0.05$) (Fig. 1A). The G1 had a peak of fever (40.9 ºC) at 8 dpi whereas in G2 (41.3 ºC) and G3 (41.4 ºC) it was observed at 7 dpi. No statistical differences between 11 and 28 dpi were observed.

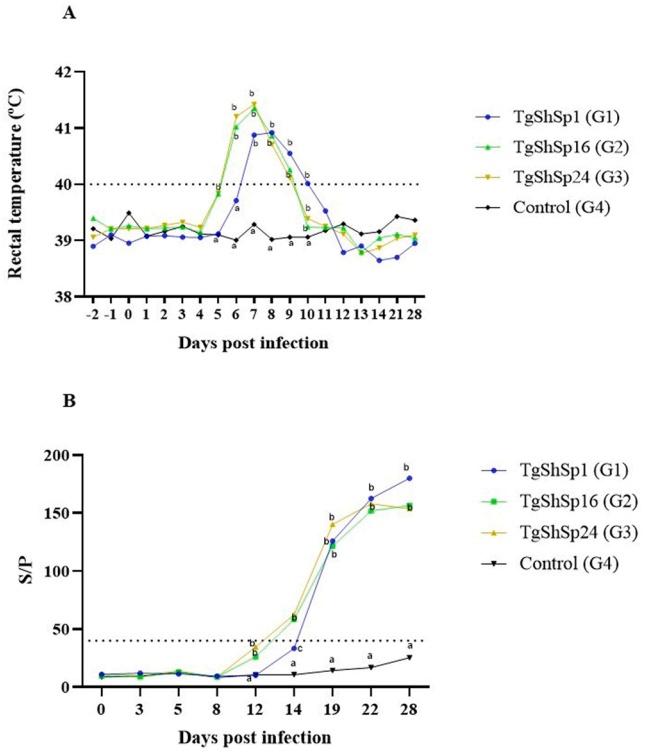

Fig. 1. Clinical observations in G1 (TgShSp1), G2 (TgShSp16), G3 (TgShSp24) and G4 (control) groups following *Toxoplasma gondii* orally infection with 10 sporulated oocysts of each isolate. **A.** Mean rectal temperature. **B.** Dynamics of antibody levels. Each point represents the mean value at the different sampling times for each group. The horizontal dashed line in A and B indicates 40 ºC and the cut off of the ELISA test, respectively. *Toxoplasma gondii* IgG titers are read at 405 nm and expressed according to the formula: S/P = (OD405 sample – OD405 negative control) / (OD405 positive control – OD405 negative control) × 100. Different superscript letters indicate statistically significant differences between isolates (Dunn's multiple comparison) ($p \leq 0.05$).

3.1.2. Reproductive losses

Outcome for the pregnancy has been summarised in Table 2. Briefly, early abortions occurred as follows: four sheep from G1 (20%) aborted between 11 and 12 dpi, four sheep from G2 (20%) aborted on 12 dpi and six sheep of G3 (32%) aborted between 10 and 12 dpi. No statistical differences were observed between infected groups ($p > 0.05$).

Regarding the findings at serial euthanasia, at 14 dpi, no foetal death was found in G1 while one sheep from G2 presented 2 mummified foetuses, and another sheep delivered a dead foetus together with a viable one. In addition, there was one sheep of G3 with 2 dead foetuses (Table 2). However, at 28 dpi, foetal death was found in all infected groups. Specifically, in G1 two sheep had one non-viable foetus along with viable ones, in G2 one sheep had 2 death foetuses along with a viable one and in G3 two sheep presented one death foetus along with another viable one (Table 2).

Regarding late abortions from remaining sheep (6 from G1, 6 from G2 and 3 from G3) took place as follows: from G1 four sheep aborted at 49 and 53 dpi (4/6, 67%), from G2 one sheep aborted at 44 dpi (1/6, 16.6%) and from G3 no sheep aborted (0/3) (Table 2). Sheep that did not aborted were left until delivery: in G1 two sheep lambed but one had weak offspring; in G2 five lambed and two had weak lambs and in G3, three lambed and one had weak lambs. The remaining four control sheep lambed ten healthy lambs.

If all the sheep with reproductive losses are considered in each infected group (unifying animals of both approaches), regardless the time of abortion, the identification of foetal death or stillbirths, sheep from G1 had suffered a 50% of reproductive failure (out of 20 initial sheep, 4 suffered early abortions, 2 with dead foetuses at day 28 dpi and 4 with late abortions or stillbirths), sheep from G2 had suffered 40% of reproductive failure (out of 20, 4 showed early abortions, 2 had dead foetuses at day 14 dpi, 1 at day 28 dpi and 1 suffered a late abortion) and G3 had a 47% of reproductive failure (6 out of 19 sheep had early abortions, one sheep had two dead foetuses at 14 dpi and 2 sheep had dead foetuses at 28 dpi) (Table 2).

3.2. Anti-Toxoplasma gondii specific IgG responses

All infected sheep became seropositive at 12–14 dpi onwards. An exponential increase in antibody levels was observed from 14 to 19 dpi in all infected groups. The dynamics of antibodies was similar in G2 and G3, in which the increase was detected from 12 dpi ($p < 0.05$) (Fig. 1B). However, the antibodies levels of G1 started to rise two days later (S/P: 33), at this time point, G2 and G3 double the S/P value of G1 (S/P: 60), becoming at 19 dpi similar in all groups (S/P: 120–140). From 28 dpi onwards the antibody levels in all infected groups reached a plateau (data not shown). All control animals remained seronegative throughout the study.

Regarding serological results of foetuses from the serial euthanasia, only one foetus from a sheep of G1 euthanized at 14 dpi was seropositive by IFAT with a titer of 1:64, whereas all foetuses of G2, G3 and G4 were seronegative. At 28 dpi three out of eight foetuses from two sheep of G1

Table 2
Clinical outcome for the pregnancy after oral infection with 10 sporulated oocysts of three different *Toxoplasma gondii* isolates in pregnant sheep at 90 days of gestation.

Isolates (inoculation group)	Early abortions	Serial euthanasia[a]		Late abortions or stillbirths	Lambs	Total
		14 dpi	28 dpi			
TgShSp1 (G1)	4/20	0/5	2/5	4/6	2/6	20
TgShSp16 (G2)	4/20	2/5	1/5	1/6	5/6	20
TgShSp24 (G3)	6/19	1/5	2/5	0/3	3/3	19
Control (G4)	0/14	0/5	0/5	0/4	4/4	14

[a] Number of sheep with at last one dead foetus/total euthanized sheep

(38%) were seropositive with IFAT titers that ranged from 1:64–1:128 and seven out of nine foetuses from the five sheep of G3 (78%) were seropositive with IFAT titers ranging from 1:64–1:512. All foetuses of G2 and G4 were seronegative at 28 dpi (Table 3).

