Tesis doctoral

INTERACCIÓN HOSPEDADOR/PARÁSITO EN LA TOXOPLASMOSIS OVINA: INFLUENCIA DE LA VIRULENCIA DEL PARÁSITO SOBRE LA RESPUESTA INMUNITARIA Y LA PATOGENIA DE LA ENFERMEDAD

PhD Thesis

HOST/PARASITE INTERACTION IN OVINE TOXOPLASMOSIS: INFLUENCE OF PARASITE VIRULENCE ON IMMUNE RESPONSE AND DISEASE PATHOGENESIS

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A LOS QUE SE HAN IDO, POR ENSEÑARME A LLORAR, A PERDER EL MIEDO Y QUE LA VIDA ES MUY CORTA PARA NO DISFRUTARLA.

"Define el éxito en tus propios términos

y vive una vida que te haga sentir orgullosa"

Anne Sweendey

"Todo lo que hice fue vivir sin pensar en el mañana

Intentando conseguir todo aquello que un día soñaba.

He pisado suelo, he volado hasta los cielos

He creído estar perdido y he encontrado mi camino"

Ander Valverde (Green Valley)

"Mientras tú gastas saliva en escupir prejuicios

***ellas** vuelan alto cual edificio"*

Mafalda

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TABLE OF ABBREVIATIONS

APP	Amyloid precursor protein	Proteína precursora amiloidea
ARN/RNA	Ribonucleic acid	Ácido ribonucleico
ASF	Average size of focus	Tamaño medio del foco
BBB	Blood-brain barrier	Barrera hematoencefálica
CD	Cluster of differentiation	Cúmulos de diferenciación
cDNA	Complementary DNA	ADN complementario
cm^2	Square centimetre	Centímetro cuadrado
CNS/SNC	Central nervous system	Sistema nervioso central
CSF	Macrophage colony-stimulating factor	Factor estimulador de macrófagos
CSIC	Spanish National Research Council	Consejo Superior de Investigaciones Científicas
DAPI	4',6-diamidino-2-phenylindole	4',6-diamidino-2-fenilindol
DNA/ADN	Deoxyribonucleic acid	Ácido desoxirribonucleico
dpi	days post infection	días post infección
ELISA	Enzyme-linked Immunosorbent Assay	Ensayo por inmunoabsorción ligado a enzimas
FITC	Fluorescein isothiocyanate	Isotiocianato de fluoresceína
g	Gram	Gramo
G1	Experimental group 1 (TgShSp1)	Grupo experimental 1 (TgShSp1)
G2	Experimental group 2 (TgShSp16)	Grupo experimental 2 (TgShSp16)
G3	Experimental group 3 (TgShSp24)	Grupo experimental 3 (TgShSp24)
G4	Experimental group 4 (Control)	Grupo experimental 4 (Controles)
GFAP	Glial fibrillary acidic protein	Proteína gliofibrilar ácida
GRAs	Dense Granule Antigen	Antígeno de Gránulos Densos
HD	Definitive Host	Hospedador Definitivo
H-E	Hematoxylin and Eosin	Hematoxilina y Eosina
HI	Intermediate Host	Hospedador Intermedio
HIER	Heat-induced antigen retrieval	Desenmascaramiento de antígeno inducido por calor
hpi	Hours post infection	Horas post infección
Iba1	ionized calcium binding adaptor molecule I	Molécula adaptadora de unión a calcio ionizado I
IDO	Indoleamine 2,3-dioxygenase	Indolamina 2,3-dioxigenasa
IFAT	Immunofluorescence antibody test	Técnica de inmunofluorescencia indirecta
IFN-γ	Interferon gamma	Gamma Interferón
Ig	Immunoglobulin	Inmunoglobulina
IHC	Immunohistochemistry	Inmunohistoquímica
IL	Interleukin	Interleuquina
IMC	Inner membrane complex	Complejo de la membrana interna
iNOS	Inducible nitric oxide synthase	Óxido nítrico sintetasa inducible
ITS	Internal transcribed spacer	Espaciador transcrito interno
L	Lesion	Lesión
MAT	Microscopic agglutination test	Microaglutinación con antígenos vivos
mg	Miligramo	Miligramo
MHC	Major histocompatibility complex	Complejo mayor de histocompatibilidad
MIC	Microneme protein	Proteina de micronemas
min	Minute	Minuto

ml	Milliliter	Milílitro
MLS	Multilocus sequence typing	Tipificación multilocus de secuenciado
mm	Milimetre	Milímetro
mm²	Square milimetre	Milímetro cuadrado
MOI	Multiplicity of Infection	Multiplicidad de la infección
mRNA	Messenger RNA	ARN mensajero
MS	Microsatellite	Microsatélites
MyD88	Myeloid differentiation primary response 88	Proteína adaptadora 88
N. caninum	*Neospora caninum*	*Neospora caninum*
n/a	Not available	No disponible
NETs	Neutrophil Extracelular Traps	Trampas Extracelulares de Neutrófilos
NK	Natural Killer (cells)	Asesinas Naturales (células)
NL	No lesion	Sin lesión
NLR	Nod-like receptor	Receptor tipo NOD
NO	Nitric oxide	Óxido Nítrico
No.	Number	Número
NOD	Nucleotide-binding oligomerization domain	Dominio de oligomerización de unión a nucleótidos
PAMPS	Pathogen associated molecular pattern	Patrones moleculares asociados al patógeno
PAS	Periodic acid-Schiff	Ácido periódico de Schiff
Pb/pb	Base pairs	Pares de bases
PBMCs	Peripheral blood mononuclear cells	Células mononucleares de sangre periférica
PBS	Phosphate-buffered saline	Tampón fosfato salino
PCR	Polymerase chain reaction	Reacción en cadena de la polimerasa
pi	Post infection	Post infección
PPAR	Peroxisome proliferator- activated receptor	Receptores activados por proliferadores peroxisomales
PRR	Pattern Recognition Receptors	Receptores de Reconocimientos de Patrones
PRU	Pruginoid	
PRU II-A	Type II PRU-abortion origin isolates (TgShSp1 and TgShSp3)	Aislados tipo II PRU procedentes de abortos (TgShSp1 and TgShSp3)
PRU II-C	Type II PRU-myocardium of chronic infection origin isolates (TgShSp11 and TgShSp16)	Aislados tipo II PRU procedentes de infecciones crónicas (TgShSp11 and TgShSp16)
qPCR	Quantitative PCR	PCR Cuantitativa
QT	Tisular cyst	Quiste tisular
R.D.	Royal decree	Real Decreto
RFLP	Restriction fragment length polymorphism	Polimorfismos de longitud de fragmentos de restricción
ROI	Region of interest	Región de interes
ROP	Rhoptry bulb protein	Proteína de cuerpo de roptrias
ROS	Reactive oxygen species	Especies reactivas de oxígeno
SAG	Surface antigen	Antígeno de superficie
SCID	Severe combined immunodeficiency	Inmunodeficiencia combinada grave
SNPs	Single-nucleotide polymorphism	Polimorfismo de nucleótido único
SRS	SAG1-related sequence	Secuencia SAG1 relacionada
STAT	Signal transducer and activator of transcription	Transductor de señal y activador de la transcripción
t	Tachyzoites	Taquizoítos

T. gondii	*Toxoplasma gondii*	*Toxoplasma gondii*
TAL	Total area of lesion	Área total de lesión
TAS	Total area studied	Área estudiada total
Tc	Cytotoxic T lymphocytes	Linfocitos T citotóxicos
TCR	T cell receptor	Receptor de células T
TGF	Transforming growth factor	Factor de crecimiento transformador
TgIST	*T. gondii* inhibitor of STAT1 transcriptional activity	Inhibidor de *T. gondii* de la actividad transcripcional de STAT1
Th	Helper T lymphocytes	Linfocitos T colaboradores
TLR	Toll-like receptor	Receptor tipo Toll
TNF-α	Tumor necrosis factor alpha	Factor de Necrosis Tumoral alfa
Treg	Regulatory T cells	Células T reguladoras
TRITC	Tetramethylrhodamine	Tetrametilhodamina
TY	Tachyzoite yield	Recolección de taquizoitos
VP	Parasitophorous vacuole	Vacuola parasitófora
WGS	Whole genome sequencing	Secuenciación del genoma completo
+	Positive	Positivo
%LES	Percentage tissue affected	Porcentaje de tejido afectado
°C	Degrees centigrade	Grados centígrados
µg	Microgram	Microgramo
µl	Microliter	Microlitro
µm	Micrometer	Micrómetro

INTRODUCTION

Toxoplasma gondii is a protozoan parasite of great importance both in Europe and globally. Toxoplamosis is considered a major disease in livestock, mainly in sheep, as well as one of the main zoonosis (Dubey et al., 2020a). Despite this importance, ovine toxoplasmosis pathogenesis still has many knowledge gaps, especially in relation to abortions and the influence of the variability of *T. gondii* isolates on the disease (Benavides et al., 2017; Calero-Bernal et al., 2022). Therefore, this PhD Thesis is focused on the study of the relation between *T. gondii* and sheep and includes an evaluation of the influence of *T. gondii* genetic variability on ovine macrophages, a descriptive study of the normal morphology and physiological findings in the placenta and fetuses of a pregnant ovine model, a study of the consequences of the infection with different *T. gondii* isolates in this model and a detailed characterization of the histological lesions and changes in resident central nervous system cells in ovine infected fetuses.

The present PhD Thesis has been arranged in four independent chapters. Each chapter has been written so that it could be understood independently of the remaining parts of the Thesis. In this sense, each chapter contains its own summary, introduction, material and methods, results, discussion and conclusion. Moreover, this PhD Thesis includes a bibliographical review, which provides information on the current context of *T. gondii* and more specifically from ovine toxoplasmosis, a general material and methods section, explaining the two experimental studies developed, a global discussion, which evaluate the four chapters as a whole and points out the scientific opportunities opened as a consequence of the results herein collected, the conclusions obtained in this PhD Thesis, a summary and finally the references, which contain all the publications cited throughout the work.

INTRODUCCIÓN

Toxoplasma gondii es un protozoo con gran importancia tanto en Europa como a nivel mundial. La toxoplamosis provoca grandes perdidas económicas en el ganado, principalmente ovino, y es además una de las principales zoonosis que existen (Dubey et al., 2020a). A pesar de esto, en la patogénesis de la toxoplasmosis ovina aún existen muchas incógnitas, especialmente en relación a los abortos y la influencia que la variabilidad de los aislados de *T. gondii* pueda tener en la enfermedad (Benavides et al., 2017; Calero-Bernal et al., 2022). Por ello, esta Tesis Doctoral se centra en el estudio de la relación parásito/hospedador en la toxoplasmosis ovina, incluyendo un estudio sobre la influencia de los aislados de *T. gondii* en macrófagos ovinos, un estudio descriptivo de la morfología y hallazgos normales en la placenta y fetos sanos de un modelo ovino gestante, un estudio sobre la influencia de diferentes aislados de *T. gondii* sobre el desarrollo de la enfermedad en este modelo y una caracterización detallada de las lesiones histológicas y cambios en las células residentes del sistema nervioso central en fetos ovinos infectados.

La presente Tesis Doctoral contiene una revisión bibliográfica, que aporta información sobre el contexto actual de *T. gondii* y más concretamente de la toxoplasmosis ovina; un apartado general de material y métodos, en el que se explican los dos estudios experimentales desarrollados; cuatro capítulos principales; una discusión general, en la que se evalúan los cuatro capítulos en su conjunto y se señalan las oportunidades científicas que surgen como consecuencia de los resultados obetenidos; las conclusiones obtenidas en esta Tesis Doctoral; un resumen y finalmente las referencias, que contienen todas las publicaciones citadas a lo largo del trabajo. Los cuatro capítulos principales se han redactado para que puedan entenderse de forma independiente al resto de las partes de la Tesis Doctoral. En este sentido, cada capítulo incluye su propio resumen, introducción, material y métodos, resultados, discusión y conclusión.

REVISIÓN BIBLIOGRÁFICA

1. Toxoplasma gondii

1.1. TAXONOMÍA

El agente etiológico responsable de la toxoplasmosis es un protozoo intracelular obligado denominado ***Toxoplasma gondii***. Desde el descubrimiento de *T. gondii* en 1908 (Nicolle & Manceaux, 2009), la clasificación taxonómica de este parásito ha sido modificada en múltiples ocasiones. *T. gondiii* es un organismo unicelular que ha pertenecido al Reino Protozoa (Schoch et al., 2020) hasta que recientemente ha pasado a formar parte del **Reino llamado Chromista** (Figura 1) (Delgado et al., 2022), Subreino Harosa, y Superfilo Alveolata, llamado así por la presencia de vesículas o alvéolos debajo de la membrana plasmática. Forma parte del Filo Myxozoa e infrafilo Apicomplexa (Delgado et al., 2022), caracterizado por agrupar parásitos intracelulares obligados que poseen un complejo apical, y que incluye géneros de gran importancia en medicina humana y veterinaria como *Plasmodium* spp*, Cryptosporodium* spp *o Theileria* spp, entre otros (Swapna & Parkinson, 2017). Más concretamente, *T. gondii* pertenece a la superclase Sporozoa y clase Coccidiomorphea, y ya no se considera dentro de la superfamilia Coccidia (Delgado et al., 2022). Por último, pertenece al Orden Eimeriida y a la **Familia *Sarcocystidae***, junto con *Neospora* spp, *Besnoitia* spp y *Hammondia* spp, parásitos formadores de quistes, con ciclos heteroxenos, donde los ooquistes esporulan en el exterior (Tenter et al., 2002; Shaapan, 2016). *T. gondii* es la única especie incluida en el género ***Toxoplasma*** por tener proteínas, antígenos y características biológicas diferentes a los otros parásitos (Figura 1).

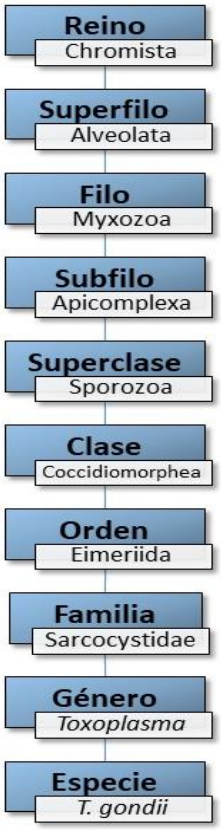

Figura 1. Clasificación taxonómica de *Toxoplasma gondii*.

1.2. ESTADIOS PARASITARIOS Y SU MORFOLOGÍA

T. gondii tiene cuatro formas parasitarias invasivas (Figura 2): merozoítos, taquizoítos, bradizoítos en el interior de quistes tisulares y esporozoítos en el interior de ooquistes esporulados.

Los **merozoítos** se dividen dentro de la célula del hospedador definitivo (HD), principalmente en el intestino delgado, formando esquizontes o merontes que lisan la célula hospedadora y liberan merozoítos a la luz intestinal, capaces de infectar nuevas células (Attias et al., 2020). Tras varias multiplicaciones, dan lugar a la formación de los gametocitos masculinos y femeninos, cuya unión (gametogonia) originará la formación de los ooquistes.

Los **taquizoítos** (Figura 2A) son los responsables de la fase aguda de la enfermedad y se diseminan por vía linfática y hemática por todo el organismo (Unno et al., 2008), pudiendo infectar a casi todos los tipos celulares (hepatocitos, miocitos,

astrocitos, enterocitos, etc) (Portes et al., 2020). Son estadios de multiplicación rápida mediante reproducción asexual (endodiogenia) responsables de la lisis celular y posterior egresión de los parásitos para invadir nuevas células, lo que se asocia a la fase clínica de la enfermedad. La conversión de taquizoíto a bradizoíto tiene lugar a los 10-14 días post infección (dpi) y se ha relacionado, en estudios *in vitro,* con cambios en el pH, presencia de NO o choque térmico en el medio (Lyons et al., 2002). Además, es reversible, y tiene lugar principalmente en individuos infectados inmunodeprimidos, donde se asocia a la reactivación de la enfermedad (Lyons et al., 2002).

Los **bradizoítos** son estadios parasitarios de replicación lenta asociados a la fase crónica de la enfermedad ya que se encuentran en el interior de quistes tisulares intracitoplasmáticos, principalmente en células del sistema nervioso central (SNC) y del musculo esquelético, donde se establece una infección latente (Figura 2B) (Cerutti et al., 2020). Los quistes tisulares tienen una forma redonda a ovalada, con una pared fina y un diámetro de alrededor de 100 µm, y pueden llegar a contener hasta 200 bradizoítos en su interior (Dubey et al., 1998; Hill & Dubey, 2002).