3.3. Pathology and lesions

3.3.1. Gross lesions

Early and lately aborted foetuses that were expelled, regardless of the infected group, were shrunken, dark and with high degree of autolysis and friable viscera (mummified). In most of these cases, the placenta was not found or, when present, was severely autolytic, especially the placentomes (Supplementary Figure 1A).

Regarding the findings during serial euthanasia, three foetuses from G2 and two foetuses from G3 were found dead at 14 dpi, and two foetuses from each group were dead at 28 dpi. These dead foetuses showed different macroscopic changes, from subcutaneous oedema, suggesting a recent death, to those with advanced autolysis and mummification. Two foetuses from G1 and one foetus from G3, both from 28 dpi, were too autolytic for further histologic or PCR evaluation. Only two placentomes from the same sheep, belonging to G3 and euthanized at 28 dpi, with a non-viable foetus at the time of euthanasia showed macroscopic changes, denoted by multifocal white pinpoint foci in the interdigitate area of the placentome (Supplementary Figure 1B). No lesions were found in the non-infected control animals.

3.3.2. Microscopic lesions

3.3.2.1. Placenta and lymph nodes. Microscopic lesions were only evaluated at the placentas from those animals euthanized at 14 and 28 dpi, as placentas from sheep that suffered early or late abortions or from stillbirths were too autolytic to be properly evaluated. Placentas at 14 dpi did not show lesions in any of the studied groups, whereas histologic changes consistent with *T. gondii* infection were found in placentas from sheep euthanized at 28 dpi. There were two types of microscopic lesions, *i.e.* necrotic or infiltrative. The necrotic lesions were characterised by a variable number, frequently only one, of necrotic foci affecting both maternal and foetal components of the placentome (necrotizing placentitis). These foci were denoted by homogeneous eosinophilic material admixed with cellular and karyorrhectic debris and occasionally eosinophilic fibrillar material (fibrin) (Fig. 2A). The infiltrative lesions were formed by the infiltration of a minimal or low number of mononuclear inflammatory cells, mainly located in the chorionic connective tissue of the foetal connective tissue (Fig. 2B).

A placentome was considered damaged by *T. gondii* when any of above types of lesions were found. Microscopic lesions are summarized in Table 3. Four sheep of G1 (80%), one of G2 (20%) and two from G3 (40%) had at least one damaged placentome ($p < 0.05$). Specifically, a total of 17 out of 45 placentomes (38%) examined were affected in sheep of G1, corresponding to 0/9, 2/9, 9/9, 1/9, 5/9 placentomes of each sheep; all of them with infiltrative lesions (17/17) and only three with necrosis (3/17), belonging to two different sheep. Six out of 45 placentomes from G2 showed lesions (6/45, 13.5%), which belonged to the same sheep, the only one affected in this group, and all of them had necrotic lesions but not infiltrative ones. It is important to remark that this sheep had two macerated foetuses together with a viable one. Regarding G3, there were lesions in 6 placentomes (6/45, 13.5%) that belonged to two sheep (2/5, 40%). The type of lesions found in these sheep was as follows: one sheep with two placentomes with infiltrative and necrotic lesions (2/9) and another two (2/9) only with necrosis; in the other sheep, only two out of nine placentomes had lesions, specifically the infiltrative ones.

Additionally, and only in the samples at 28 dpi, vascular lesions affecting mainly the maternal villi were found in 2/5, 3/5 and 1/5 sheep from G1, G2 and G3, respectively, with similar characteristics. These

Table 3
Foetal death, IFAT results, histopathologic lesions, and *Toxoplasma gondii* DNA detection in placentomes, lymph nodes and foetal tissues from serial euthanasia (at 14 dpi and 28 dpi).

Isolates (Inoculation group)	Placentomes				Lymph nodes				Foetuses															
					Jejunal mesenteric		Ileofemoral																	
	14 dpi		28 dpi		14 dpi	28 dpi	14 dpi	28 dpi	14 dpi				28 dpi											
	HE	PCR	HE	PCR	PCR	PCR	PCR	PCR	Foetal death	HE[a]	PCR[a]	IFAT	Foetal death	HE					PCR				IFAT
														Brain	Liver	Heart	Skeletal muscle	Lung	Brain	Liver	Heart	
TgShSp1 (G1)	0/45	5/45 *11.1%*	17/45 *37.8%*	43/45 *95.6%*	1/5 *20%*	2/5 *40%*	0/5 *0%*	0/5 *0%*	0/11 *0%*	0/11 *0%*	0/11 *0%*	1/11 *9.1%*	2/10[b] *20%*	6/8 *75%*	8/8 *100%*	7/8 *87%*	4/8 *50%*	4/8 *50%*	5/8 *62.5%*	6/8 *75%*	6/8 *75%*	3/8 *37.5%*	
TgShSp16 (G2)	0/45 *0%*	0/45 *0%*	6/45 *13.3%*	3/45 *6.7%*	0/5 *0%*	2/5 *40%*	0/5 *0%*	2/5 *40%*	3/9 *33.3%*	0/9 *0%*	0/9 *0%*	0/9 *0%*	2/10 *20%*	0/10 *0%*	2/10 *20%*	0/10 *0%*	0/10 *0%*	0/10 *0%*	2/10 *20%*	0/10 *0%*	0/10 *0%*	0/8[c] *0%*	
TgShSp24 (G3)	0/45 *0%*	0/45 *0%*	6/45 *13.3%*	21/45 *46.7%*	0/5 *0%*	1/5 *20%*	0/5 *0%*	0/5 *0%*	2/10 *20%*	0/ 10 *0%*	0/10 *0%*	0/10 *0%*	2/10[b] *20%*	3/9 *33.3%*	4/9 *44%*	0/9 *0%*	2/9 *22.2%*	3/9 *33.3%*	5/9 *55.5%*	5/9 *55.5%*	5/9 *55.5%*	7/9 *77.8%*	
Control (G4)	0/45 *0%*	0/45 *0%*	0/45 *0%*	0/45 *0%*	0/5 *0%*	0/5 *0%*	0/5 *0%*	0/5 *0%*	0/5 *0%*	0/5 *0%*	0/5 *0%*	0/5 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	0/6 *0%*	

Proportions (n/N): number of placentomes, lymph nodes or fetal tissues showing lesions or T. gondii-DNA positive samples among the total number of tested samples; a All foetal tissues are included. b Two foetuses from G1 and one from G2 were too autolytic to allow proper analysis by HE, PCR or IFAT; c Uncoagulated blood or thoracic fluid unavailable for IFAT analysis in two fetuses.

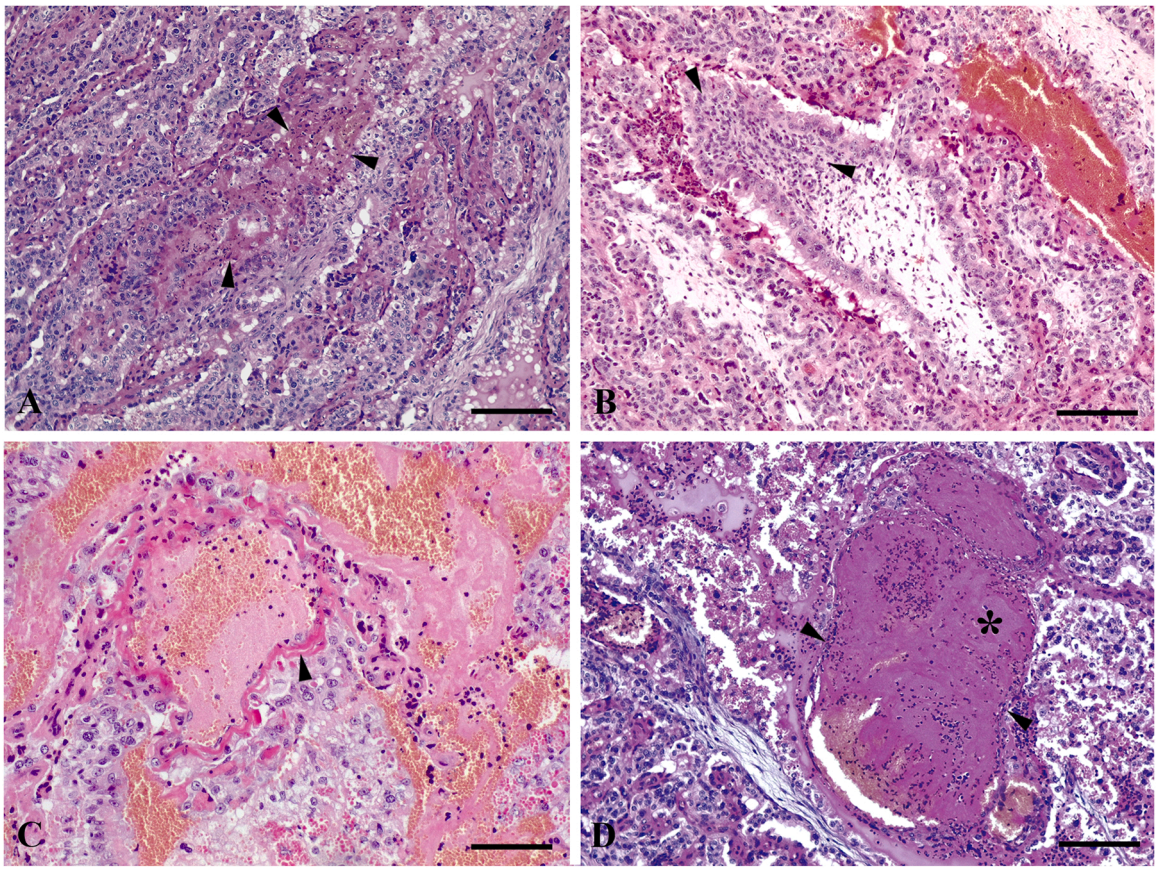

Fig. 2. Microscopic lesions in the placenta of sheep at 28 days post infection. **A**. Foci of necrosis at the interdigitate area of the placentome (necrotizing placentitis) from a sheep infected with TgShSp1 isolate characterized by homogeneous eosinophilic material admixed with numerous degenerated cells and karyorrhectic debris (arrowheads). H-E. Scalebar 100 μm. **B.** Hypercellularity at the mesenchymal tissue of a foetal villus from a sheep infected with TgShSp1 isolate. There is an increased number of mononuclear cells morphologically consistent with lymphocytes and, to a lesser extent, macrophages. H-E. Scalebar 100 μm **C.** Venule at the interdigitate area of a placentome from a sheep infected with TgShSp1 isolate. The blood vessel wall is transmurally replaced by a brightly eosinophilic material (hyalinization; arrowhead) admixed with cellular debris. The left and upper side of the picture shows multifocal haemorrhages and eosinophilic globular proteinaceous material. H-E. Scalebar 50 μm. **D.** Venule at the interdigitate area of a placentome from a sheep infected with TgShSp16 isolate. The blood vessels wall is infiltrated by degenerated neutrophils and karyorrhectic cellular debris admixed with eosinophilic material (necrotizing vasculitis; arrowheads), attached to the endothelial wall completely or partially occluding the lumen of the vessels, there are fibrin thrombi admixed with necrotic debris (asterisk). H-E. Scalebar 100 μm.

lesions went from thickening of the vessel wall with deposition of brightly eosinophilic homogeneous material (hyalinization) to loss of the endothelium with moderate to severe necrosis of the vascular wall, characterised by homogeneous eosinophilic fibrillar material admixed with degenerated neutrophils (necrotizing vasculitis) (Fig. 2C). Frequently, affected vessels were partially or completely occluded by organised eosinophilic fibrillar material attached to the endothelium of the vessel (thrombus) that were commonly intermingled with fibroblasts and inflammatory cells (organising thrombus) (Fig. 2D).

Multifocal foci of granular to homogenous basophilic material, interpreted as mineralization, where frequently seen scattered throughout the interdigitated area of the placentomes, however, this was not considered because of infection as it was found in infected as well as in control animals.

Finally, jejunal mesenteric and ileofemoral lymph nodes had normal histological features. Frequently follicles had prominent germinal centers within all infected groups, together with scattered tingible body macrophages (activated lymphoid tissue).

3.3.2.2. Foetuses and lambs. Samples of the foetuses from early abortion showed variable degree of autolysis and oedema. βAPP accumulation at the white matter denoting leukomalacia (Fig. 3A) was detected in all foetuses from G1 (11/11, 100%), in seven out of eight of G2 (87.5%) and all foetuses from G3 (12/12, 100%). There were no significant differences in the frequency or severity of the lesions when comparing the infected groups ($p > 0.05$).

Regarding the foetuses from the serial euthanasia at 14 and 28 dpi, those collected at 14 dpi showed no lesions. The degree of autolysis of two foetuses from G1 and one foetus from G3 at 28 dpi, all of them with gross lesions suggesting foetal death before euthanasia, hampered a proper histological and PCR evaluation. In the rest of the foetuses, the lesions were characterised by multifocal foci of gliosis (Fig. 3B), some of them with central area of necrosis. Tissue cysts were occasionally observed in the brain with crescent shaped basophilic bradyzoites (Fig. 3B-inset). The liver showed perivascular mononuclear inflammatory cells and multifocal, randomly distributed, foci of inflammation frequently with a central area of eosinophilic material (lytic necrosis) (Fig. 3C); whereas lungs showed peribronchiolar and perivascular inflammatory mononuclear infiltrates that occasionally extends into the adjacent alveoli. Skeletal and cardiac muscle showed foci of mononuclear inflammatory cells most likely lymphocytes, mainly around vessels, expanding endomysium and separating myocytes (Fig. 3D).