Los **esporozoítos** se localizan en el interior de los ooquistes esporulados y son la fase de resistencia del parásito. Los ooquistes son eliminados sin esporular con las heces del HD (Figura 2C) (Di Genova et al., 2019) y necesitan estar esporulados (Figura 2D) para tener capacidad infectiva. Este proceso, llamado esporogonia, tiene lugar bajo unas condiciones específicas de humedad y temperatura, y suele tardar entre 1 y 5 días desde que los ooquistes son excretados en las heces. Cuando esporulan, presentan en su interior 2 esporoquistes con 4 esporozoítos cada uno (2x4) (Webster, 2010). Estos ooquistes esporulados miden alrededor de 11 µm de diámetro (Dubey et al., 1998; Hill & Dubey, 2002).

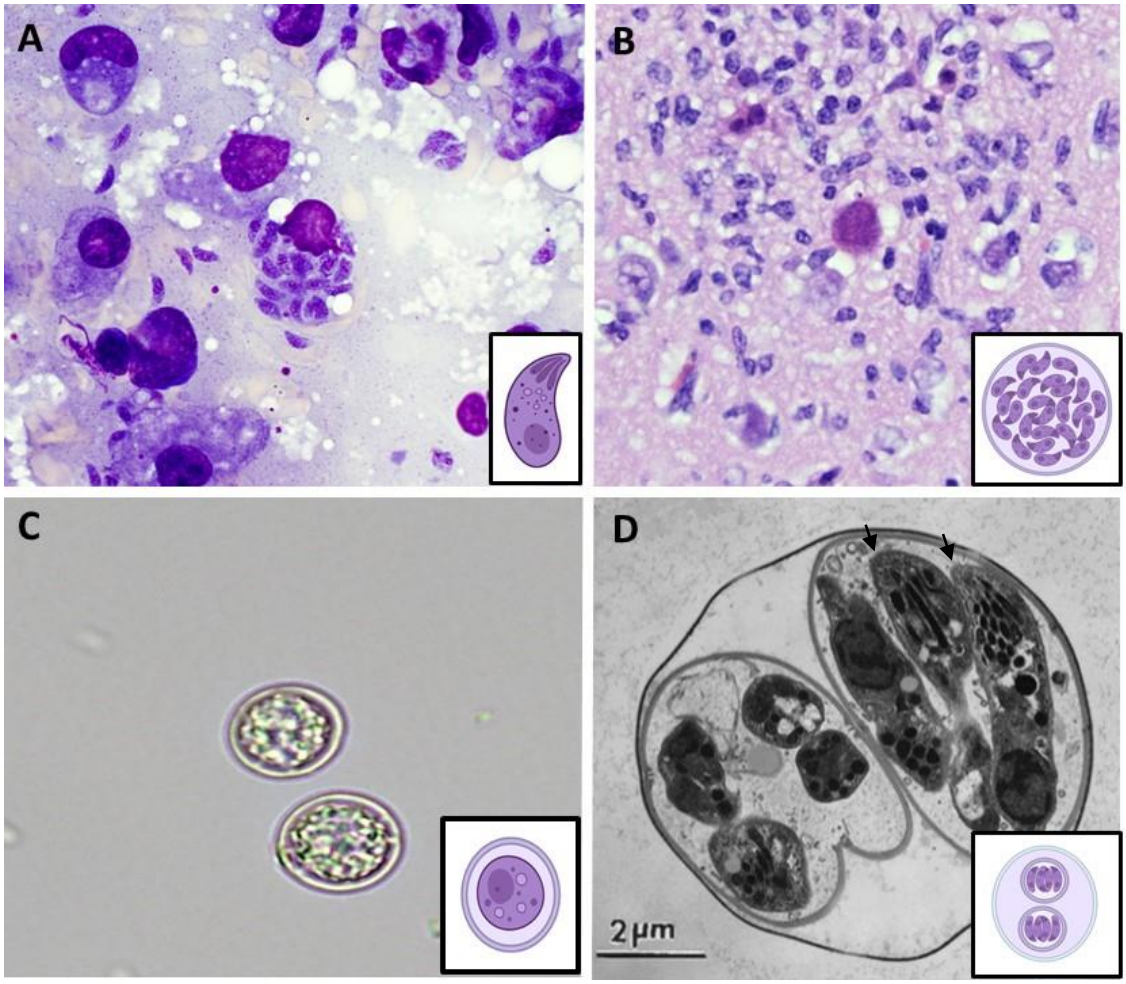

Figura 2. **Representación gráfica y microscópica de los diferentes estadios parasitarios de *Toxoplasma gondii*. A.** Imagen microscópica de múltiples taquizoítos intracitoplasmaticos y escasos extracelulares (May-Grünwald Giemsa) (Pritt, 2022) y representación gráfica de un taquizoíto (recuadro inferior). **B.** Un quiste tisular con bradizoítos en su interior localizado en el encéfalo de una oveja (H-E) y su representación gráfica (recuadro inferior). **C.** Un ooquiste no esporulado (ooquiste obtenido por flotación) y su representación gráfica (recuadro inferior). **D.** Ooquiste esporulado visualizado mediante microcopia electrónica de transmisión con ocho esporozoítos, donde se puede observar el complejo apical electrón-denso en el extremo de estos esporozoítos en sección longitudinal (flechas) (Hamie, 2020). Representación gráfica de ooquiste esporulado (recuadro inferior). Imágenes del recuadro inferior obtenidas de BioRender.com.

Todas estas formas parasitarias móviles e invasivas, llamadas conjuntamente zoítos, tienen forma elongada (de media luna o plátano) y un tamaño de 2 x 5-7 µm (Delgado et al., 2022). Los zoítos tienen una ultraestructura (Figura 3) similar al resto de las células eucariotas con un núcleo central, un aparato de Golgi junto al polo apical del núcleo, una única mitocondria y un centrosoma necesario para la división del parásito (Delgado et al., 2022). Además, estos zoítos presentan estructuras especializadas (Figura 3) que son los **apicoplastos**, el **complejo de la membrana**

interna (IMC), **los complejos apicales y basales** (Delgado et al., 2022) y los **gránulos secretores**. Dentro de estas estructuras destacan los gránulos densos (Ferguson & Dubremetz, 2014) secretores de proteínas (GRAs) esenciales para el mantenimiento de la vacuola parasitófora (VP), y que tienen capacidad de modificar la respuesta inmunitaria del hospedador (Venugopal & Marion, 2018). Por último, poseen también un complejo apical que da el nombre al subfilo Apicomplexa (indicado en el apartado 1.1), compuesto por un conjunto de microtúbulos dispuestos en espiral (**conoide**), **micronemas y roptrias** (Ferguson & Dubremetz, 2014; Attias et al., 2020). Estas dos últimas tienen vesículas secretoras de factores de virulencia, esenciales para la diseminación y la supervivencia del parásito (Sanchez & Besteiro, 2021). En el caso de los micronemas, tienen funciones de adhesión a las células del hospedador, y las roptrias, secretan proteínas (ROPs) relacionadas con la formación de la VP y también moduladoras de la respuesta inmunitaria del hospedador (Venugopal & Marion, 2018).

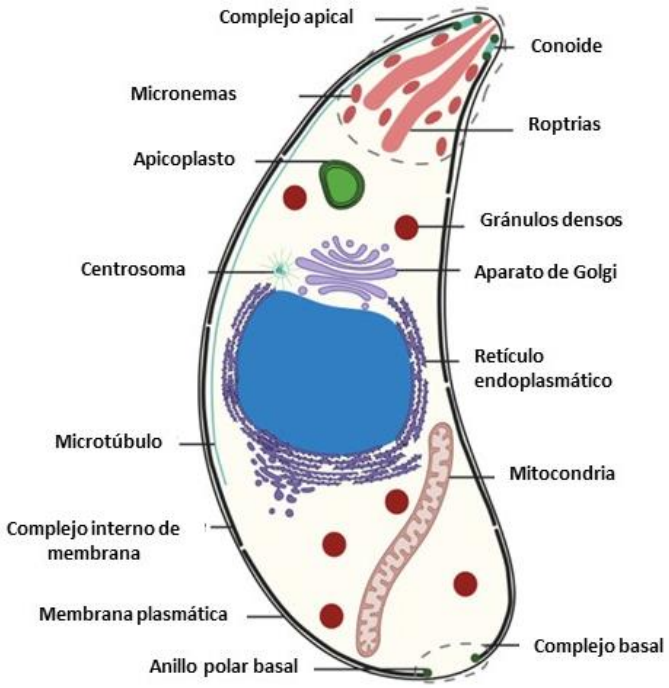

Figura 3. Morfología de un zoíto de *Toxoplasma gondii*. Esquema que representa un corte transverso de un taquizoíto con sus principales estructuras y organelas. Los complejos apical y basal se encuentran rodeados por líneas intermitentes. Creado con Biorrender.com por Delgado et al., 2022 y traducido al castellano.

1.3. CICLO BIOLÓGICO Y TRANSMISIÓN

El ciclo biológico de *T. gondii* es **heteroxeno facultativo**, ya que los ooquistes pueden infectar tanto a los HD, felinos domésticos y salvajes, como a los HI, todo animal de sangre caliente (Attias et al., 2020). El HD se puede comportar, por lo tanto, como un HD o como un HI. En el HD pueden tener lugar las fases sexuales (gametogonia) y asexuales (merogonia) de multiplicación del parásito y en el hospedador intermediario (HI) únicamente la fase de replicación asexual (merogonia) (Attias et al., 2020). Estas fases se engloban dentro de la **fase endógena** del ciclo biológico de *T. gondii*, en que también existe una **fase exógena** que tiene lugar en el medio ambiente (esporogonia) (Attias et al., 2020). El ciclo biológico de *T. gondii* queda gráficamente representado en la Figura 4.

El HD se infecta mediante **transmisión horizontal**, por la ingesta de presas (roedores, pájaros…) que contienen quistes con bradizoítos, que son liberados en el intestino por la acción de enzimas y ácidos, tras lo cual invaden las células epiteliales de la mucosa del intestino delgado exclusivamente (Innes et al., 2009). Una vez establecidos, tiene lugar una **reproducción asexual** por esquizogonia dentro de dichas células dando lugar a merozoítos. Después tiene lugar la **reproducción sexual** (gametogonia) donde los merozoítos se diferencian en microgametos haploides (masculinos) y macrogametos haploides (femeninos) que se fusionaran para formar ooquistes diploides que salen a la luz intestinal tras la lisis de las células infectadas, y al exterior por las heces del animal para, de esta forma, contaminar el medio ambiente (Attias et al., 2020). Estos ooquistes esporulan al cabo de 1 a 5 días mediante un proceso denominado esporogonia y se forman esporozoítos haploides (Attias et al., 2020). Los ooquistes maduros pueden resistir en el medio ambiente durante largos periodos de tiempo (fase exógena) y son la principal fuente de infección del HI que los ingiere normalmente mediante comida, agua o pastos contaminados (Shapiro et al., 2019). Además, animales invertebrados como cangrejos, caracoles marinos, almejas, erizos de mar o mejillones, y peces, se consideran vectores de la enfermedad al acumular estos ooquistes en su organismo (Morozińska-Gogol, 2021).

Tras la ingesta por parte del HI (la oveja o el ser humano) de ooquistes o, menos comúnmente, de quistes tisulares, los esporozoítos (o bradizoítos), se liberan e

invaden las células epiteliales del intestino donde se diferencian rápidamente a taquizoítos y forman una roseta dentro de la VP. Ahí son altamente proliferativos por endodiogenia, e invasivos, de forma que se diseminan y son los responsables de la fase aguda de la toxoplasmosis (Tilley et al., 1997; Innes et al., 2019; Attias et al., 2020). Los animales inmunocompetentes controlarán la enfermedad y los taquizoítos se diferenciarán a bradizoítos de crecimiento lento dentro de quistes parasitarios, evadiendo el sistema inmunitario, donde se dividen también por endodiogenia (Webster, 2010). Como se ha mencionado en el apartado 1.2, los quistes tisulares se encuentran principalmente en el músculo esquelético y SNC (Cerutti et al., 2020). El ciclo biológico volvería a empezar cuando las presas con los quistes tisulares son ingeridas por el HD o el HI. Por tanto, la transmisión puede tener lugar de HI a HI, de HD a HD, de HI a HD y de HD a HI, de forma que no son necesarios ambos para la transmisión de la enfermedad (Tenter et al., 2000). Existen casos en humanos en los que la infección ha tenido lugar por una transfusión de sangre que contenía taquizoítos o por órganos trasplantados con quistes tisulares (Morozińska-Gogol, 2021). Aparte de la forma de contagio horizontal, es importante destacar otra forma que tiene lugar cuando el animal se encuentra en estado de gestación: la vertical o materno-fetal, que ocurre mediante una **infección transplacentaría,** donde tiene lugar una transmisión de la madre al feto (Borges et al., 2019). En el caso concreto del ganado ovino, esta transmisión sucede cuando el animal gestante se infecta por primera vez (**primoinfección**) y puede llegar a ocasionar el aborto o el nacimiento de corderos débiles (Innes, 1997).

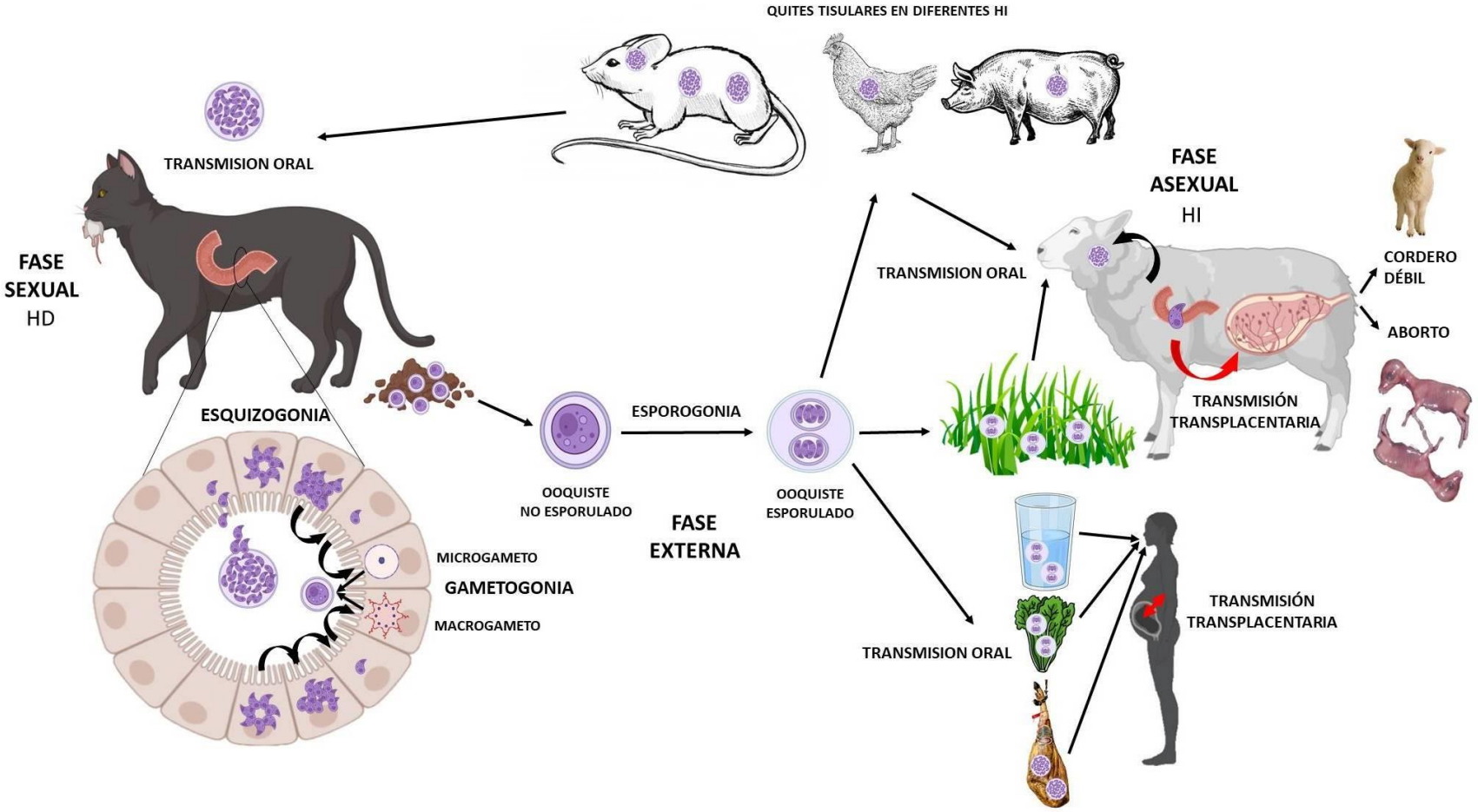

Figura 4. Ciclo biológico de *Toxoplasma gondii* y vías de transmisión. HD: hospedador definitivo. HI: hospedador intermediario. Realizado con Biorrender.com.

2. VARIABILIDAD GENÉTICA DE T. gondii

2.1 ESTUDIO DE LA VARIABILIDAD GENÉTICA DE *T. gondii*

Recientemente se ha demostrado que el cariotipo de *T. gondii* está formado por un total de 13 cromosomas (Xia et al., 2021) que albergan más de 8300 genes, muchos de los cuales codifican para proteínas relacionadas con la invasión o proteínas de superficie que controlan la virulencia y la interacción con las células del hospedador (Reid et al., 2012). De estas proteínas, las más importantes en la virulencia de *T. gondii* son las que codifican para proteínas secretoras, como MIC, GRA y ROP, o adhesinas como SRS (Lorenzi et al., 2016).

A lo largo de los años, para estudiar la variabilidad genética de este parásito, se han utilizado diferentes técnicas moleculares, entre las cuales destacan:

- El análisis de marcadores en los polimorfismos identificados mediante análisis de la longitud de los fragmentos de restricción (**PCR-RFLP**) y **secuenciación por PCR**, que se fundamenta en la detección de polimorfismos de un único nucleótido (SNPs), presentes en los productos de PCR analizados mediante endonucleasas (Su et al., 2006). El marcador SAG2 ha sido tradicionalmente el más utilizado, probablemente por ser de los primeros en describirse (Sibley & Boothroyd, 1992; Howe & Sibley, 1995). El mayor inconveniente de esta técnica es que un 40 % de los estudios realizados en Europa únicamente han empleado un locus para la tipificación del parásito (Fenández-Escobar et al., 2022a). Por esta razón, recientemente se ha establecido la necesidad de analizar un mínimo de cuatro locus para que los estudios puedan ser evaluados correctamente (Fernández-Escobar et al., 2022a).
- Métodos de genotipado por micrcosatélites (**MS**). Los MS son secuencias de ADN de escasos pares de bases (2-6 pb) que se repiten de manera consecutiva y pueden sufrir variaciones en el número de repeticiones (Vieira et al., 2016). Los marcadores más utilizados han sido B18, TUB2, TgM-A, W35 y B17 (Fernández-Escobar et al., 2022a). En MS se suele utilizar un

panel de cinco a ocho marcadores, por lo que no tiene los inconvenientes de la técnica anterior (Fernández-Escobar et al., 2022a).

- Tipificación multilocus de secuencias o técnica de secuenciación multilocular (**MLS**). Esta técnica es capaz de analizar toda la secuencia de ADN de una serie de genes y sus variaciones pertenecientes a una región concreta, a diferencia de los dos métodos anteriores que se basan en mutaciones en posiciones conocidas (Vargas-Villavicencio et al., 2016).

Los aislados de *T. gondii* se pueden clasificar principalmente en tres **líneas clonales**, tomando como referencia el método RFLP tras el análisis únicamente del gen SAG2: **tipo clonal I, tipo clonal II y tipo clonal III** (Hunter et al., 1995; Dumètre et al., 2006). Los aislados RH, ME49 y VEG se utilizan en estudios científicos como referencia de los genotipos II, II y III, respectivamente (Bernstein et al., 2020). A pesar de esto, gracias a las técnicas de secuenciación multilocular y a la aparición de nuevos marcadores para PCR-RFLP y MS, se ha demostrado que la variabilidad genética es más amplia, pese a ser los tipos clonales I II y III los que predominan (Sibley et al., 2009). Actualmente existen, por un lado, los llamados **aislados recombinantes,** que aparecen por la recombinación de dos aislados diferentes pertenecientes a las 3 líneas clonales principales y por otro, los **aislados atípicos o exóticos** que están formados por un polimorfismo único de su genoma en un locus concreto que no se detecta en los tres tipos principales, constituyendo menos de un 1% del total de aislados estudiados (Ajzenberg et al., 2004). Además, en Norteamérica se ha descrito el tipo clonal XII (HG12), previamente clasificado como un aislado atípico o recombinante (Khan et al., 2011).

Sudamérica se caracteriza por tener una diversidad genética mayor que Europa y Norteamérica, ya que coexisten poblaciones clonales con aislados atípicos y recombinantes, y esta mayor diversidad se relaciona con una mayor virulencia (Pena et al., 2008). Más concretamente, en Brasil se ha identificado un total de 48 genotipos que han sido clasificados en cuatro tipos clonales distintos llamados **BrI, BrII, BrIII y BRIV**. Curiosamente, en este mismo estudio, no se identificó ningún aislado tipo II, que es el más frecuente en Europa y Norteamérica (Pena et al., 2008), como se explicará en el apartado 2.2. Estas diferentes variabilidades genéticas entre regiones se explicarían de forma que la expansión del parásito por medio de la reproducción sexual en las

células epiteliales del intestino delgado de los felinos y la consiguiente expulsión de ooquistes, que serán ingeridos por cualquier animal de sangre caliente, daría lugar a una posible recombinación mediante cruces meióticos y por tanto a una mayor variabilidad genética (Sibley & Ajioka, 2008). Por el contrario, cuando el parásito se transmite a través del HI, donde únicamente se produce la reproducción asexual, la variabilidad genética es mínima por la expansión clonal del parásito (Sibley & Ajioka, 2008).

De manera sucinta, las técnicas PCR-RFLP y secuenciación por PCR, y MS, son las más utilizadas en los estudios epidemiológicos. La principal diferencia de la técnica MS con la PCR-RFLP es que permite, aparte de clasificar los aislados de *T. gondii* en los tipos clonales I, II y III o aislados recombinantes, identificar genotipos específicos como por ejemplo *Africa 1*, *Caribbean 2* y *Caribean 3* (Fernández-Escobar et al., 2022a). Además, existen limitaciones a la hora de clasificar aislados mediante estas técnicas. Hay que tener en cuenta que la clasificación de los aislados no es definitiva y depende principalmente del número de marcadores utilizados, de modo que, cuando estos son escasos, hay probabilidades de perder variabilidad genética (Fernández-Escobar et al., 2022a). Existe una base de datos llamada ToxoDB (https://toxodb.org/toxo/app) donde se recogen multitud de aislados y variables genéticas, clasificados teniendo en cuenta la combinación de alelos de 11 marcadores caracterizados mediante PCR-RFLP, donde un número concreto es asignado a cada aislado (ToxoDB#) (Gajria et al., 2008). De esta forma se puede realizar una comparación entre genomas que probablemente explicaría finalmente las diferencias entre los aislados de *T. gondii*.

Europa y América son los continentes donde más estudios relacionados con la variabilidad genética de *T. gondii* se han llevado a cabo (Fernández-Escobar et al., 2022a). No obstante, su número es todavía bajo y los resultados obtenidos no son exactamente representativos de la variabilidad genética que probablemente exista en estos lugares. Todos los estudios realizados obtienen los aislados de cuatro fuentes principales: humanos, animales domésticos como gatos y ovejas, animales salvajes, o del medio ambiente (Fernández-Escobar et al., 2022a).

2.2. AISLADOS EN EUROPA Y ESPAÑA

En **Europa**, el mayor número de estudios se ha realizado a partir de animales domésticos y son las gallinas, los cerdos y las ovejas los más estudiados. En relación con la especie ovina, se han realizado estudios en Francia, España, Italia, Suiza, y Austria y se ha encontrado que los aislados tipo II son los dominantes, seguidos del tipo III y aislados recombinantes (Dumètre et al., 2006; Berger-Schoch et al., 2011; Chessa et al., 2014). Resultados similares se han observado también de forma general en otros animales domésticos, humanos, animales salvajes y en el medio ambiente (Fernández Escobar et al., 2022a). De forma global, en todos los estudios realizados en Europa, se ha demoestrado que los aislados de **tipo II son el grupo más frecuentemente detectado**, entre un 79,5 % y un 87,7 %; los de tipo III, que son más frecuentes en el Sur de Europa comparado con otras partes del continente, entre un 8,1 % y un 4,2 % (Kuruca et al., 2019; Uzelac et al., 2021); los aislados de tipo I entre un 1,7 % y un 2,4 % y por último los aislados atípicos o recombinantes e infecciones mixtas suponen entre un 10,7 % y un 4,7 %; dependiendo de si la técnica utilizada era PCR-RFLP/secuenciación PCR o MS, respectivamente (Fernández-Escobar et al., 2022a). Un trabajo reciente realizado en delfines reflejaba una frecuencia de genotipos similar a los circulantes en Europa, previamente mencionados (Fernández-Escobar et al., 2022b). No obstante, un estudio medioambiental en mejillones del mediterráneo en el sur de Italia demostró una predominancia de aislados de tipo I (Santoro et al., 2020). Los aislados atípicos, recombinantes o infecciones mixtas fueron más comunes en animales salvajes, aunque estos datos son preliminares, ya que habría sido necesario un número de marcadores más alto y un mayor número de muestras para comprobar la veracidad de los resultados obtenidos (Fernández-Escobar et al., 2022a). Todos los países europeos incluidos en estos estudios se indican en la Figura 5, con una gran variabilidad en los resultados obtenidos entre ellos. Aún son muchos los países donde no se han realizado estudios para evaluar la diversidad genética de *T. gondii* (Fernández-Escobar et al., 2022a).

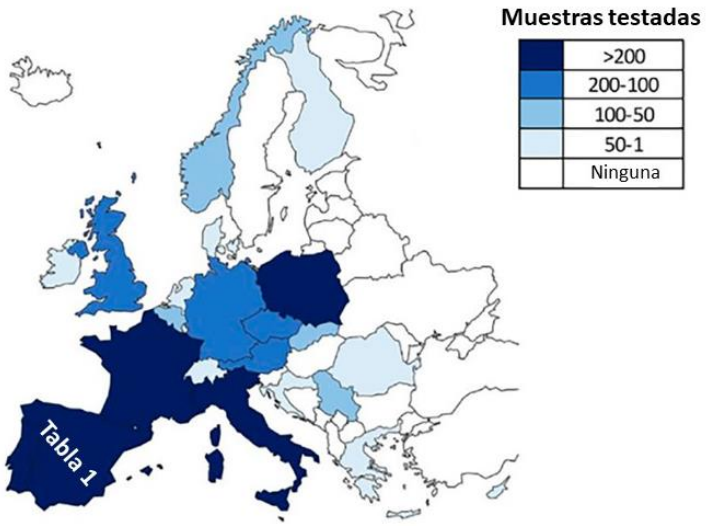

Figura 5. Distribución geográfica de los países en los que se han genotipado aislados de *T. gondii* en Europa. Únicamente están incluidos aquellos estudios donde se han analizado como mínimo 4 marcadores por PCR-RFLP/PCR, o 5 marcadores por MS. Modificada y traducida al castellano a partir de Fernández-Escobar et al., 2022a. Los datos concretos de España se incluyen en la Tabla 1.

En España, de forma general, se ha observado un predominio de aislados tipo II, y en menor medida de los de tipo I (Tabla 1), a diferencia de lo que ocurre en Europa donde los segundos genotipos más frecuentes son los aislados tipo III (Fernández-Escobar et al., 2022a). Se han llevado a cabo estudios en animales salvajes (Calero-Bernal et al., 2015; Gamble et al., 2019; Ebani et al., 2022), en gatos (Montoya et al., 2008) y, recientemente, en animales domésticos como ovejas y cerdos, utilizando 11 marcadores mediante PCR-RFLP donde se caracterizaron aislados de tipo II PRU (Pruginaud) y tipo III clonal (Fernández-Escobar et al., 2020a; Fernández-Escobar et al., 2020b). El mayor número de aislados genotipados pertenecía a la especie ovina donde se obtuvieron 31 aislados de *T. gondii* que procedían de restos de abortos o de adultos crónicamente infectados. Se observó que los aislados tipo II PRU (ToxoDB#3), como por ejemplo TgShSp1, TgShSp3, TgShSp11 y TgShSp16, representaban un 90.3 % del total de muestras analizadas. Únicamente dos aislados pertenecían al tipo III (ToxoDB#2): TgShSp24 y TgShSp25, y un aislado pertenecía al tipo II clonal (ToxoDB#1): TgShSp2. Se observó también en una pequeña proporción de infecciones dobles y una variabilidad genética baja, en concordancia con lo que ocurre en Europa (Fernández-Escobar et al., 2020a).

Es importante tener en cuenta que, debido a la globalización actual y al intercambio mercantil entre países, estos resultados pueden cambiar en los próximos años, por lo que serían necesarios nuevos estudios (Galal et al., 2019). Asimismo, es importante lograr una estandarización de los métodos de caracterización molecular para poder comparar de forma correcta los diferentes estudios de diversidad genética de *T. gondii* (Fernández-Escobar et al., 2022a). Recientemente, el análisis del genoma completo de este parásito (**WGS**) ha cobrado mayor importancia en el estudio de esta diversidad genética y constituye una de las claves para entender las diferencias entre aislados, por su gran potencial; sin embargo, el número de aislados en los que esta técnica se ha utilizado en Europa sigue siendo reducido (Yucesan et al., 2021; Fernández-Escobar et al., 2022a).

Tabla 1. Estudios disponibles de genotipado de aislados de *Toxoplasma gondii* obtenidos en España

Grupo de origen de las muestras	Especie	Marcadores moleculares utilizados	Nº marcadores	Tipo I	Tipo II	Tipo III	Aislados divergentes*	ND	Total	Referencia
Animales domésticos	*Sus scrofa* (cerdo)	PCR-RFLP (SAG1, SAG2 (5'-SAG2 y 3'-SAG2), altSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico y CS3) y PCR-Seq (CS3)	12	-	3	2	-	-	5	(Fernández-Escobar et al., 2020b)
	Ovis aries (Oveja)	PCR-RFLP (SAG1, SAG2 (5'-SAG2 y 3'-SAG2), altSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico y CS3) y PCR-Seq (SAG3, GRA6, GRA7)	12	4	164	2	4	1	175	(Fernández-Escobar et al., 2020a)
	Felis catus (Gato)	PCR-RFLP (SAG2 (5'-SAG2 y 3'-SAG2))	1	12	34	-	-	1	47	(Montoya et al., 2008)
Animales Salvajes	*Rattus rattus (Rata)*	PCR-RFLP (SAG1, SAG3, GRA6, c22-8 y Apico)	5	-	-	-	1	-	1	(Fernández-Escobar et al., 2020c)
	Ungulados salvajes	PCR-RFLP (SAG1, SAG3, BTUB y GRA6)	2	-	13	2	1	-	16	(Calero-Bernal et al., 2015)
	Sus scrofa ferus (Jabalí)	PCR-RFLP (SAG1, SAG2 (5'-SAG2 y 3'-SAG2), SAG3, BTUB y GRA6)	4	2	2	-	3	-	7	(Calero-Bernal et al., 2015)
		PCR-RFLP (SAG1, SAG2 (5'-SAG2 y 3'-SAG2), SAG3, BTUB y GRA6)	5	1	-	1	2	-	4	(Calero-Bernal et al., 2013)
	Larus michah ellis (Aves salvaje)	MS (TUB2, TgM-A, W35, B17, B18, M33//IV.1, XI.1, M48, M102, N60, N82, AA, N61, N83)	15	-	1	-	-	-	1	(Gamble et al., 2019)
	Stenella coeruleoalba y Tursiops truncatus (Delfines)	PCR-RFLP (SAG1, SAG2 (5'-SAG2 y 3'-SAG2), altSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico y CS3)	13	-	8	2	1	-	12	(Fernández-Escobar et al., 2022b)
TOTAL				19	225	9	12	2	268	

*Aislados divergentes incluyen las categorías de infecciones mixtas, aislados recombinantes (mezcla de tipo I, II o II como consecuencia de recombinaciones) o aislados atípicos (incluyen polimorfismos únicos en un loci).

2.3. VIRULENCIA DE LOS AISLADOS DE *T. gondii*

Existen múltiples factores que influyen en la patogenia de la toxoplasmosis, como son el periodo de gestación, la especie del hospedador, su sistema inmunitario, el estadio parasitario infectivo y la virulencia del aislado de *T. gondii* que está produciendo la infección (Mukhopadhyay et al., 2020). Este último aspecto tiene un alto impacto en el desenlace y la patogenia de esta enfermedad.