Foetuses from serial euthanasia at 28 dpi showing microscopic lesions compatible with *T. gondii* in at least one organ were significantly more frequent in G1 (8/8; 100%) than in G2 (2/10; 20%) ($p < 0.05$), whereas G3 had six foetuses with lesions (6/9; 67%) (Table 3); moreover, lesions were more severe, in terms of frequency, in G1 compared with G2 and G3 ($p < 0.01$). In the brain, six foetuses from four sheep of

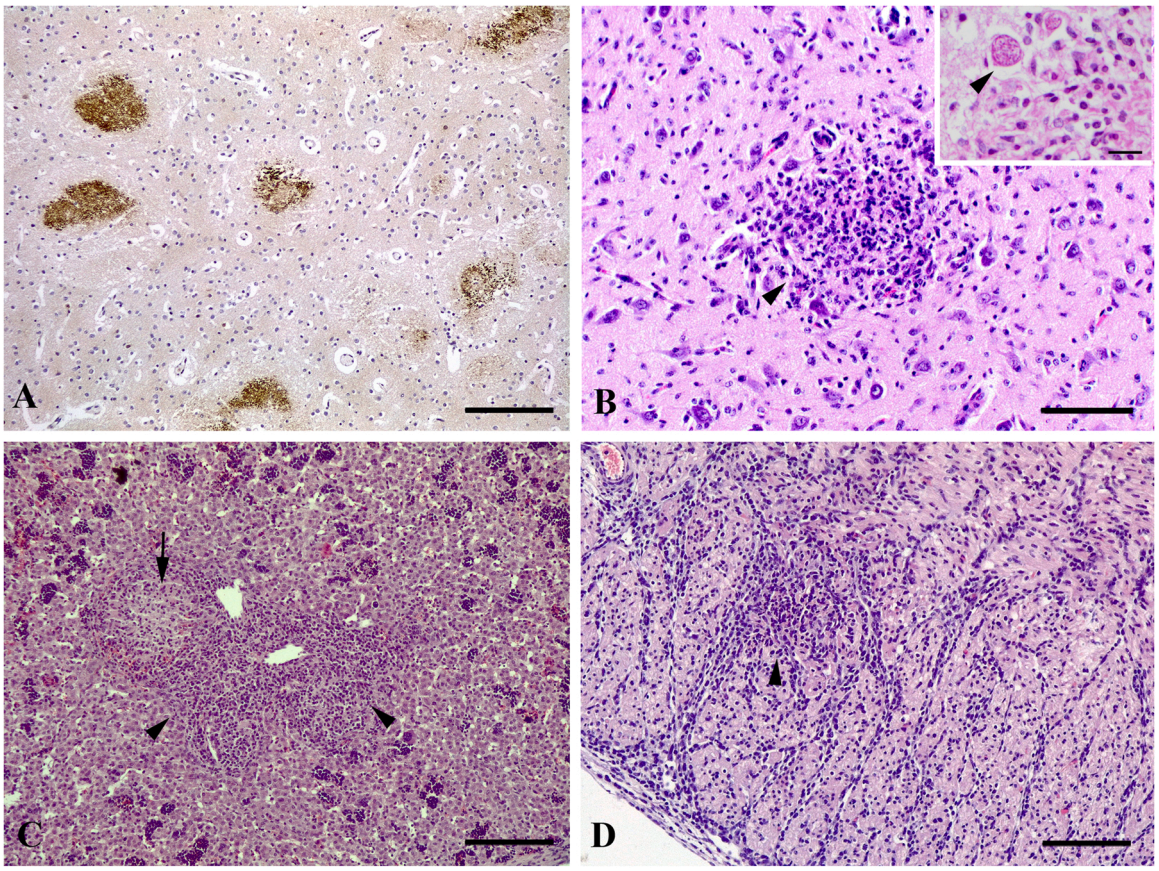

Fig. 3. Microscopic lesions found in infected foetuses. **A**. Foetal brain from an early abortion from G1. Multiple areas of white matter necrosis (leukomalacia) labelled in brown signal. βAPP IHC. H-E. Scalebar 100 μm. **B-D:** Microscopic lesions in foetuses from sheep euthanized at 28 days post infection (G1). **B**. Brain. Foci of gliosis. Notice the absence of necrosis. H-E. Scalebar 50 μm. Inset: Brain. Foci of eosinophilic cellular debris (necrosis) at the lower-right corner of the picture. It is surrounded by inflammatory mononuclear cells and a round to oval 12–15 μm in diameter tissue cyst-like structure with numerous basophilic crescent shaped bradyzoites inside (arrowhead). H-E. Scalebar 20 μm. **C.** Liver. Portal hypercellularity denoted by mixed inflammatory infiltrate surrounding central arteries (arrowheads) with a foci of necrosis at the left of the image (arrow) characterized by loss of hepatocytes and their replacement by eosinophilic material admixed with cellular and karyorrhectic debris. Physiological haematopoiesis is present throughout the organ. H-E. Scalebar 100 μm **D**. Heart. Mononuclear inflammatory cells (arrowheads) are infiltrating, separating and effacing cardiomyocytes (myocarditis) affecting the pericardium (pericarditis). H-E. Scalebar 100 μm.

G1 (6/8, 75%) showed multifocal encephalitis. In contrast, in G3 only three foetuses from the same sheep had lesions in the brain (3/9, 33%) (Table 3). Scant tissue cysts containing bradyzoite-like structures were seen in the brain of one foetus from G1 and other from G3. Neither microscopic lesions nor tissue cysts were found in any of foetal brain of G2. Again, G1 had more severe lesions in the liver than G2 or G3 ($p < 0.05$), as all foetuses of G1 had lesions in the liver (8/8; 100%), whereas in G3 they were found in four foetuses belonging to four different sheep (4/9, 44.4%) and two foetuses from G2 had lesions (2/10, 20%). Similarly, there were significant differences regarding the lesions in the heart, since seven out of 10 foetuses (70%) of G1 presented inflammatory cells infiltrating the myocardium and, occasionally, the pericardium (7/8, 87.5%) and no lesions were seen in foetuses from G2 or G3 ($p < 0.01$). Regarding the skeletal muscle, a similar pattern is found, where lesions were more common in G1 than in G3 ($p < 0.05$) and both than G2 (no lesions, $p < 0.05$). Specifically, four foetuses from G1 (4/8, 50%) and two from G3 (2/9, 22%) showed non-purulent myositis. Finally, the lungs of four foetuses from G1 (4/8, 50%) and three from G3 (3/9, 33%) were affected by a mild non-purulent multifocal pneumonia, whereas no lesions were found in G2 ($p < 0.05$). It is important to mention that, when viable and dead foetuses were found in the same sheep (*i.e.* siblings), none of the foetuses that were found dead at 28 dpi and suitable for histological evaluation showed any lesions compatible with *T. gondii* infection, whereas the viable ones of the same sheep, as dead foetuses had always at least one viable sibling, frequently had compatible lesions.