T. gondii posee una serie de **factores de virulencia,** como la unión móvil, encargada de que las VP no se fusionen con los endosomas o lisosomas del hospedador (Mordue et al., 1999), o las roptrias y gránulos densos que modulan la respuesta inmunitaria o actúan como mecanismos de defensa mediante la secreción de proteinas ROP y posteriormente de GRAs. Las ROPs y GRAs varían dependiendo del aislado de *T. gondii* (Ihara & Nishikawa, 2021; Sanchez & Besteiro, 2021). Como se ha indicado en el apartado 1.2, las **ROPs** son kinasas que activan diferentes vías de transducción en el núcleo de la célula del hospedador y que controlan la respuesta inmunitaria y la apoptosis (Venugopal & Marion, 2018). Como ejemplo el ROP16 inhibe la secreción de citoquinas proinflamatorias y producción de NO para favorecer el crecimiento del parásito (Butcher et al., 2011) y se encuentra activo en los aislados de tipo I pero no en los de tipo II (Saeij et al., 2007; Jensen et al., 2013). El ROP18 se expresa en los aislados de tipo I pero no en los de tipo III, con funciones de protección de la membrana de la VP (Niedelman et al., 2012). Por otro lado, los **GRAs** se encuentran normalmente dentro de la VP, contribuyendo a la nutrición del parásito (Rastogi et al., 2020) y algunos serán secretados, como por ejemplo el GRA16 que modula la P53, inhibiendo la apoptosis y promoviendo la supervivencia de las células en condiciones de estrés y, por lo tanto, la del parásito (Bougdour et al., 2013). El GRA15 estimula la producción de citoquinas proinflamatorias mediante la vía NF-kB, activando macrófagos, y se expresa en mayor medida en aislados de tipo II que de tipo III (Rosowski et al., 2011; Ihara et al., 2020). Por tanto, la virulencia del parásito está influida por los polimorfismos de estos efectores, por cómo actúan entre ellos y cuánto están expresados, por lo que los distintos aislados podrían llegar a tener diferentes consecuencias en el hospedador (Mukhopadhyay et al., 2020). La cantidad de proteínas efectoras que se han caracterizado en los últimos años y que actúan de

forma diferente en cada especie animal, demuestran la complejidad de *T. gondii* (Sanchez & Besteiro, 2021).

El **grado de virulencia** de los diferentes genotipos de *T. gondii* ha sido tradicionalmente caracterizado atendiendo únicamente a un marcador fenotípico: la **mortalidad acumulada** que producía en modelos murinos, de forma que se caracterizaban como muy virulentos, con un 100 % de mortalidad acumulada, a los aislados de tipo I; de una virulencia intermedia a los aislados de tipo II, con una mortalidad acumulada que va desde 33 % al 30 %; y como no virulentos a los aislados de tipo III, con una mortalidad acumulada menor del 30 % (Sibley & Boothroyd, 1992; Verma et al., 2015). Calero-Bernal et al. (2022) realizaron una revisión de 62 estudios (311 aislados), que únicamente consideraba la mortalidad en ratones, en estudios con taquizoítos, y no con ooquistes, valorando la virulencia de los aislados a nivel mundial. En esta revisión, pese a estar sesgada debido a que más de tres cuartas partes de los estudios pertenecían a Sudamérica y que África únicamente contaba con un trabajo, se observó que en Sudamérica y Norteamérica se encontraban aislados de alta virulencia (37 % y 45 %) en mayor proporción que en Europa y Asia (18 % y 15 %), donde predominaban los no virulentos (Figura 6).

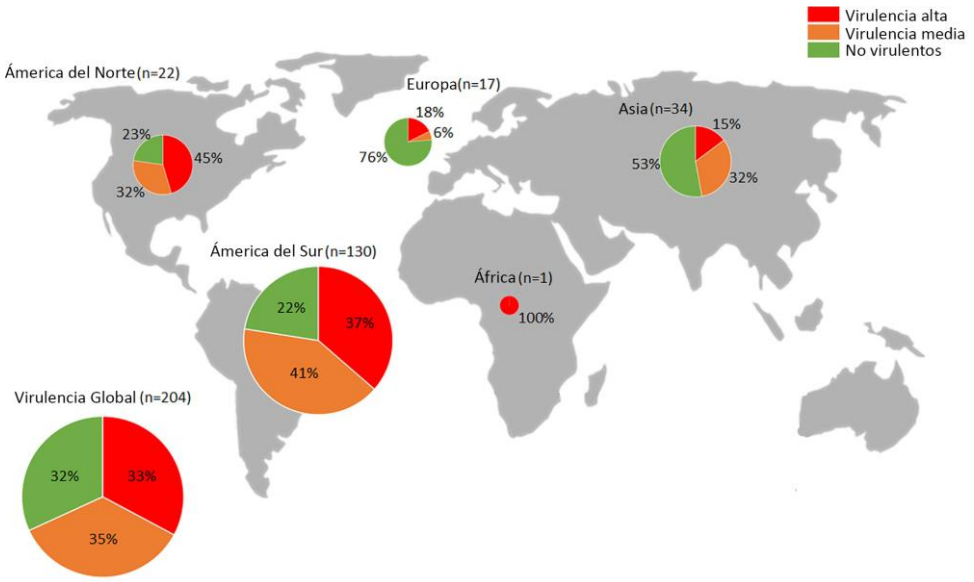

Figura 6. Distribución de la diversidad fenotípica de *Toxoplasma gondii* a nivel mundial basada en el análisis de un único marcador fenotípico (la mortalidad acumulada en ratones), y que incluye únicamente estudios con taquizoítos. En el mapa de Europa se muestra el porcentaje de aislados con virulencia alta (100 % de mortalidad), virulencia intermedia (99-30 % de mortalidad) y no virulentos (<30 % de mortalidad). El tamaño del gráfico circular se corresponde con el número total de aislados analizados (n). Traducida al castellano y modificada a partir de Calero-Bernal et al., 2022.

Sin embargo, estos grados de virulencia han sido cuestionados después de estudiar diferencias entre aislados dentro del mismo genotipo (Fernández-Escobar et al., 2020b; 2021). Además, no hay demasiada información sobre cómo la virulencia en modelos murinos puede extrapolarse a otros hospedadores, ya que existen muy pocos estudios donde se utilice el mismo aislado en diferentes especies animales. En estos trabajos, se han demostrado diferencias entre especies (Taniguchi et al., 2018 ; Hassan et al., 2019; Sánchez-Sánchez et al., 2019a ; Xia et al., 2020). Por ejemplo, se ha visto que la virulencia observada en ratón no necesariamente se puede correlacionar con la que aparece tras la infección experimental en ovinos, dado que un aislado tipo II (TgShSp1) provoca fallo reproductivo en ganado ovino, pero no es patógeno en modelos murinos (Sánchez-Sánchez et al., 2019a). Asimismo, se han observado brotes de abortos en ovejas por un aislado clonal tipo III en el sur de Brasil, pese a considerase los aislados tipo III como no virulentos en ratones (Henker et al., 2022). Se ha demostrado que la vía de inoculación, la dosis infectiva y el número de pases en el laboratorio del parásito también pueden llegar a influir en la virulencia que se observa de un determinado aislado en estudios experimentales (Sánchez-Sánchez et al., 2019a). Por último, existen aislados no pertenecientes a los principales tipos clonales, como los aislados atípicos de América del Sur, que han sido caracterizados por tener una virulencia muy alta y ser más diversos genéticamente (Grigg & Suzuki, 2003; Calero-Bernal et al., 2022).

Por todo ello, estudiar la virulencia de los diferentes aislados es mucho más complejo de lo que se creía y es necesario evaluar marcadores fenotípicos y genotípicos, y no únicamente la mortalidad acumulada en ratones (Calero-Bernal et al., 2022). La **caracterización genotípica** se realiza mediante marcadores genéticos, ya explicados en apartados anteriores, como MS (Johannsen, 2014) o RFLP-PCR (Ajzenberg et al., 2002; Fernández-Escobar et al., 2020a; 2020b), que además de estudiar la diversidad genética de *T. gondii*, se utilizan para relacionar las características genéticas con la virulencia de los diferentes aislados. Para esta carcaterización se utilizan **marcadores fenotípicos** mediante estudios ***in vitro*** como es el caso de la invasión celular, la tasa de proliferación de parásito, la formación de quistes parasitarios o de placas; o mediante estudios ***in vivo,*** analizando, además de la tradicionalmente utilizada mortalidad acumulada en animales ratones, otros

parámetros no letales como la respuesta inmunitaria del hospedador frente al parásito, la presentación clínica, la carga parasitaria, las lesiones histológicas, la expresión de citoquinas o los niveles de IgGs frente a *T. gondii* o los cambios en el comportamiento (Saraf et al., 2017; Calero-Bernal et al., 2022). Por lo tanto, el uso de diferentes marcadores y el empleo de modelos animales es esencial para caracterizar de manera correcta la virulencia de los diferentes aislados de *T. gondii* (Calero-Bernal et al., 2022).

3. MODELOS EXPERIMENTALES

El conocimiento sobre la toxoplasmosis se basa principalmente en el uso de modelos experimentales, tanto *in vitro* como *in vivo*, que han permitido un mayor conocimiento de la patogenia de la enfermedad, la caracterización de la virulencia de los diferentes aislados, el tratamiento o su control.

3.1. MODELOS *IN VITRO*

Los modelos *in vitro* son una gran herramienta para el estudio de la toxoplasmosis, ya que *T. gondii* es un parásito intracelular obligado y además representan una alternativa a los modelos animales, al minimizar y reemplazar su uso. Actualmente, existe una gran diversidad de tipos celulares donde realizar ensayos *in vitro,* que pueden proceder de diferentes hospedadores (humano, ratón, oveja, etc). Además, tienen otras ventajas, como el menor coste económico y el permitir una mayor cantidad de réplicas (Chatterton et al., 2002). Sin embargo, también presentan serios inconvenientes, como el hecho de que los resultados obtenidos pueden no corresponderse con el comportamiento del parásito en modelos *in vivo*, debido principalmente a que no se produce la interacción con otros tipos celulares dentro del mismo órgano, o con células del sistema inmunitario, como ocurre en el animal (Calero-Bernal et al., 2022). Otra desventaja es la falta de estandarización de métodos a la hora de realizar las infecciones experimentales en estos modelos *in vitro,* con una gran diversidad en la cantidad de taquizoítos utilizados en la infección, o en las horas post infección a las que se valoran los resultados, lo que hace imposible comparar unos estudios con otros (Calero-Bernal et al., 2022).

Para llevar a cabo estudios *in vitro* se han utilizado **líneas celulares primarias,** donde las características de los tejidos de los que derivan permanecen en su mayoría, como astrocitos, células dendríticas, trofoblastos, monocitos o macrófagos derivados de monocitos (Scheidegger et al., 2005; Contreras-Ochoa et al., 2012; Mammari et al., 2014; Pacheco et al., 2020), y **líneas celulares inmortalizadas,** células que pueden proliferar indefinidamente y que se diferencian más de los tejidos originales que las anteriores, como Hela (células de adenocarcinoma cervical humano), Vero (células epiteliales de riñón de mono), BeWo o AH-1 (trofoblastos humanos y ovinos) (Da Silva et al., 2017; Jabari et al., 2018; Fernández-Escobar et al., 2021).

Los modelos *in vitro* se han utilizado principalmente para estudiar la relación entre el parásito y diferentes células del hospedador, centrándose sobre todo en el estudio del llamado ciclo lítico de *T. gondii* (Blader et al., 2015; Hortua Triana et al., 2018; Kim et al., 2022), pero también en la acción de fármacos o vacunas (Mikaeiloo et al., 2016; Basto et al., 2017; Da Silva et al., 2021b; Guo et al., 2021; Khamesipour et al., 2021), la obtención de antígenos parasitarios (Hughes et al., 1986), el estudio de efectores parasitarios (Khan et al., 1988; Hakim et al., 1991; Boyle & Radke, 2009; Wang et al., 2020a) y la caracterización fenotípica de aislados de *T. gondii*, que tiene especial relevancia en la evaluación de su virulencia (Brenier-Pinchart et al., 2010; Sánchez-Sánchez et al., 2019a; Bernstein et al., 2020; Ribeiro- Uzelac et al., 2020; Fernández-Escobar et al., 2021).

Para la caracterización fenotípica *in vitro* de los aislados de *T. gondii* se han utilizado diferentes parámetros (Tabla 2), como la tasa de invasión parasitaria, la cinética de proliferación del parásito, la formación de placas, la conversión de taquizoítos a bradizoítos o la producción de taquizoítos (Calero-Bernal et al., 2022). Gracias a estas técnicas *in vitro,* se han realizado múltiples avances en el conocimiento de la virulencia de los aislados, que han permitido caracterizar tanto aislados típicos de las líneas clonales (tipo I-RH, II-ME49 y III-VEG), como aislados recientemente conseguidos de casos naturales, como por ejemplo los obtenidos en España de casos naturales en ganado ovino (Fernández-Escobar et al., 2021). En muchos de estos estudios (Tabla 2) se ha demostrado que la virulencia determinada mediante marcadores fenotípicos *in vitro* no coincide con la observada en modelos *in vivo*, por lo que resulta necesario llevar a cabo estudios de virulencia en modelos animales para

poder tener una idea más clara de cómo pueden influir los diferentes aislados en las infecciones naturales.

Tabla 2. Principales estudios *in vitro* relacionados con la caracterización fenotípica de aislados de *Tonxoplasma gondii*.

Línea celular	Parámetros para la caracterización fenotípica	Resultados principales obtenidos	Referencia
AH-1 (trofoblasto ovino)	Tasas de invasión y proliferación.	El aislado TgShSp1 (tipo II) obtuvo los valores más bajos y el aislado TgShSp24 (tipo III) los más altos. Se vieron diferencias entre aislados del mismo genotipo y entre genotipos, que no pudieron asociarse a cambios genéticos en los marcadores moleculares examinados.	(Fernández-Escobar et al., 2021)
Vero	Tasas de invasión y proliferación.	El aislado ME49 (tipo II) mostró los valores más altos, seguido de TgSb (genotipo atípico) y VEG (tipo III). Los aislados RH (tipo I) y TgMr (aislado atípico) mostraron los resultados más bajos. Sin embargo, en su estudio en ratones, los aislados atípicos fueron los más virulentos y con mayor carga parasitaria.	(Bernstein et al., 2020)
	Tasas de invasión y proliferación. Formación de placas.	Se estudian cuatro aislados tipo III prevalentes en Serbia, donde se obtienen resultados menores que los obtenidos con RH (tipo I), excepto un aislado tipo III (K1) que obtiene un mayor valor en la formación de placas. Estos aislados demostraron virulencia intermedia en ratones.	(Uzelac et al., 2020)
HFF	Tasas de invasión. Formación de quistes.	Los resultados más altos se obtuvieron con los aislados RH (tipo I) y ME49 (tipo II) en comparación con los aislados japoneses estudiados (CatJpOk1-4). Sin embargo, el aislado CatJpOk4 mostró una alta virulencia junto con RH en modelos murinos.	(Fukumoto et al., 2020)
	Tasas de invasión y proliferación. Formación de quistes.	Se estudiaron cuatro aislados tipo II pertenecientes a infecciones congénitas en humanos. La capacidad de formación de quistes variaba entre aislados con una mayor capacidad invasiva en los dos procedentes de casos sintomáticos.	(Brenier-Pinchart et al., 2010)
	Tasas de invasión y proliferación.	Dos aislados obtenidos en China (TgCtwh3 y 6) mostraron valores similares al aislado RH (tipo I) en los estudios *in vitro*. En ratones, el aislado TgCtwh6 fue virulento y el TgCtwh3 no virulento, coincidiendo con una mayor expresión de GRA3.	(Li et al., 2014)
	Formación de quistes.	El aislado COUG demuestra alta capacidad de formar quistes.	(Fux et al., 2007)
HFF	Tasa de proliferación.	Comparan el aislado ME49 (tipo II) con TgShSp1 (tipo II) obteniendo una mayor formación de quistes en este último, pero una menor proliferación y formación de placas. No se observó mortalidad en ratones con TgShSp1. No se vieron diferencias en un modelo de oveja gestante entre los dos aislados.	(Sánchez-Sánchez et al., 2019a)
MARC-145	Formación de quistes.		
VERO	Formación de placas.		
HFF y macrófagos peritoneales murinos	Tasa de proliferación. Formación de quistes.	Estudian un aislado tipo II obtenido de heces de gato (TgCatJpObi1) donde se observó una tasa de proliferación baja y que tenía la habilidad de formar quistes. En ratones mostró una virulencia menor que el aislado ME49 (tipo II).	(Salman et al., 2021)
Macrófagos peritoneales de rata	Tasa de proliferación y muerte celular.	Estudian el aislado atípico GUY008-ABE virulento en la especie humana comparándolo con un aislado tipo II PRU. Ninguno proliferaba en macrófagos de rata, no correspondiéndose con la muerte del 100 % observada en estudios *in vivo* con ratas.	(Loeuillet et al., 2019)
Sarcoma 180	Tasa de proliferación.	Comparan KI-1 con RH y observan resultados similares.	(Chai et al., 2003)
MARC-154	Formación de quistes.	Estudian tres aislados recombinantes, y los aislados RH, y ME49. Se observaron diferencias entre los aislados recombinantes y los dos típicos. Además, se observaron diferencias entre los aislados recombinantes.	(Ribeiro-Andrade et al., 2019)

3.2. MODELOS *EX VIVO*

Recientemente han comenzado a utilizarse modelos ***ex vivo*** que permiten llevar a cabo experimentos en condiciones controladas y evitan una de las principales desventajas de los modelos *in vitro,* que es la falta de interacción de las células utilizadas con las demás del organismo. Estos modelos utilizan **tejido vivo que ha sido creado de manera artificial en un laboratorio o extraído de un organismo vivo**, en este último caso manteniendo sus funciones por un corto periodo de tiempo. En el caso de *T. gondii,* la utilización de explantes de placenta humana ha sido ensayada en varias ocasiones (Da Silva et al., 2017; Franco et al., 2019; Holthaus et al., 2021), ya que se ha demostrado que estos explantes mantienen la diferenciación celular y secretan citoquinas y hormonas similares a las condiciones fisiológicas (Miller et al., 2005). Sin embargo, el uso de explantes de placenta en rumiantes para el estudio de parásitos como *T. gondii* y *Neospora caninum* es casi inexistente (Pastor-Fernández et al., 2021). Hay un estudio reciente, de *T. gondii* y *Trypanosoma cruzi,* que compara el daño tisular y la eficacia de la infección mediante el empleo de explantes de placentas humanas, caninas y ovinas (Liempi et al., 2020).