Regarding late abortions and lambs, microscopic lesions in at least one tissue sample were found in 79%, 78% and 67% of G1, G2 and G3, respectively, without significant differences between them ($p > 0.05$) (Table 4). Glial foci with or without a central area of necrosis were observed in the brain of infected lambs or late abortions as follows: 10/14 (71%) in G1, 3/9 (33%) in G2 and 2/3 (67%) in G3. Liver was affected in 4/14 (29%) in G1, 4/9 (44%) in G2 and 0/3 (0%) in G3 and the lungs presented lesions in 4/14 (29%) in G1, 5/9 (56%) G2 and 2/3 (67%) in G3. Regarding cardiac muscle, microscopic lesions were present in 9/14 (64%) in G1, in 2/9 (22%) G2 and no microscopic lesions were found in G3. Skeletal muscle only presented microscopic lesions in 4/14 (29%) animals from G1. No statistically significant differences were found between groups when considering each organ.

3.4. Parasite detection and quantification

All placentomes and lymph nodes of the control group as well as all tissue samples from early aborted foetuses were negative for *T. gondii* DNA presence. Regarding samples of serial euthanasia, *T. gondii* DNA was not detected at 14 dpi in any placentome from G2 or G3 whereas five placentomes belonging to three out of 5 sheep of G1 were positive (Table 3), being statistically significant compared with the other two groups ($p < 0.05$). Regarding lymph nodes, only the jejunal mesenteric lymph node of one sheep from G1 was positive at this time point whereas

Table 4
Toxoplasma gondii DNA detection and histopathological lesions detected in late abortions, stillbirths, and lambs.

Isolates (Inoculation group)	Brain		Liver		Heart	Lung	Skeletal muscle
	HE	PCR	HE	PCR	HE	HE	HE
TgShSp1 (G1)	10/14	14/14	4/14	14/14	9/14	4/14	4/14
	71.4%	*100%*	*28.6%*	*100%*	*64.2%*	*28.6%*	*28.6%*
TgShSp16 (G2)	3/9	5/9	4/9	2/9	2/9	5/9	0/9
	33.3%	*55.5%*	*44.4%*	*22.2%*	*22.2%*	*55.5%*	*0%*
TgShSp24 (G3)	2/3	2/3	0/3	2/3	0/3	2/3	0/3
	66.7%	*66.7%*	*0%*	*66.7%*	*0.0%*	*66.7%*	*0%*
Control (G4)	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	0%	*0%*	*0%*	*0%*	*0%*	*0%*	*0%*

Proportions (n/N): number of animals showing lesions or resulted positive for T. gondii DNA among the total number of foetal tissues tested

all iliofemoral lymph nodes were negative. All samples from foetuses of infected sheep (G1, G2 and G3) euthanized at 14 dpi resulted negative including controls animals (Table 3).

In the second serial euthanasia at 28 dpi, *T. gondii*-DNA was detected in placentomes and foetuses of all infected groups. In G1, every sheep had positive placentomes and most of their placentomes resulted positive (43/45; 96%), showing statistically significant differences ($p < 0.0001$) with G2 and G3 (Table 3). In contrast, three out of five