3.3. MODELOS *IN VIVO*

3.3.1. Modelo murino

Los ratones son sensibles a la infección por *T. gondii* y juegan un papel crucial en el ciclo biológico del parásito, por lo que el estudio de la enfermedad en esta especie animal ha generado gran interés. Los modelos murinos presentan multitud de ventajas frente a otros modelos *in vivo* por su facilidad a la hora de manejar estos animales, su bajo coste, su pequeño tamaño, su corto periodo de gestación y el gran número de crías (Calero-Bernal et al., 2022). Además, existen multitud de cepas disponibles, como por ejemplo C57BL/6J (Taniguchi et al., 2018), CD1 (Sánchez-Sánchez et al., 2019a) o BALB/c que se ha considerado resistente a la infección crónica (Subauste, 2012) y que incluyen, además, modelos de inmunodeficiencia como son los ratones SCID (Johnson, 1992) o ratones transgénicos donde se expresan ciertas

proteínas ("knock in") o se inhiben ("knock out"). Los ratones SCID y transgénicos se utilizan para el estudio de las funciones de diferentes receptores en la patogenia de la toxoplasmosis (Taylor et al., 2000).

Los ratones se han utilizado para estudiar la cinética de distribución del parásito durante las fases aguda y crónica de la enfermedad, la función de diferentes efectores proteicos (Rochet et al., 2019) o para testar vacunas (Arcon et al., 2021) y medicamentos (Sina et al., 2021). Además, son el **modelo *in vivo* más utilizado para la caracterización de aislados** (Howe et al., 1996; Taniguchi et al., 2018; Sánchez-Sánchez et al., 2019a; Fernández-Escobar et al., 2020a; 2020b; Fukumoto et al., 2020; Chiebao et al., 2021; Fernández-Escobar et al., 2021; Salman et al., 2021), tradicionalmente evaluada únicamente mediante la mortalidad acumulada (Saraf et al., 2017). Actualmente, como se ha explicado en el apartado 2.3, se emplean otros marcadores fenotípicos no letales como la carga parasitaria, las lesiones histológicas o los signos clínicos (Calero-Bernal et al., 2022), lo que permite un estudio de la virulencia mucho más preciso. De esta forma, se han caracterizado aislados obtenidos en España, donde se ha observado, además de una mortalidad significativamente más elevada en dos aislados concretos (TgShSp16 y TgShSp24, tipo II PRU y tipo III, respectivamente), una mayor pérdida de peso, signos neurológicos, carga parasitaria y lesiones histológicas en los diferentes órganos en comparación con otros aislados de tipo II (Fernández-Escobar et al., 2021).

Es importante destacar **el modelo murino gestante**, al ser el fallo reproductivo uno de los problemas más importantes que acarrea esta enfermedad (Innes et al., 2009). Aunque el uso de ratones gestantes ha sido muy utilizado, por el corto periodo de gestación de estos animales y el alto número de crías a estudiar, existen múltiples diferencias inmunológicas y morfológicas con otras especies afectadas como el ganado ovino o porcino (Calero-Bernal et al., 2022), entre las que destaca el tipo de placentación. Los ratones poseen una placenta discoidal y hemocorial, a diferencia de los rumiantes que tienen una placentación cotiledonaria sinepiteliocorial (Sammin et al., 2009) (apartado 3.3.2.2), y los cerdos que es difusa y epiteliocorial (Wooding & Burton, 2008). También son relevantes las diferencias con los humanos y otras especies relacionadas con la duración de la gestación y el número de crías que se obtienen (Flexner & Gellhorn, 1942; Carter, 2007). En un estudio donde se comparan

dos aislados tipo II (ME49 y TgShSp1) en modelos murinos y en modelos ovinos, se observa que la diferencia de virulencia con ambos aislados en modelos murinos desaparecía al estudiar estos mismos aislados en ovejas (Sánchez-Sánchez et al., 2019a). Por ello es importante valorar el modelo a utilizar según el objetivo del estudio que se vaya a realizar, ya que no son extrapolables los resultados obtenidos de unas especies a otras (Taniguchi et al., 2018; Sánchez-Sánchez et al., 2019a).

3.3.2. Modelo ovino

Uno de los mejores modelos para el estudio de la toxoplasmosis es el ovino, al ser la oveja uno de los principales HI de *T. gondii* (Innes et al., 2009). Se ha utilizado para el estudio de la respuesta inmunitaria, la transmisión vertical y la patogenia de la enfermedad (Tabla 3). Además, existen otras muchas ventajas en el uso de este modelo, como son el pequeño tamaño y el fácil manejo de estos animales en comparación con otros rumiantes como el ganado bovino. No obstante, también presenta desventajas, como son la necesidad de instalaciones adecuadas para su manejo y necropsia, los condicionantes de ética animal, que obligan a que el número de animales utilizado se deba reducir lo máximo posible, aunque ello suponga una menor potencia estadística de los resultados, si tenemos en cuenta la propia variabilidad individual de cada animal (Castaño, 2019).

A lo largo de los años, en los diversos estudios experimentales con ganado ovino recogidos en la Tabla 3, se han evaluado distintos aspectos de la toxoplasmosis, como la patogenia del aborto inducido por *T. gondii* usando un **modelo gestante en ovejas** infectadas por primera vez en varios periodos de gestación (Castaño et al., 2014), el comportamiento del parásito en ovejas **adultas no gestantes** (Verhelst et al., 2014) o **en corderos** (Benavides et al., 2011), la eficacia de vacunas (Katzer et al., 2014) y, más recientemente, la influencia de la variabilidad de los aislados de *T. gondii* en la toxoplasmosis mediante la evaluación de diferentes marcadores fenotípicos (Sánchez-Sánchez et al., 2019a).

Los abortos tempranos descritos previamente en ovejas (Owen et al., 1998; Castaño et al., 2014) hacen aún más interesante este modelo, considerando que este tipo de presentación clínica podría ocurrir también en la especie humana, por lo que el

modelo ovino gestante puede ser utilizado para entender su patogenia, que hasta la fecha sigue siendo una incógnita (Arranz-Solís et al., 2021).

En cuanto al genotipo de *T. gondii*, los datos recogidos en la Tabla 3 demuestran que las infecciones experimentales se han realizado con los tres tipos de aislados principales, e incluso con aislados fuera de estos tres grupos, como son los aislados brasileños. Sin embargo, los aislados de tipo II han sido hasta la fecha los más utilizados, en consonancia con ser el tipo más común causante de abortos en infecciones naturales (Kuruca et al., 2019; Uzelac et al., 2021).

Aparte del aislado, existen múltiples factores que pueden influir en los resultados de una infección experimental, como son el estadio parasitario con el que se infecta a los animales, la vía de administración, la dosis infectiva utilizada, la edad y raza de los animales, y el periodo de gestación, si se emplean modelos gestantes.

Otro hecho a considerar es el número de pases en cultivo celular o en ratón a los que se ha sometido al aislado utilizado ya que pueden influir en su virulencia, de forma que se puede ver incrementada con un mayor número de pases (Sánchez-Sánchez et al., 2019a). Debido a la variabilidad en el curso de la enfermedad que producen todos los factores mencionados, es necesaria una estandarización del modelo ovino para poder comparar resultados de los diferentes estudios realizados. En el siguiente apartado se exponen los principales factores que influyen en estudios experimentales de toxoplasmosis en la especie ovina, si bien la mayoría de estos factores son aplicables a cualquier otro modelo *in vivo*.

3.3.2.1. FACTORES DETERMINANTES

Las **vías de administración** empleadas en los diferentes estudios han sido la oral, la subcutánea, la intraperitoneal, la intramuscular o la intrauterina (Tabla 3). Mediante las vías subcutánea e intramuscular se inoculan taquizoítos (Mévélec et al., 2010; Katzer et al., 2014) y por vía oral ooquistes, en el modelo ovino gestante (Tabla 3). En los escasos estudios que han utilizado la vía sexual, mediante la inoculación intrauterina de semen contaminado con taquizoítos de *T. gondii* en el momento de cubrición, ha demostrado ser efectiva en el establecimiento de la enfermedad y la aparición de fallo reproductivo (De Moraes et al., 2010; Consalter et al., 2017).

En relación al estadio **parasitario utilizado,** se han empleado ooquistes esporulados, quistes tisulares con bradizoítos en su interior y taquizoítos (Tabla 3). Todos han logrado la infección de los animales. Sin embargo, la ingestión de ooquistes esporulados presentes en alimentos o agua contaminada, es la forma de infección más común en las infecciones naturales (Buxton, 1990), y por lo tanto más empleada en los estudios experimentales. El estadio parasitario puede afectar al momento en el que aparece el parásito en sangre (parasitemia) tras la infección de las ovejas, de forma que se ha observado que en la infección con quistes tisulares comenzaría dos días antes si se produce con ooquistes (Verhelst et al., 2014).

La **dosis infectiva** es uno de los factores que más puede influir en las consecuencias de una infección experimental con *T. gondii* (Sánchez-Sánchez et al, 2019a). Las dosis de taquizoitos y quistes parasitarios utilizados suelen ser más altas que las de ooquistes (Tabla 3). Pese a que anteriormente se consideraban 200 ooquistes esporulados como la cantidad mínima necesaria para poder desencadenar el aborto en el ganado ovino, se ha observado que con dosis infectivas mucho menores, se consigue la infección de los animales, de forma que empleando únicamente 10 ooquistes se logra producir abortos tempranos (Sánchez-Sánchez et al., 2019a). También se ha comprobado que a una mayor dosis infectiva aumenta el número de abortos en fase aguda, se adelanta la seroconversión de las ovejas, aumenta la mortalidad de los fetos y se incrementa la carga parasitaria en la placenta y en los diferentes órganos fetales (Esteban-Redondo & Innes, 1998; Esteban-Redondo et al., 1999; Castaño et al., 2014; Sánchez-Sánchez et al., 2019a).

El **periodo de gestación** puede influir también en el desenlace de la enfermedad, como se expone en el apartado 4.2 (Castaño et al, 2016). En la mayor parte de los estudios realizados hasta el momento en ovejas gestantes, recogidos en la Tabla 2, se ha escogido el segundo tercio de gestación para llevar a cabo la infección experimental posiblemente porque es entonces cuando se logra que aparezcan las lesiones más graves en la madre y en los fetos (Buxton & Finlayson, 1986). Sin embargo, el periodo de hipertermia es mayor cuando la infección tiene lugar en el primer tercio de gestación, los abortos tempranos son más frecuentes cuando la infección se produce en el segundo tercio y las lesiones en feto y placenta aparecen

antes cuando la infección tiene lugar en el tercer tercio de gestación (Castaño et al., 2016).

Todas las **razas** son susceptibles a la infección por *T. gondii*, y el empleo de una u otra ha dependido principalmente de la zona geográfica en la que se realiza el estudio, como por ejemplo en España, donde se han utilizado las razas Churra y Rasa Aragonesa (Castaño et al., 2016; Sánchez-Sánchez et al., 2019a). No se ha llevado a cabo un número suficiente de estudios con el objetivo concreto de comprobar la susceptibilidad de las diferentes razas ovinas a la infección por *T. gondii*, en concreto para valorar la posibilidad de que ciertas razas sean más susceptibles que otras a la transmisión vertical y a cómo esto puede influir en las infecciones experimentales (Stelzer et al., 2019). Hasta ahora existen indicios de que la raza Charolais es más susceptible a la transmisión vertical que otras razas (Morley et al., 2008) de forma que se produce una mayor cantidad de abortos (Williams et al., 2005) o un menor grado de inmunidad adquirida en la primera infección (Morley et al., 2008). Por otro lado, en la raza Churra se ha observado una mayor cantidad de abortos tempranos que en otras razas (Castaño et al., 2014), aunque no se han realizado estudios comparativos en las mismas condiciones.

Además de todos los factores citados anteriormente, los resultados pueden depender a su vez del tipo de aislado utilizado, como se ha indicado en el apartado 2.3 y del número de pases que este tenga (Sánchez-Sánchez, et al., 2019a). También el estado fisiológico del animal puede influir en la patogenia de la enfermedad, en función de si se encuentra gestante o inmunodeprimido, como se ha explicado en apartados anteriores.

Tabla 3. Estudios experimentales utilizando como modelo animal la especia ovina.

Aislado de *T. gondii*	Vía infección	Estadio parasitaria	Dosis infectiva^	Edad	Periodo de gestación	Referencia
S48 (tipo I)	sb	T	$10^5 – 10^7$	-	-	(Innes et al., 1995a)
	sb	T	1×10^5	-	-	(Innes et al. 1995b)
	sb	T	-	-	-	(Buxton et al., 1991)
RH (tipo I)	sb	T	1×10^6	14 meses	-	(Lopes et al., 2009)
	sb	T	1×10^6	14 meses	-	(Lopes et al. 2011)
	iu	T	4×10^7	Adultos	-	(Consalter et al., 2017)
ME49 (tipo II)	vo	O	$10^1 – 5 \times 10^1 – 5 \times 10^2$	Adultos	90 dg	(Sánchez-Sánchez, et al., 2019a)
	vo	O	1×10^3	3 meses	-	(Thomas et al. 2022)
M1 (tipo II)	sb	QT	200	Adultos	40 dg – 60 dg – 90 dg	(Buxton & Finlayson, 1986)
	vo	O	2×10^3	Adultos	Entre los 80 y 90 dg	(Owen et al., 1998)
M3 (tipo II)	vo	O	$10^5 – 10^4 – 10^3$	9 meses	-	(Esteban-Redondo & Innes, 1998)
	vo	O	$10^3 – 10^5$	Adultos	-	(Esteban-Redondo et al., 1999)
M4 (tipo II)	vo	O	3×10^3	Adultos	90 dg	(Gutierrez et al., 2010)
	vo	O	$5 \times 10^3 – 5 \times 10^5$	2 meses	-	(Benavides et al., 2011)
	vo	O	3×10^3	Adultos	90 dg	(O'Donovan et al., 2012)

Aislado de *T. gondii*	**Vía infección**	**Estadio parasitaria**	**Dosis infectiva^**	**EDAD**	**Periodo de gestación**	**Referencia**
M4 (tipo II)	vo	O	3×10^3	Adultos	90 dg	(Marques et al., 2012)
	vo	O	$5 \times 10^2 – 2 \times 10^3$	Adultos	120 dg -- 90 dg	(Castaño et al., 2014)
	vo	O	50	Adultos	40 dg – 90 dg – 120 dg	(Castaño et al., 2016)
	vo	O	5×10^5	4 meses	-	(Katzer et al., 2014)
PRU (tipo II)	vo	O	$1 \times 10^2 – 4 \times 10^2 – 4 \times 10^2$	Adultos	Mitad de la gestación	(Mévélec et al., 2010)
	vo	QT	3×10^3	7 semanas	-	(Verhelst et al., 2014)
	vo	QT	3×10^3	No especificada	-	(Verhelst et al., 2015)
TgShSp1 (tipo II)	vo	O	$10^1 – 5 \times 10^1 – 5 \times 10^2$	Adultos	90 dg	(Sánchez-Sánchez, et al. 2019a)
CZ-Tiger (tipo II)	vo	O	1×10^4	6 meses	-	(Glor et al., 2013)
CZ-Tiger (tipo II)	vo	O	1×10^4	5-7 meses	-	(Juránková et al., 2015)
VEG (tipo III)	vo	O	$2{,}5 \times 10^3$	Adultos	40 dg – 80 dg – 120 dg	(Dos Santos et al. 2016)
TgCatBr71 (Tipo BvI) y TgCatBr60 (Tipo BvIII)	vo	O	2×10^3	Adultos	Antes y 60 dg	(Chiebao et al., 2019)
No especificado	iu	T	$6.5 \times 10^4 - 4 \times 10^7$	Adultos	-	(De Moraes et al., 2010)

O: ooquistes esporulados. / T: taquizoítos. / QT.: quistes tisulares. / vo: vía oral. / sb.: vía subcutánea. / iu: intrauterina

3.3.2.2. PLACENTA OVINA ÓRGANO DIANA

La placenta ovina tiene una gran importancia en el estudio de la toxoplasmosis, al ser uno de los órganos diana de este parásito (Benavides et al., 2017; Pastor-Fernández et al., 2021). Es un órgano complejo, que varia a lo largo gestación, sobre cuyas características morfológicas existen escasos estudios que, sin embargo, son necesarios para poder evaluar correctamente los cambios producidos por la inoculación de agentes infecciosos abortivos.