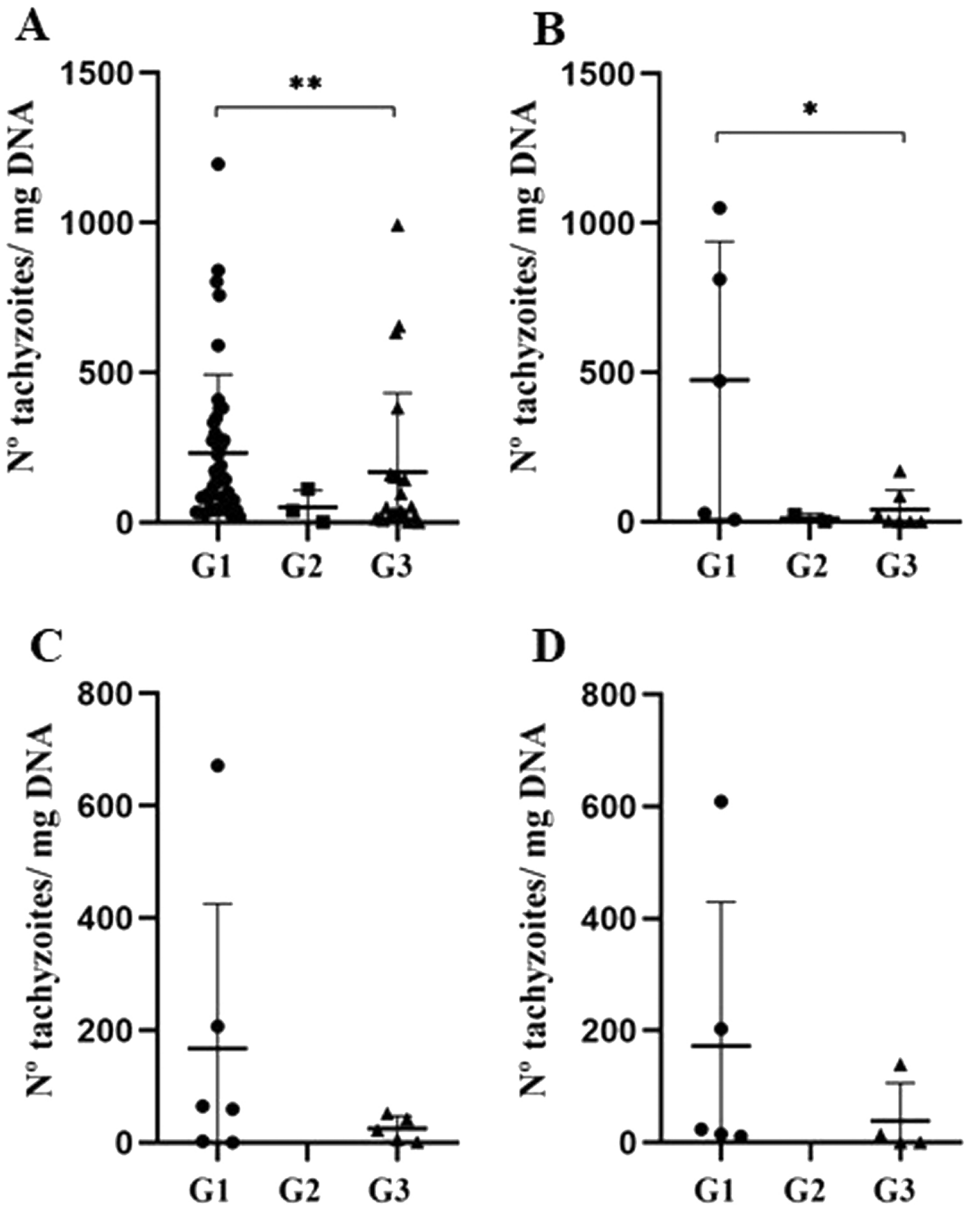

Fig. 4. Dot-plot graphs of *Toxoplasma gondii* burdens at 28 days post-infection in placental and foetal tissues from sheep infected with *T. gondii* oocysts of each isolate. **A**. Placentomes. **B.** Foetal brain. **C.** Foetal liver. **D.** Foetal heart. Each dot represents individual values of parasite burden. Error bars represent standard deviation. Significant differences between infected groups are indicated with asterisk where * and ** denote $p < 0.05$ and $p < 0.01$, respectively.

sheep of G2 with one placentome each were positive (3/45, 7%) and in G3 all sheep were positive in almost half of their placentomes (21/45, 46.6%). Regarding jejunal lymph nodes: two from G1, two from G2 and one from G3 were positive whereas, only two iliofemoral lymph nodes resulted positive in G2 (Table 3). Regarding parasite detection in foetuses, all foetuses in G1 (8/8; 100%) and eight out of nine in G3 (87%) resulted positive. Moreover, 75% of foetuses from G1 and 55% from G3 had more than one positive tissue sample. In contrast, only two out of 10 foetuses, both belonging to the same sheep, resulted positive in G2 (20%), having statistically significant differences with the other two groups ($p < 0.01$). When analysing the tissue distribution of the parasite, the brain resulted only positive in five foetuses from G1 (5/8, 63%) and G3 (5/9, 55%), having differences with G2 where none was affected ($p < 0.01$). The liver resulted positive in six foetuses from G1 (6/8, 75%), five foetuses from G3 (5/9, 55%) and no *T. gondii*-DNA was detected in foetal livers from G2 ($p < 0.05$). Finally, cardiac muscle resulted positive in six foetuses in G1 (6/8, 75%) belonging to four infected sheep, five in G3 (5/9, 55%) belonging to five infected sheep and none from G2 ($p < 0.05$).

Concerning the parasite burden at 28 dpi, the mean of tachyzoites/mg of DNA in placentomes was the highest in G1, followed by G3 and G2, showing significant differences between G1 and G3 ($p < 0.01$) (Fig. 4). Regarding foetal organs, G1 had the highest parasite burden in all three organs analysed whereas G3 had lower parasite burdens having significant differences in the brain with G1 (Fig. 4) ($p < 0.05$). Brain samples of foetuses from G2 showed the lowest parasite burden (11.9 tachyzoites/mg of DNA) (Fig. 4).

Regarding parasite detection in foetuses aborted later than 28 dpi, stillbirths or lambs DNA-*T. gondii* was detected in all foetuses of G1 (14/14, 100%), five of G2 (5/9, 55%) and three of G3 (3/3, 100%) belonging to 6, 6 and 3 sheep, respectively (Table 4). The frequency of parasite detection in G2 was significantly lower than in G1 and G3 ($p < 0.001$) (Table 4). In relation to parasite distribution, 100% (14/14), 55% (5/9) and 66% (2/3) of the brain samples from groups G1, G2 and G3, respectively were positive (Table 4), with statistically significant differences between G1 and G2 ($p < 0.05$). These foetuses with positive brain samples belonged to 4/4, 5/6 and 2/3 sheep from each group respectively. Finally, the liver of all G1 animals (14/14, 100%), two from G2 (2/9, 22%) and two from G3 (2/3, 66%) were positive (Table 4), with statistically significant differences between G1 and G2 ($p < 0.0001$).

4. Discussion

Genotypic diversity of *T. gondii* isolates has been proposed as responsible factor for variations in the clinical presentations of toxoplasmosis in different hosts, including humans (Boothroyd and Grigg, 2002). The cumulative mortality rate in murine models has been used to classify the virulence of the different isolates as highly-, intermediate-, and non-virulent and linking them to the archetypal types I, II and III (Su et al., 2002). Besides, there is a growing evidence that virulence degree observed in mice model do not necessarily correlate with other animal models (Taniguchi et al., 2018; Fernández-Escobar, 2021; Largo de la Torre et al., 2022). In fact, the current study confirms what has been stated previously: results obtained in murine models might not be directly extrapolated to sheep (Sánchez-Sánchez et al., 2019). Actually, it has been suggested that several variables, and not only cumulative mortality in mice, should be considered as phenotypic markers when characterising *T. gondii* isolates (Calero-Bernal et al., 2022), and the findings from the current study fit into that statement.

The experimental design carried out involved two different approaches that enabled the evaluation of the clinical outcome of ovine toxoplasmosis from the infection to end of gestation occurred either by abortion or lambing, and the other approach which allow for the detailed study of microscopic lesions, parasite distribution and parasite burden in both maternal and foetal tissues when infected ewes were euthanized at 14 and 28 dpi. In addition, an oral dose of 10 sporulated oocysts was used to reproduce ovine toxoplasmosis expecting the lowest percentage of early abortions, similarly to previous studies (Sánchez-Sánchez et al., 2019). Furthermore, three recently obtained *T. gondii* isolates with a low, and known, number of passages lower than 10 in cell culture were compared in this experimental infection in pregnant sheep. This is a relevant point to drive conclusions into the clinical outcomes in experimental models, as it has been established that a high number of cell culture passages can alter the behaviour and virulence of the isolates in *in vitro* and *in vivo* models (Jerome et al., 1998; Howe et al., 1996; Harmer et al., 1996; Sánchez-Sánchez et al., 2019). The influence of the number of passages might explain why microscopic lesions found in the 14 and 28 dpi placentas and foetuses in this study were milder than previous studies with a similar experimental design but that employed laboratory-adapted *T. gondii* isolates that had gone through a high number of passages in cell culture, which could have enhanced their virulence (Sánchez-Sánchez et al., 2019). In this sense, remains unclear the effect of reproducing the life cycle using the cat as definitive host on the *in vivo* virulence of laboratory-adapted *in vitro* isolates. Nevertheless, to compare these results with previous ones (Dubey, 2009; Gutierrez et al., 2010; Castaño et al., 2014, 2016), it is necessary to consider not only the number of passages but also the stage of gestation when infection occurs, the parasite stage, the dose of infection and the genotype, all of them key players in the outcome of the infection. The great heterogeneity of experimental designs in the published studies hamper this comparison (Dubey et al., 2020).