La placenta ovina es de tipo **cotiledonario,** ya que está formada por múltiples placentomas donde tiene lugar el intercambio de sustancias entre la madre y el feto. Los placentomas están compuestos por una parte fetal (**cotiledón**) formado por la fusión del corion y el alantoides (membranas fetales) y por una parte materna (**carúncula**) (Sammin et al., 2009). Además, es un tipo de placentación **sinepiteliocorial** y, pese a presentar todas las capas (endotelio, tejido conjuntivo y epitelio tanto materno como fetal) en la zona intercotiledonaria, en los placentomas el epitelio materno es sustituido por una capa sincitial de células materno-fetales (Wooding, 1992). A nivel histológico existen dos zonas bien diferenciadas dentro del placentoma: la **zona de interdigitaciones,** en cuyo examen donde se centran la mayor parte de estudios enfocados a caracterizar lesiones (Castaño et al., 2016; 2020) y la **zona hematomata** donde se produce una acumulación de sangre materna que se puede observar como una zona rojiza a nivel macroscópico (Sammin et al., 2009).

Al estudiar el fallo reproductivo producido por agentes infecciosos o parasitarios como *T. gondii*, es importante diferenciar el estudio que se realiza en placentomas obtenidos a través de sacrificios previos al aborto en infecciones experimentales y el estudio del cotiledón obtenido tras la expulsión de la placenta en caso de abortos tanto en casos naturales como experimentales. Por todo lo mencionado, el estudio preciso del aspecto histológico normal de la placenta ovina es necesario para poder, posteriormente, conocer cómo influye la infección por *T. gondii,* al tratarse de su principal órgano diana.

3.3.3. Otros modelos

3.3.3.1. OTROS ROEDORES

Además del modelo murino, se han utilizado otros roedores para el estudio experimental de la toxoplasmosis, aunque en menor medida. Las **ratas**, a diferencia de los ratones, se consideran resistentes a la toxoplasmosis, por lo que representan un buen modelo para el estudio de la resistencia a *T. gondii* en humanos (Dubey & Sheen, 1991). Para el estudio de la toxoplasmosis congénita, además de los modelos gestantes ovinos y murinos, recientemente se han utilizado las **cobayas** (Grochow et al., 2021). Los **gerbos** han demostrado ser también un buen modelo para conocer la patología hepática producida por *T. gondii* (Kahyaoglu & Atmaca, 2022).

3.3.3.2. MODELO PORCINO

El uso de modelos porcinos es de gran utilidad pese a no ser tan frecuentes los casos naturales como en la especie ovina (Stelzer et al., 2019). *T. gondii* puede causar abortos en cerdas gestantes y provocar una infección transplacentaria (Basso et al., 2015). Estudios recientes, en los que se intentaba establecer un modelo de cerda gestante para el estudio de la toxoplasmosis, no han obtenido el éxito esperado (Basso et al., 2017). Sin embargo, el uso de cerdos (Largo-de la Torre et al., 2022), y de **"minipigs" y "microminipigs"**, seleccionados genéticamente para tener un menor tamaño, un fácil manejo y coste; sí han demostrado ser un buen modelo para estudiar la virulencia de diferentes aislados (Taniguchi et al., 2018, 2019; Xia et al., 2020), por lo que son modelos mejor caracterizado que los cerdos corrientes (Jungersen et al., 2001; Miranda et al., 2015; Dubey et al., 2020b).

El cerdo se ha utilizado como modelo experimental por dos razones principales: como ya se ha mencionado, es susceptible a la enfermedad (Joachim & Schwarz, 2015) y además el parásito es capaz de establecer una infección crónica en esta especie (Wingstrand et al., 1997). Se han realizado estudios para evaluar la eficacia de la vacunación (Burrells et al., 2015) o para estudiar la patogenia en esta especie (Jungersen et al., 1999). Por otra parte, la similitud de su sistema circulatorio con el de los humanos, ha hecho que se haya empleado el modelo porcino para estudiar la toxoplasmosis humana (Miranda et al., 2015; Nau et al., 2017), con éxito, al reproducir la enfermedad tal y como se presenta en la especie humana (Miranda et al., 2015).

Al igual que en el ganado ovino, se ha demostrado que en muchos casos resulta imposible extrapolar lo observado en la especie murina a la especie porcina y que la edad es un factor importante que afecta al desenlace de la enfermedad, de manera que los cerdos jóvenes son los que tienen una mayor susceptibilidad a la infección (Jungersen et al., 1999; Taniguchi et al., 2018).

3.3.3.3. OTROS RUMIANTES

Otros rumiantes, aparte de la oveja, se han utilizado a lo largo de los años para el estudio de la toxoplasmosis, como son las cabras y las vacas (Stalheim et al., 1980). La especie **caprina** ha sido empleada en múltiples estudios experimentales ya que, al igual que la oveja, es susceptible a la infección por *T. gondii* y ocasiona fallo reproductivo (Stelzer et al., 2019). No obstante, hay una clara diferencia entre ambas especies ya que las cabras pueden abortar en múltiples ocasiones tras sucesivas infecciones por *T. gondii* y no como en el caso de la oveja, que sólo aborta si tiene lugar una primoinfección (Innes, 1997). Al igual que en la especie ovina, la vía oral y el uso de ooquistes esporulados han sido los procedimientos más empleados, simulando lo que ocurre en condiciones naturales (Santana et al., 2010). Esta especie también se ha empleado para el estudio de la transmisión venérea del parásito, y se ha comprobado que esta vía es la que menos respuesta immunitaria produce (Santana et al., 2010; Wanderley et al., 2013). También se ha demostrado que los cabritos son más susceptibles a la enfermedad que el animal adulto y que la placentitis y la enteritis son las lesiones características tras la infección con ooquistes parasitarios (Dubey, 1989).

La especie **bovina** se considera susceptible a la infección, aunque es poco frecuente que curse con aborto, tanto en infecciones naturales (Innes, 1997), como en experimentales (Dubey, 1983). El número de estudios que existe es mucho menor que en la especie caprina, ovina y porcina, pero aún así se han utilizado tanto vacas gestantes (Dubey, 1983; Costa et al., 2011) como no gestantes (Burrells et al., 2018) y terneros (Dubey, 1983).

3.3.3.4. AVES

Pese a que los pollos **broiler** se consideran resistentes a la toxoplasmosis y existen pocos casos en los que se hayan observado signos clínicos de la enfermedad, se ha visto recientemente una alta seropositividad a la toxoplasmosis (Khan et al., 2020),

lo que indica que se pueden infectar. Existen escasos estudios que hayan empleado estos animales, donde se ha observado que diferentes aislados y la edad pueden influir en el desarrollo de la enfermedad (Wang et al., 2014).

4. TOXOPLASMOSIS OVINA

4.1. EPIDEMIOLOGÍA, IMPACTO ECONÓMICO Y RELEVANCIA EN SALUD PÚBLICA

La toxoplasmosis en el ganado ovino, al igual que en humanos y en muchos animales domésticos (Hajimohammadi et al., 2022), es una enfermedad frecuente cuya prevalencia se ve afectada por varios factores de riesgo, como son la edad de los animales, el sexo, las características geográficas y regionales, el manejo de la granja, las medidas de higiene, el tamaño del rebaño o la presencia de otros animales, como por ejemplo los gatos (Stelzer et al., 2019). Existen varios estudios (Dubey & Kirkbride, 1989; Katzer et al., 2011; Yan et al., 2020; Ahaduzzaman & Hasan, 2022), donde se ha visto que la seroprevalencia aumenta con la edad, denotando que la mayor parte de los contagios se producen por transmisión horizontal y no vertical, y que estos contagios suelen ocurrir antes de los 4 años de edad. A su vez, en explotaciones intensivas o con presencia de gatos, el ganado se encuentra más afectado que en explotaciones semiintensivas o sin presencia de gatos. También se ha observado una mayor seroprevalencia en hembras que en machos.

A continuación, se exponen diferentes datos epidemiológicos de la toxoplasmosis ovina a nivel mundial, en Europa y en España.

La seroprevalencia en ovejas a **nivel mundial** es alta pero variable entre las regiones y los estudios realizados. A nivel mundial, se ha citado una seroprevalencia del 33,86 % en la última revisión publicada, valorando estudios en diferentes regiones mediante métodos de análisis sistemático y meta-análisis (Ahaduzzaman & Hasan, 2022). Más concretamente, en el mismo estudio, se refiere una seroprevalencia media del 54,48 % en Oceanía, 41,01 % en Europa, 38,48 % en África, 31,48 % en Norte América, 30,27 % en Sudamérica y 28,48 % en Asia (Ahaduzzaman & Hasan, 2022). Por otro lado, después de búsquedas sistemáticas y meta-análisis publicadas

recientemente, la prevalencia a nivel mundial de fetos abortados o muertos al nacimiento es del 42 % mediante métodos moleculares y del 16% mediante test serológicos (Nayeri et al., 2021). La prevalencia en animales adultos sacrificados en matadero incluyendo únicamente métodos de detección directa (bioensayos, PCR y técnicas microscópicas), es del 14.7 % (Belluco et al., 2016). En Europa y Estados Unidos, se estima que entre el 10 y el 23 % de los abortos infecciosos son provocados por *T. gondii* (Dubey, 2009) y es la segunda causa de aborto infeccioso más común, después del aborto enzoótico causado por *Chlamydia abortus* (Buxton & Henderson, 1999; Borel et al., 2014).

Comparado con el resto de los continentes, Europa es donde se observa una mayor seroprevalencia individual, del 41 % (Ahaduzzaman & Hasan, 2022). En la Figura 7 se recogen los datos obtenidos en diferentes estudios realizados en **Europa** que valoran la seroprevalencia de la toxoplasmosis ovina en distintas zonas geográficas (Ahaduzzaman & Hasan, 2022). Además, recientemente se ha observado en Suiza una seroprevalencia del 66.3 % a nivel individual, pero de un 90.9 % a nivel de rebaño (Basso et al., 2022); y en Portugal central una seroprevalencia del 58 % y el 69 % a nivel individual, dependiendo del año estudiado (Almeida et al., 2021).

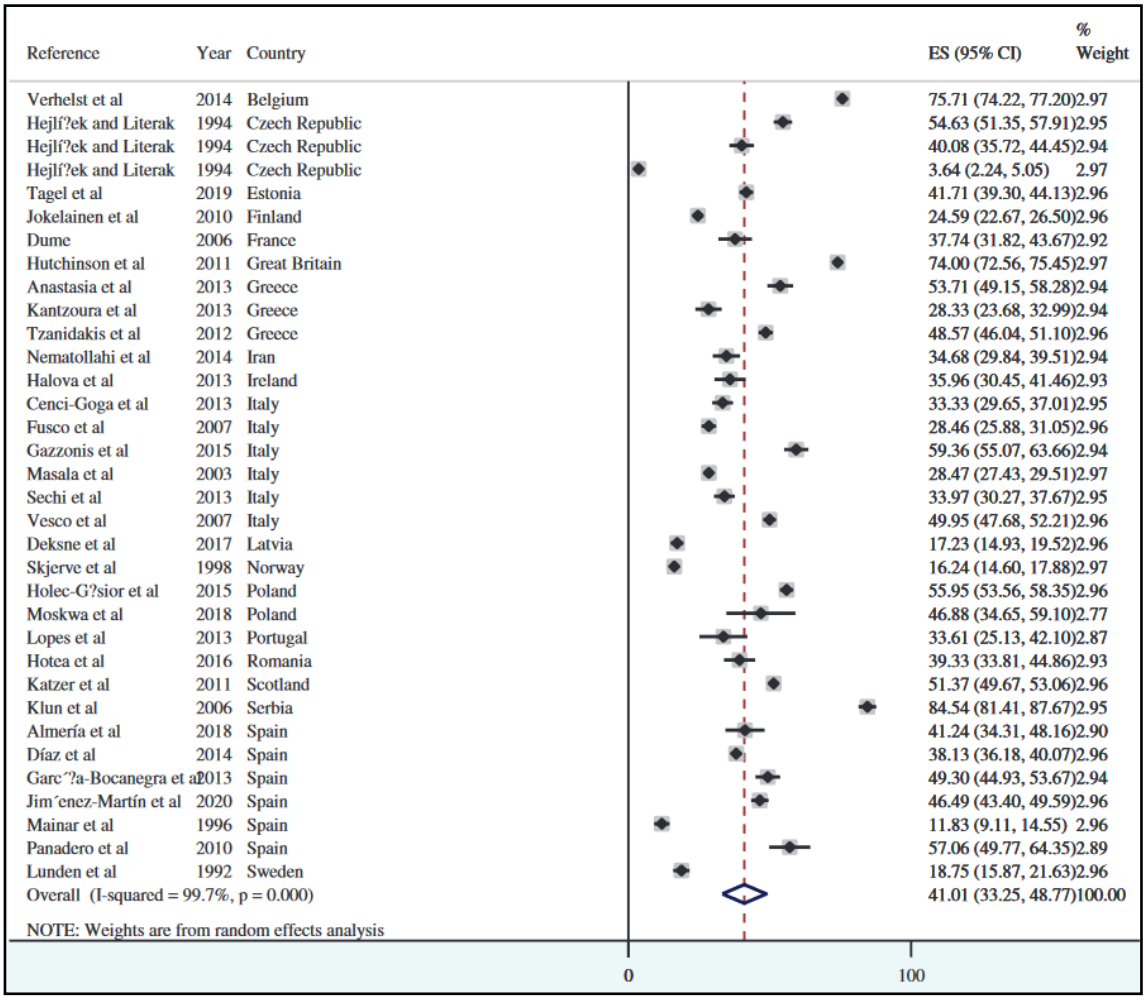

Figura 7. Diagrama donde se muestra la seroprevalencia regional de la *Toxoplasma gondii* en ovinos estimada mediante meta-análisis de los estudios realizados en Europa. Figura extraida de Ahaduzzaman & Hasan, 2022.

En **España** la seroprevalencia de *T. gondii* varía considerablemente según los estudios. En el sur del país, oscila entre el 46,5 % y el 49,3 % a nivel individual y del 98,4 % y el 84,7 % a nivel de rebaño, similar a otros países mediterráneos (García-Bocanegra et al., 2013; Jiménez-Martín et al., 2020). En rebaños de la costa mediterránea se encontraron porcentajes parecidos con una seroprevalencia del 41,2 % de las ovejas (Almería et al., 2018). En el noreste de España se han citado resultados similares, con un 48 % de las ovejas y un 74 % de los rebaños del estudio, positivos (Díaz et al., 2016). En el noroeste, Galicia, la seroprevalencia individual fue del 57 % (Panadero et al., 2010). Por último, en Madrid la seroprevalencia de rebaño observada fue del 11,8 % en un estudio realizado hace ya más de 25 años (Vázquez-Boland, 1996). No obstante, se estima que la seroprevalencia de *T. gondii* en el ganado ovino es

todavía mayor debido a la variabilidad de los test serológicos empleados (Shaapan et al., 2008).

A pesar de la cantidad de datos publicados en relación con la prevalencia y seroprevalencia de esta enfermedad, la **variabilidad** es muy alta incluso en las mismas regiones (Belluco et al., 2016), que puede ser debida al tipo de estudio experimental, a las técnicas utilizadas en el análisis, a la recogida de muestras y su estado, a las diferentes edades en los grupos utilizados y al tamaño de muestras, lo que hace que muchas veces la comparación entre trabajos sea complicada o imposible (Ahaduzzaman & Hasan, 2022; Nayeri et al., 2021). Además, existen multitud de regiones de las que no existen datos y por tanto se desconoce si la infección está presente (Ahaduzzaman & Hasan, 2022.).

Esta enfermedad tiene una gran importancia dadas las **pérdidas económicas** que produce (Ortega-Mora et al., 2007). En ellas influye el tamaño del rebaño afectado, el sistema de producción, la tasa de abortos y las medidas de control implantadas tras un brote. Existe poca información acerca de las pérdidas económicas producidas en una explotación ganadera a consecuencia de la infección por *T. gondii*. En la Unión Europea, se estima que alrededor de 1,5 millones de abortos anuales se deben a la infección por *T. gondii* (Innes et al., 2009). Además, un estudio reciente realizado en España ha calculado unas pérdidas de 171,8 y 63,6 euros por aborto en una granja intensiva de leche y una semi-intensiva de carne, respectivamente, donde se observaron tasas de abortos del 12,6 % y el 33,3 % (Gutiérrez-Expósito et al., 2021). A su vez, se ha estimado en Gran Bretaña y Uruguay que esta enfermedad supone pérdidas anuales de entre 5 y 15 millones de dólares estadounidenses por país (Stelzer et al., 2019). Sin embargo, las pérdidas económicas producidas están seguramente infravaloradas debido a la falta de diagnósticos definitivos en muchos casos de aborto en el ganado ovino, al envío inadecuado de muestras, a la falta de informatización y de especificidad de los test serológicos, a que la enfermedad no produce una clínica característica en la madre o a que en los abortos en fase aguda por *T. gondii* no se detecta el parásito (Stelzer et al., 2019).

La **salud pública** es otra de las grandes preocupaciones en relación con *T. gondii* por su transmisión zoonótica a los seres humanos a partir del consumo de carne infectada o alimentos contaminados. Como ejemplo, se ha observado la presencia de

quistes tisulares o de ADN de *T. gondii* por PCR en **carne** destinada al consumo humano en Turquía (Dubey et al., 2020a). Recientemente, también se ha detectado una alta cantidad de ADN parasitario en músculo esquelético de corderos, particularmente en las partes comestibles como en los músculos cuádriceps femoral, intercostales o las patas delanteras y traseras, por lo que, en este estudio, concluyen que comer carne cruda de corderos es arriesgado para la salud pública, debiéndose cocinar o congelar antes de su consumo (Thomas et al., 2022). Conjuntamente, también está cobrando importancia la posible transmisión por consumo de **leche,** ya que se ha detectado la presencia de ADN de *T. gondii* mediante PCR en leche cruda de oveja, en estudios realizados en diferentes países (Luptakova et al., 2015; Sadek et al., 2015; Saad et al., 2018), o incluso se han llegado a encontrar taquizoítos en leche (Sadek et al., 2015). Afortunadamente se ha demostrado recientemente que *T. gondii* no sobrevive a las enzimas utilizadas en los procesos de fabricación del queso (Ranucci et al., 2020).

4.2. PATOGENIA, SIGNOS CLÍNICOS Y LESIONES

4.2.1. Patogenia

El conocimiento acerca de la patogenia de la toxoplasmosis ovina ha avanzado notablemente gracias a las infecciones experimentales que se han llevado a cabo en el modelo ovino desde los años 80 a la actualidad (Buxton & Finlayson, 1986). Existen diversos factores que la pueden afectar como son, por un lado, el aislado del parásito que está produciendo la infección y, por otro, las características del hospedador, incluyendo su genética y estado inmunitario (Benavides et al., 2017). La oveja se considera una especie resistente en la que el parásito sólo provoca síntomas leves. No obstante, cuando su sistema inmunitario se ve afectado, por ejemplo en una enfermedad inmunodepresora, o es inmaduro como en los fetos, la infección puede tener consecuencias graves (Innes, 1997). Las ovejas y cabras gestantes son las que se consideran más susceptibles desde el punto de vista del fallo reproductivo, seguidas del ganado porcino, mientras que los equinos y bovinos, aun siendo susceptibles a la infección, serían muy resistentes al aborto (Stelzer et al., 2019).

La infección tiene lugar tras la ingestión de ooquistes esporulados o quistes tisulares presentes en el agua o alimento contaminado (Innes et al., 2009). El

esporozoíto (o el bradizoíto), llega al intestino e invade la mucosa intestinal, en concreto las células epiteliales de revestimiento, donde puede ser detectado a partir de los 4 dpi (Sanchez & Besteiro, 2021). A continuación, se distribuye por todo el organismo, a partir de los 5 dpi, tanto por vasos sanguíneos como por vasos linfáticos y tiene lugar la fase de multiplicación rápida (taquizoítos) durante alrededor de una semana (Innes et al., 2009), coincidiendo con un aumento de la temperatura corporal por encima de los 40 grados (fiebre), que desaparece a los 10-12 dpi (Innes et al., 2009). Una vez diseminado, *T. gondii* puede infectar cualquier célula, y llega a la placenta alrededor de los 15 dpi si la hembra se encuentra gestante (Buxton & Finlayson, 1986; Benavides et al., 2017). El aborto tiene lugar si la oveja se infecta por primera vez (**primoinfección**) de forma que el parásito atravesaría las criptas maternas del placentoma (carúncula), entrando por los vasos sanguíneos, hasta llegar a los trofoblastos fetales (cotiledón) y posteriormente distribuirse por los diferentes tejidos fetales (Buxton & Finlayson, 1986; Webster, 2010). Esto se debe a que en la oveja, a diferencia de las cabras (Marshall et al., 2004), se produce una inmunidad adquirida tras esta primoinfección (Buxton, 1990).

Tras la llegada del parásito al feto, el aborto suele ocurrir alrededor de dos semanas después (**aborto tardío**), por lo que los abortos suelen observarse **4 semanas** después de la infección (Webster, 2010). Sin embargo, no se conocen con exactitud los mecanismos que lo ocasionan. Durante los dos primeros tercios de la gestación, se cree que la multiplicación del parásito en órganos fetales puede provocar lesiones suficientes como para provocar la muerte fetal (Buxton, 1998) o que una respuesta inmunitaria materna exacerbada de tipo proinflamatorio, que provocaría un desequilibrio en la barrera materno-fetal (Th1), podría causar el aborto (Buxton et al., 2007). En el tercer tercio de gestación las lesiones en los placentomas debido a la presencia del parásito podrían limitar su función y provocar una muerte fetal por falta de oxigenación en el feto (Buxton et al., 1982). Por todo ello, es importante tener en cuenta el periodo de la gestación en el cual tiene lugar la infección para poder entender la patogenia de la enfermedad (Castaño et al 2014).

Es importante destacar que en diferentes estudios (Owen et al., 1998; Castaño et al., 2014; Sánchez-Sánchez et al., 2019a) se han descrito abortos entre los días 7 y 14 pi (**aborto temprano)**, donde el parásito no es detectado ni en placenta, ni en

órganos fetales, y las ovejas no presentan anticuerpos frente a *T. gondii* en el momento del aborto (Owen et al., 1998). A excepción de la detección del parásito en un bajo número de placentomas pertenecientes a una oveja (1/8) en un único estudio (Castaño et al., 2014), el hecho de no detectar el parásito sugiere que la patogenia de este tipo de abortos es diferente a la expuesta en los abortos tardíos por multiplicación del parásito en placenta o en fetos. Inicialmente, se sugirió que el aborto ocurría a consecuencia de la pirexia provocada en la madre por la infección (Trees et al., 1989; Owen et al., 1998). Sin embargo, en estudios experimentales posteriores, la temperatura resultó ser igual en las ovejas abortadas en fase aguda y en las no abortadas (Castaño et al., 2014; Arranz-Solís et al., 2021) y el examen histológico de las placentas planteó la hipótesis de que el aborto podría ser una consecuencia del daño vascular causado por un mecanismo diferente a la replicación del parásito.

A pesar de todos los trabajos llevados a cabo para el estudio de la patogenia de la enfermedad, son muchos los aspectos que se desconocen acerca de cuáles son los mecanismos que desencadenan el aborto, ya que tanto el periodo de la gestación como la respuesta inmunitaria de la madre y del feto juegan un papel crucial en el desenlace de la infección.

4.2.2. Signos clínicos

La infección por *T. gondii en* el ganado ovino suele ser subclínica, a excepción de que se produzca una **primoinfección** en una hembra gestante, como se ha explicado en el apartado anterior. En animales adultos o en corderos los signos clínicos son inespecíficos y normalmente inaparentes, como por ejemplo la apatía, falta de apetito o letargia, pero el más importante es la fiebre, que se puede acompañar de disnea, fatiga o diarrea (Benavides et al., 2011; Dos Santos et al., 2016), lo que hace que puedan pasar desapercibidos con frecuencia para el ganadero.

En las **ovejas gestantes**, además de estos signos inespecíficos, se produce fallo reproductivo que se manifiesta como reabsorción fetal, abortos o nacimiento de corderos débiles que en muchos casos no llegan a alimentarse, se levantan con dificultad, no maman y pesan menos (Esteban-Redondo & Innes, 1997). Una presentación u otra depende del momento de gestación en el que se produce la infección (Innes et al., 2009): las infecciones en el **primer tercio** de gestación suelen ser

más graves y causan la muerte embrionaria o fetal, debido a que el sistema inmunitario del feto no está desarrollado y, como consecuencia, se produce una reabsorción o expulsión del feto; en el **segundo tercio** de gestación, provoca abortos que se presentan como fetos momificados que son expulsados, donde se observan las lesiones más graves tanto en placenta como a nivel fetal (Buxton & Finlayson, 1986) y por último, en el **tercer tercio** de gestación tienen lugar abortos junto con el nacimiento de mortinatos, corderos débiles o corderos clínicamente sanos pero infectados (Buxton, 1990; Castaño et al., 2016), ya que estos empiezan a ser inmunocompetentes (Silverstein et al., 1963).

4.2.3. Lesiones

Tanto en casos de abortos naturales (De Moraes et al., 2011; Shahbazi et al., 2019) como en estudios experimentales (O'Donovan et al., 2012; Castaño et al., 2016), las lesiones producidas por *T. gondii* tienen lugar tanto en la placenta, donde se presentan principalmente en los placentomas y cotiledones, como en los fetos, de forma particular el encéfalo (Benavides et al., 2017).

4.2.3.1. LESIONES MACROSCÓPICAS

La placenta, a nivel macroscópico, en abortos tardíos, puede presentar lesiones en el cotiledón, caracterizadas por **múltiples focos blancos** correspondientes a zonas de inflamación y necrosis, que se pueden observar también en los placentomas completos en infecciones experimentales de *T. gondii* (Figura 8B) y que constituyen una lesión característica de esta enfermedad. La zona intercotiledonaria no muestra lesiones, excepto, en ocasiones, un leve edema. (Buxton, 1990; Webster, 2010).

En el caso de los abortos tempranos, los placentomas presentan **zonas multifocales de coloración rojiza con forma piramidal** o de cuña, consistentes con infartos (Figura 8A) (Castaño et al., 2014; Benavides et al., 2017). La base de la pirámide se encuentra cerca del endometrio uterino y la zona estrecha cerca de la zona del hilio, donde el tejido corio-alantoideo fetal entra en el placentoma.

En cuanto a los fetos abortados, es frecuente la aparición de momificación fetal, con un color marrón-negruzco de los fetos, junto con edema subcutáneo, acúmulo de líquido serosanguinolento en las cavidades torácica y abdominal y, en casos muy graves, un punteado multifocal blanquecino en el hígado correspondiente a

focos de necrosis. Los mortinatos y los corderos débiles generalmente no presentan lesiones macroscópicas aparentes (Benavides et al., 2017).

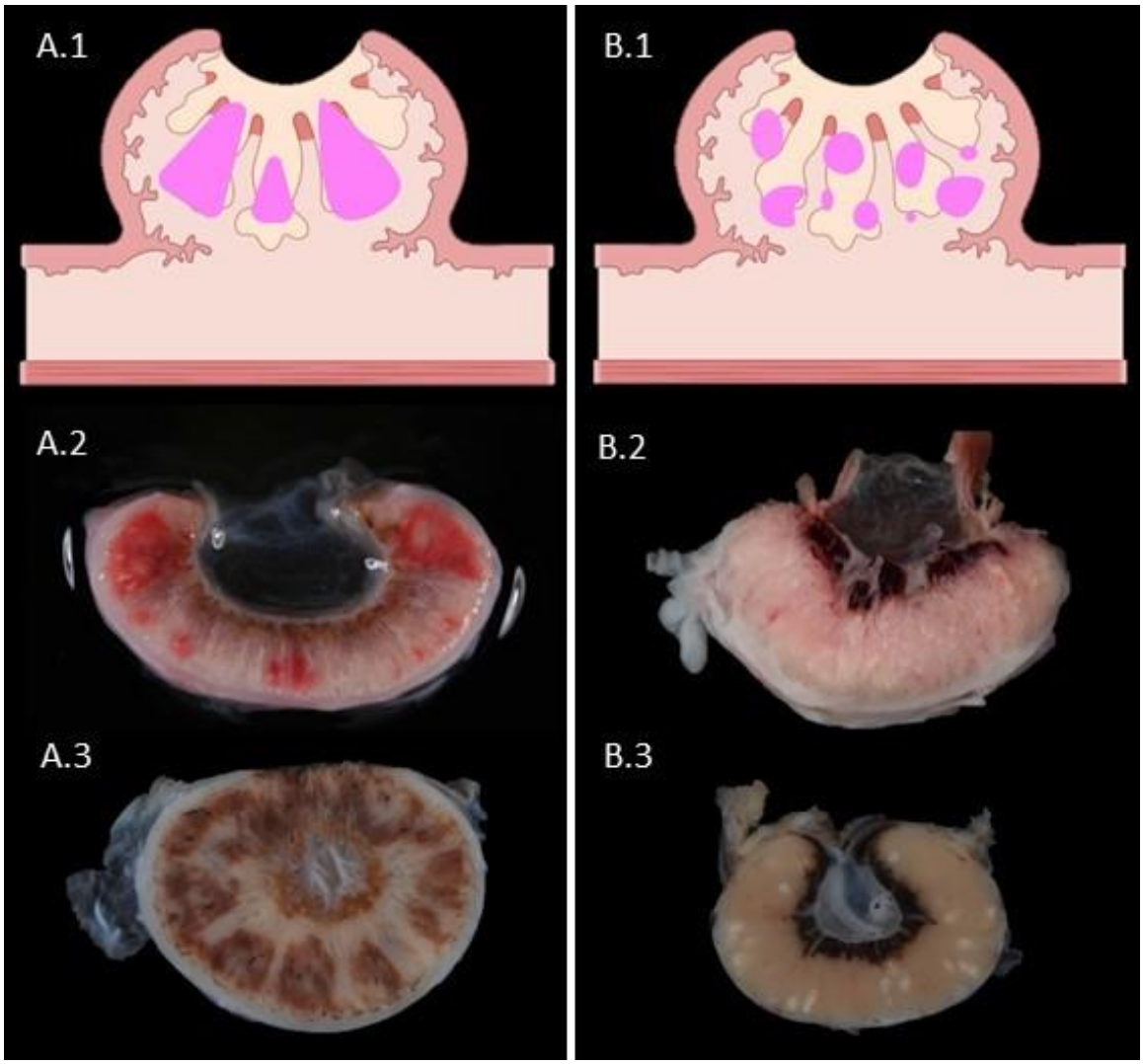

Figura 8. Lesiones macroscópicas observadas en placentomas procedentes de ovejas infectadas por *Toxoplasma gondii* en estudios experimentales. **A.** Lesiones observadas en abortos tempranos caracterizadas por la presencia de áreas en forma de cuña o pirámide de color rojo con la base cerca de la parte materna del placentoma y la punta cerca de la zona de tejido fetal corioalantoideo. A.1. Representación gráfica mediante Biorrender. A.2. Corte transversal de un placentoma ovino. A.3. Corte transversal de un placentoma fijado en formol. **B.** Lesiones observadas en abortos tardíos, caracterizadas por la presencia de un punteado blanquecino multifocal correspondiente a áreas de necrosis. B.1. Representación gráfica mediante Biorrender. B.2. Corte transversal de un placentoma ovino. B.2. Corte transversal de un placentoma fijado en formol.

4.2.3.2. LESIONES MICROSCÓPICAS EN LA PLACENTA

En el examen histopatológico, los cotiledones en caso de **abortos tardíos** (Figura 9A) y los placentomas en caso de sacrificio de la madre gestante en estudios experimentales (Figura 9B), pueden presentar focos multifocales de necrosis, donde la estructura histológica normal de la placenta se sustituye por áreas eosinófilas amorfas, que se acompañan de un infiltrado inflamatorio formado principalmente macrófagos y linfocitos (Figura 9C) (Buxton et al., 2007). Estas lesiones se observan independientemente del momento de gestación (Benavides et al., 2017), aunque las lesiones más graves, tanto en placenta como en tejidos fetales, se han encontrado en el segundo tercio (Buxton & Finlayson, 1986). Es importante reseñar que, recientemente, se ha observado que en fases tempranas de gestación, los focos de necrosis afectan en mayor medida a la parte materna del placentoma (carúncula) mientras que en el tejido conjuntivo fetal adyacente se observan células inflamatorias mononucleares, linfocitos y macrófagos, en contraste con las fases tardías en las que las células inflamatorias se encuentran tanto en la parte fetal como en la materna (Benavides et al., 2017).

En los **abortos tempranos** la placenta presenta necrosis con una forma piramidal que se asocia a trombosis (Figura 9D) y que afecta a las criptas de la parte materna principalmente (Castaño et al., 2014). Además, se ha observado la presencia de macrófagos en la parte materna, comparado con abortos tardíos donde los macrófagos y los linfocitos T aumentaban principalmente en la parte fetal (Castaño et al., 2020).