Isolates belonging to clonal type II, such as TgShSp1 and TgShSp16 (ToxoDB genotype#3), are the most prevalent among sheep flocks in Europe, considered of intermediate virulence in mice, and have been associated to multiple cases of ovine abortions (Fernández-Escobar et al., 2022). On the other hand, isolates belonging to clonal type III, such as TgShSp24 (ToxoDB#2), are considered as non-virulent in mice and it caused the highest number of early abortions (32%) in the current study, while infection with both ToxoDB#3 isolates (TgShSp1 y TgShSp16) caused 20% of abortion rate. This latter value is similar to that previously found when pregnant sheep were infected with 10 oocysts of TgME49 (ToxoDB#1) and TgShSp1 (ToxoDB#3) isolates in previous experiments (Sánchez-Sánchez et al., 2019). The fact that TgShSp24 also showed an earlier dissemination in previous studies in *in vitro* or mice and pigs experimental models is a relevant finding (Fernandez-Escobar et al., 2021; Largo-de la Torre et al., 2022). It is possible that this behaviour could be linked to the fact that ewes infected with TgShSp24 showed higher early mortality in foetuses than those infected with TgShSp1 or TgShSp16. Similarly, when analysing the number of late abortions or stillbirths, figures were higher in those sheep infected with TgShSp1 isolate than in those infected with TgShSp16 isolate, despite both belonging to the same clonal type and haplogroup. Remarkably, the genetic characterization through RFLP markers ($n = 11$) and MLST analysis ($n = 3$ polymorphic genes) of TgShSp1 and TgShSp16 isolates previously reported by Fernández-Escobar et al. (2020a), (2020b) proved to be the same. However, it is not clear whether there are other unknown genetic markers that could have participated. Thus, further genome wide association studies (GWAS) and search for specific virulence factors between isolates are needed. In this sense, and regarding inter- and intra-genotype variations, early abortions occurred in the current study with the three different isolates, even with TgShSp24 isolate, but the role of the isolate's genetic background on the occurrence of early abortions remains unclear, similarly as the pathogenesis of this clinical presentation, which has been always reported with *T. gondii* isolates genotypes belonging to clonal type II (Buxton et al., 1988; Trees, 1989; Castaño et al., 2014; Benavides et al., 2017). Regarding the incidence of abortions observed in this study, the dose, the time of gestation and the isolates used do not allow to compare these results with those previously published (reviewed by Dubey et al., 2020). However, Sánchez-Sánchez et al. (2019) did not found differences between TgShSp1 and TgME49 isolates belonging to the same type

II using two different doses (50 and 10 oocysts) and following similar experimental conditions as this study.

Rectal temperature was another parameter used to evaluate the phenotypic variability of the isolates, which showed differences during the first week post-infection, rising significantly earlier for TgShSp16 and TgShSp24 isolates than for TgShSp1 isolate. This finding has been also reported in an experimental porcine model using both TgShSp1 and TgShSp24 isolates (Largo-de la Torre et al., 2022). Considering fever as a host response against an active infection, such as *T. gondii,* that could trigger the production of endogenous pyrogens as IFNγ (Dinarello, 1999), an earlier rise of fever could suggest an earlier arrive of tachyzoites to blood of TgShSp16 and TgShSp24 isolates than TgShSp1 isolate or even a lesser immunogenicity of this latter. The production of IFNγ at lymph nodes after the invasion by *T. gondii* is a key feature in the pathogenesis of toxoplasmosis (Buxton et al., 2007; Innes et al., 2009). Under present conditions, this finding is presumably attributable to the differences between the isolates. Seroconversion occurred in all infected groups at 12–14 dpi, similarly to previous experimental studies in sheep (Buxton, 1993; Esteban-Redondo and Innes, 1998; Castaño et al., 2019; Sánchez-Sánchez et al., 2019). Nevertheless, following the same dynamics than fever, detection of specific antibodies was earlier after infection with TgShSp16 and TgShSp24 isolates than with TgShSp1 isolate (the same finding was also reported using TgShSp1 and TgShSp24 isolates in an experimentally infected piglets) (Largo-de la Torre et al., 2022), which is consistent with an earlier onset of immune response against the arrival of tachyzoites to the blood suggested above and the subsequent development of an earlier adaptive immune response (Innes et al., 2009). The monitorization of parasitaemia, which has been reported to occur between 3 and 10 dpi in sheep (Dubey and Sharma, 1980; Esteban-Redondo and Innes., 1998), could shed light on the behaviour of the different isolates after infection in future studies.

The infection with the three isolates had unexpected consequences regarding the occurrence of vascular lesions in placentomes found at 28 dpi. This is a remarkable finding, as those changes were similar among groups, despite the differences in parasite distribution and burden. Although is it well known that *T. gondii* could infect endothelial cells, mainly studied in ocular toxoplasmosis and central nervous system (Konradt et al., 2016; Smith et al., 2021), the descriptions of vascular lesions directly related to *T. gondii* are uncommon and mainly linked to an immunocompromised health status (Vidal, 2019; Mazzariol et al., 2021). The occurrence of vascular lesions in the current study is unlike to be linked to the compromise of the host immune response, as no other concomitant diseases were found in these experimental animals. However, one must bear in mind the Th1/Th2 paradigm during gestation, which would favour a T-reg, anti-inflammatory microenvironment from mid gestation (Colucci et al., 2014). It is then possible that *T. gondii* might take advantage of this modulation on the immune response to disseminate through the endothelium and cause vascular lesions. On the other hand, this paradigm into the maternal-foetal relationship during pregnancy has been mainly studied in murine models and its application to other species, such as sheep, needs to be further evaluated (Entrican, 2002; Mukhopadhyay et al., 2020).

Additionally, and leaving apart the occurrence of early abortions, which pathogenesis seems to be different to that of the classical toxoplasmosis (Owen et al., 1998; Benavides et al., 2017), oral infection with *T. gondii* sporulated oocysts takes between 15 and 28 days to cause characteristic initial lesions in the placentomes. When studying the placentomes at 28 dpi, there were clear differences in the frequency of necrotic and/or infiltrative lesions, as animals infected with TgShSp1 isolate showed histological lesions in significantly more placentomes than those infected with TgShSp16 or TgShSp24 isolates; in the same way as occur in foetuses in which lesions were more frequent in animals infected with TgShSp1 isolate. It is important to highlight that cardiac muscle, which is normally not affected in a high percentage of foetuses (Rassouli et al., 2013; Nunes et al., 2017), was the organ that showed the higher differences between isolates, where lesions were found in most foetuses infected with TgShSp1 isolate at 28 dpi whereas no lesions were found in with TgShSp16 and TgShSp24 isolates. When studying the correlation between the occurrence of histological lesions and parasite detection in placentomes, the frequency of detection by PCR was higher than that of lesions in infections with TgShSp1 and TgShSp24, which supports the hypothesis that lesions are mainly caused by the presence of the parasite suggesting that the presence of the parasite is required, but not enough, to trigger the development of lesions (Castaño et al., 2016; Gutiérrez et al., 2010). However, the occurrence of microscopic lesions was more frequent than parasite detection in sheep infected with TgShSp16 isolate; this could be because the parasite burden was not enough to be detected by PCR. This hypothesis is consistent with the occurrence of milder lesions than those described in other studies using higher doses of infection (Castaño et al., 2016; Sánchez-Sánchez et al., 2019). Another possibility is that, as different samples from the same placentome were used for different techniques, if the parasite burden was low, the samples for PCR detection did not contain any parasite. The parasite detection in placentomes at 28 dpi was markedly lower for TgShSp16 and TgShSp24 isolates (6.6% and 46.6%, respectively) than for TgShSp1 (95.5%), and there were also similar differences in the parasite burdens, even though, the severity of the lesions was similar among groups. Suggesting that, although the parasite is needed to trigger the occurrence of lesions, hence more frequent lesions when more parasites invade the placenta, a higher parasite burden does not increase the severity of the lesions, as the lesions at placentas infected with TgShSp16 had similar histologic characteristics that those infected with TgShSp1 and TgShSp24.

The three isolates used in the current study have been deeply characterized in previous *in vitro* studies, and there seems to be a lack of correlation between the capacity to multiply in ovine trophoblast cells reported (Fernández-Escobar et al., 2021) and the burden found in the ovine placentas at 28 dpi. In this sense, the *in vitro* characterization in trophoblasts and OvMOs, showed that TgShSp24 isolate had the highest parasite invasion, proliferation, and parasite burden, followed by TgShSp16 isolate, and TgShSp1 isolate showing the markedly lowest rates in all phenotypic *in vitro* markers (Fernández-Escobar et al., 2021; Vallejo et al., submitted). However, parasite behaviour in a primary or immortal cell line does not necessarily mimics the real situation in a tissue due to the lack of environmental factors, as it is shown by the high parasite burden of TgShSp1 isolate at 28 dpi does not correlate with the low burden found *in vitro* in AH-1 trophoblast cell line (Fernández-Escobar et al., 2021). Previous studies, in human placental explants, have shown differences in the growth rate between isolates, specifically a slightly slower growth of type II isolates relative to types I and III (Robbins et al., 2012). The reasons, and implications, of these differences warrant further studies on the pathogenesis of the disease.

Infection by TgShSp1 isolate resulted in an earlier invasion of the placenta, as parasite DNA was detected at 14 dpi, as well as more frequent lesions, parasite detection and higher parasite burden, in both the placenta and foetal tissues, at 28 dpi when compared with TgShSp16 and TgShSp24 isolates. These results, taken together with the earlier establishment of fever and specific serological response in the sheep infected by TgShSp16 and TgShSp24 isolates, suggest that the initial immune response developed by the host might have controlled the initial dissemination of TgShSp16 and TgShSp24 isolates, hence delaying the invasion of the placenta and foetuses; or even preventing it from occurring as there were sheep and foetuses from these isolates that were negative. Additionally, TgShSp1 isolate may be inducing a proinflammatory immune response as infected sheep showed more frequently infiltrative lesions at the placenta or foetal heart than those infected with TgShSp16 or TgShSp24 isolates. This assumption is also supported by Largo-de la Torre et al. (2022), who found a higher pro-inflammatory immune response of piglets infected with TgShSp1 than those infected with TgShSp24. Nevertheless, the specific mechanisms behind these differences need to be elucidated exploring the local immune response or even comparing capability of migration across placenta (*i.e.*, active

motility) of each isolate. In this sense, a Th1 proinflammatory immune response is related with a higher tissue damage and subsequent more severe lesions (Cohen & Denkers, 2014). In this study, the scarcer histological lesions and the lowest parasite burden found at 28 dpi in animals infected with TgShSp16 and TgShSp24 isolates suggest that these isolates might have delayed invasion of the placenta, hence it was less detected at the 28 dpi. These isolates might have favoured a permissive microenvironment, with a downregulated Th1 response. Obviously, this hypothesis regarding the relation between *T. gondii* isolates and host immune response requires further studies and characterization.

5. Conclusions

This study shows that *T. gondii* infection of sheep at 90 days of pregnancy with a low dose (ten sporulated oocysts) of two ToxoDB#3 isolates (TgShSp1 and TgShSp16 isolates) and one ToxoDB#2 isolate (TgShSp24) cause similar reproductive losses. Furthermore, it has been demonstrated that *T. gondii* isolates belonging to type III (TgShSp24) can also cause early abortions. However, inter-, and intra- genotype differences regarding other phenotypic parameters such as rise of temperature, dynamics of antibodies, parasite dissemination or frequency of microscopic lesions has been confirmed. TgShSp1 isolate (type II, #3) was more pathogenic if frequency of lesions and dissemination and parasite loads are considered, followed by TgShSp24 isolate (type III, #2). By contrast, TgShSp16 isolate (type II, #3) had a reduced ability to cause lesions and disseminate in different tissues. Thus, present study provide evidence that genotype of each *T. gondii* isolate has an important role on the outcome of infection in ovine toxoplasmosis. Additional molecular and functional investigations aimed at unravelling the machinery responsible for the observed effects is of major interest.

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CRediT authorship contribution statement

Raquel Vallejo: Investigation, Resources, Writing – original draft. **Julio Benavides**: Conceptualization, Methodology, Writing – review & editing, Project administration, Funding adquisition. **Noive Arteche-Villasol**: Investigation, Resources, Methodology. **Roberto Sánchez-Sánchez**: Investigation, Writing – review & editing. **Rafael Calero-Bernal:** Investigation, Writing – review & editing. **Ma Carmen Ferreras:** Investigation, Writing – review & editing. **Miguel Criado:** Investigation, Resources. **Valentín Pérez:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **Luis Miguel Ortega-Mora**: Conceptualization, Writing – review & editing, Project administration, Funding adquisition, Supervision. **Daniel Gutiérrez-Expósito**: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Project administration, Funding adquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2023.109889.

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