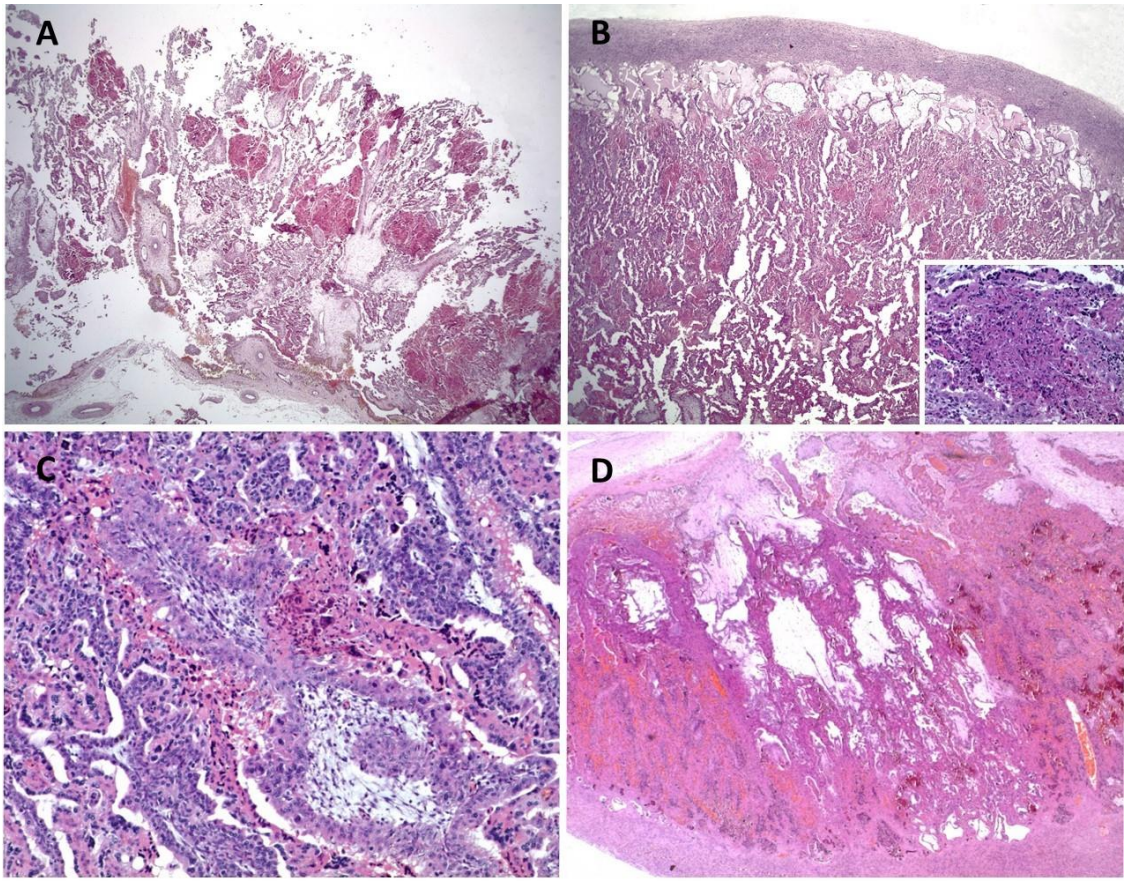

Figura 9. Lesiones microscópicas en placenta ovina provocada por *Toxoplasma gondii.* A. Placenta (cotiledón) procedente de caso natural de aborto donde se pueden observar múltiples focos de necrosis dispuestos de forma aleatoria y de diferente tamaño caracterizados por una pérdida de células remplazada por un material eosinofílico, restos cariorrécticos y fibrina (necrosis). Nótese el estado de autolisis de la muestra. 20x. **B.** Placentoma (cotiledón y carúncula) procedente de sacrificio experimental donde se pueden observar múltiples focos de necrosis. 20x. **C.** Infiltrado inflamatorio en el tejido conjuntivo fetal compuesto por células mononucleares probablemente macrófagos y linfocitos. 100x. **D.** Placentoma procedente de un caso de aborto temprano, donde se observan zonas de necrosis coagulativa en forma de cuña. 20x.

4.2.3.3. LESIONES MICROSCÓPICAS EN FETOS

Las lesiones clásicas en los **fetos** aparecen a partir de los 15 dpi (Buxton & Finlayson, 1986), aunque se encuentran con más frecuencia a partir de los 20 dpi. *T. gondii* puede afectar a múltiples tejidos, si bien el encéfalo es el órgano más comúnmente lesionado, en el que se aprecian focos de inflamación y gliosis (Figura10A), que rodean una zona central de necrosis (Figura 10B), ocasionalmente mineralizada. Las zonas más afectadas son la del tracto óptico o el tronco del encéfalo, y las menos afectadas, el cerebelo, más concretamente la porción caudal (O'Donovan et al., 2012). Otros órganos donde se pueden encontrar focos de inflamación y necrosis, son el hígado (Figura 10F), pulmones (Figura10D) y corazón (Benavides et al., 2017) y, con una menor frecuencia, riñón y músculo esquelético (Rassouli et al., 2013). Es importante destacar el predominio de lesiones necróticas en las fases tempranas de gestación frente a la presencia de infiltrados inflamatorios en las etapas más tardías (Benavides et al., 2017). Además de lesiones inflamatorias y necróticas, en la fase crónica de la enfermedad, en todos los tejidos, aunque principalmente en el encéfalo, se puede observar la formación de quistes tisulares (Figura 10E) con una pared fina y bradizoítos basófilos con forma de media luna en el interior que pueden o no estar asociados a inflamación (Benavides et al., 2017). En el caso de los abortos en fase aguda no se observa lesiones en órganos fetales, excepto **leucomalacia periventricular** (Figura 10C), asociada a hiperemia y hemorragias en la sustancia blanca del encéfalo (Castaño et al., 2014; Gutiérrez-Expósito et al., 2020a), que ha sido asociada a hipoxia fetal (Penning et al., 1994).

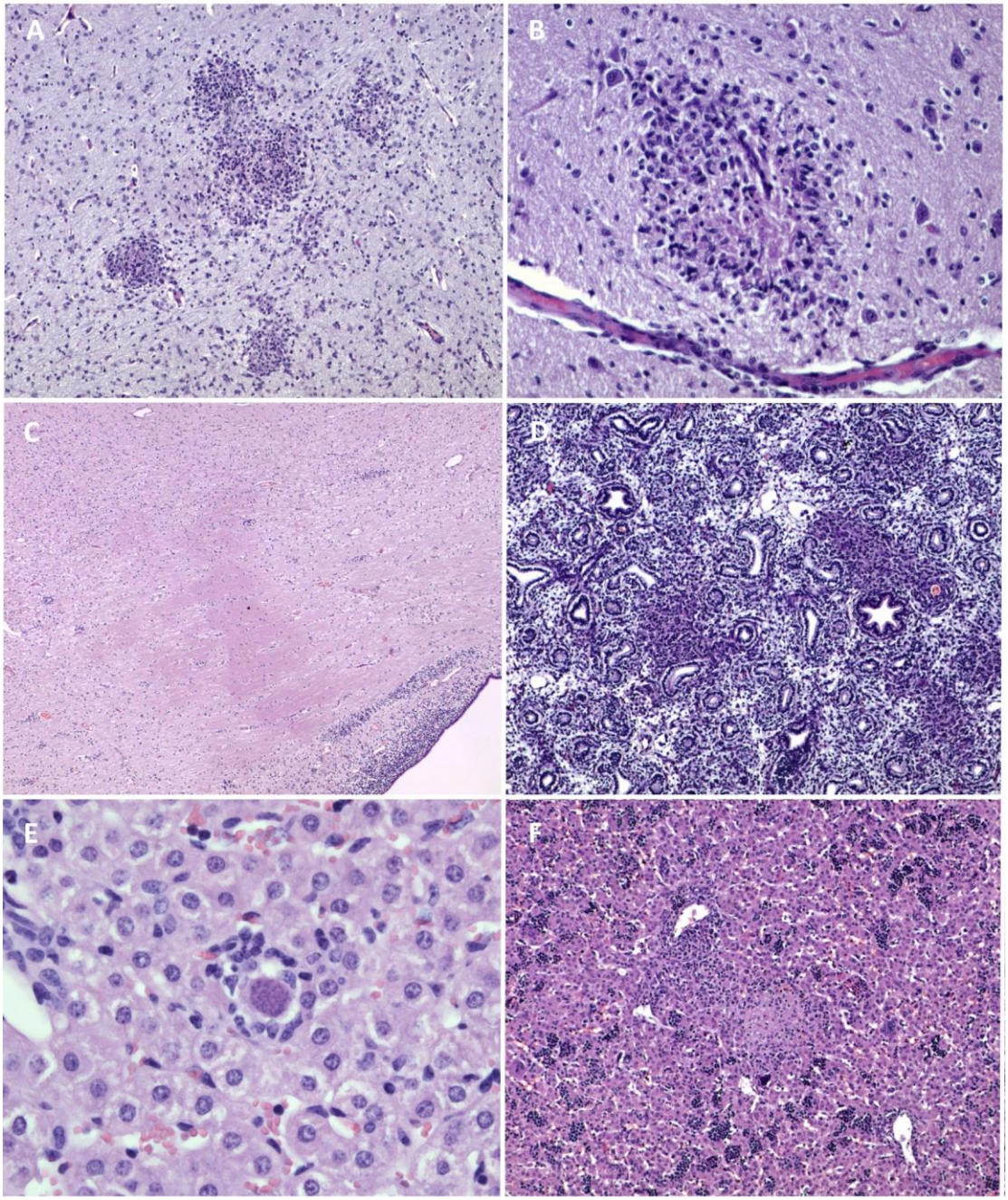

Figura 10. Lesiones microscópicas en órganos de fetales ovinos provocados por *Toxoplasma gondii*. H-E. A. Encéfalo. Múltiples focos de gliosis. 200x. **B.** Encéfalo. Foco de gliosis caraterizado por células mononucleares (linfocitos y macrófagos) con un material eosinofílico central junto a restos celulares (necrosis). 400x. **C.** Encéfalo de un feto proveniente de un aborto temprano con focos multifocales a coalescentes de material hipereosinofílico y homogéneo localizado en la sustancia blanca (leucomalacia). 40x. **D.** Pulmón. Múltiples focos de inflamación mononuclear alrededor de vasos sanguíneos y de bronquiolos. 100x. E. Hígado. Quiste tisular con bradizoítos en forma de media luna basófilos en su interior. 400x. F. Hígado con un foco de necrosis rodeado de un infiltrado inflamatorio que también está presente alrededor de los vasos portales. 100x.

4.2.3.4. DIAGNÓSTICO DIFERENCIAL

A pesar de que las lesiones que *T. gondii* provoca en fetos y placenta son características de esta enfermedad, no son completamente específicas (Benavides et al., 2017), por lo que se deben considerar varios diagnósticos diferenciales en el estudio histopatológico. En primer lugar, la infección con *N. caninum*, protozoo muy similar a *T. gondii* y de especial importancia en el ganado bovino, que está ganando importancia en los últimos años también en el ganado ovino (Nayeri et al., 2022), puede causar abortos que dan lugar a lesiones caracterizadas por focos de necrosis en los cotiledones sin afectar a la zona intercotiledonaría y encefalitis multifocal en el feto. Por lo tanto, las lesiones serían indistinguibles de aquellas producidas por *T. gondii* y sería necesario el uso de técnicas complementarias (serología, inmunohistoquímica o PCR) para llegar a un diagnóstico etiológico (Meixner et al., 2020).

Además de los abortos causados por protozoos, los agentes causales que aparecen con más frecuencia implicados en los abortos ovinos son *Chlamydia abortus, Coxiella burnetti, Campylobacter fetus fetus, Salmonella* spp *y Listeria* spp (Tirosh-Levy et al., 2022). Las lesiones macroscópicas y microscópicas pueden llegar a ser de gran utilidad para diferenciar los abortos provocados por estos agentes de los producidos por *T. gondii*. *Chlamydia abortus* y *Brucella* spp se caracteriza por provocar al igual que *T. gondii* una placentitis necrotizante, pero se encuentra afectada también la zona intercotiledonaria (Caspe et al., 2021), a diferencia de la toxoplasmosis, donde el único cambio en esta región es edema. A nivel microscópico, en el caso de *C. abortus,* la necrosis suele estar acompañada de una vasculitis prominente y se pueden observar infiltrados de neutrófilos, lesiones que no son frecuentes en infecciones por *T. gondii*, junto con agregados de bacterias Gram negativas, de 1-2 µm en el citoplasma de los trofoblastos (Borel et al., 2018). *Coxiella burnetti* daría lesiones similares a *C. abortus* pero sin vasculitis (Schlafer & Foster, 2016). *Campylobacter fetus* causa lesiones específicas en el hígado fetal, caracterizadas por focos de necrosis, al igual que en la toxoplasmosis, pero de un tamaño mucho mayor y con forma de diana (Foster, 2017). *Listeria* spp también puede causar necrosis en el hígado fetal, pero se suele acompañar de lesiones en el encéfalo, principalmente en el tronco de encéfalo caracterizadas por micro-abscesos y perivasculitis (Fentahun & Fresebehat, 2012). En el caso de los

abortos por *Salmonella* spp las lesiones son inespecíficas, con placentitis fibrinopurulenta y muerte fetal por septicemia (Schlafer & Foster, 2016). Por último, agentes fúngicos puede ser causantes también de placentitis necrotizantes con vasculitis, aunque en estos casos se pueden llegar a observar las hifas de los hongos, que pueden ser visualizadas utilizando tinciones especiales como PAS o tinciones plata. A su vez los fetos afectados suelen presentar dermatitis acompañada de hiperqueratosis o acantosis (Ali & Khan, 2006; Schlafer & Foster, 2016). Es importante indicar que se pueden dar casos de co-infección de *T. gondii* con cualquiera de estos agentes infecciosos y que, en muchas ocasiones, no se puede llegar a un diagnóstico definitivo mediante la valoración exclusiva de las lesiones (Tirosh-Levy et al., 2022).

4.3. RESPUESTA INMUNITARIA

La capacidad de *T. gondii* para establecerse en un HI como la oveja depende principalmente de la respuesta inmunitaria del hospedador y del aislado de *T. gondii* que esta causando la infección, de forma que según la regulación inmunológica que se establezca, tendrá lugar el aborto o una protección frente al parásito (Buxton et al., 2007). Casi todo el conocimiento del que se dispone de la respuesta inmunitaria frente a la toxoplasmosis se ha obtenido de estudios realizados en modelos murinos o líneas celulares murinas y humanas, aunque existen grandes diferencias entre los sistemas inmunitarios de ratones, humanos y ovejas, lo que podría indicar que la respuesta inmunitaria a esta enfermedad no sería idéntica en todas las especies (Sanchez & Besteiro, 2021). Existen dos componentes inmunitarios que actúan de forma conjunta para llevar a cabo la respuesta frente a *T. gondii*: la respuesta inmunitaria innata y la adquirida. La respuesta innata es necesaria para controlar la multiplicación del parásito en su fase aguda pero también modula y activa la respuesta adaptativa, que es la responsable de la protección frente a la infección a largo plazo (Sasai & Yamamoto, 2019).

A continuación, se exponen las características principales de ambas respuestas, en relación con los resultados obtenidos en estudios experimentales *in vitro* y estudios experimentales *in vivo* con ratones y ovejas.

4.3.1. Respuesta inmunitaria innata

4.3.1.1. CÉLULAS IMPLICADAS

Entre las células de la respuesta immune innata que producen y responden a citoquinas se encuentran los neutrófilos, macrófagos, células dendríticas y células "natural killer" (NK), todas con funciones de protección frente a *T. gondii* (Sasai & Yamamoto, 2019).

Los **neutrófilos** son las primeras células en llegar al lugar de infección y han sido reconocidos como componentes importantes en la respuesta frente a *T. gondii* por su contribución a la eliminación del parásito, al que neutralizan mediante la formación de NETs (trampas extracelulares de neutrófilos) (Debierre-Grockiego et al., 2020), y en menor medida de fagocitosis. En el ganado ovino se ha observado en estudios *in vitro* que esta formación de NETs aumentaba con el tiempo y el número de taquizoítos, y sería capaz de inmovilizar el parásito, pero no de matarlo (Yildiz et al., 2017).

Las **células dendríticas** son las principales células que presentan antígenos a los linfocitos T durante la respuesta inmunitaria innata en la toxoplasmosis. Se ha demostrado en ratones que estas células son esenciales para la protección frente al parásito por su producción de IL-12 (Liu et al., 2006) y más recientemente por su papel en la diseminación del parásito por el fenotipo hipermigratorio que *T. gondii* induce en estas células (Bhandage & Barragan, 2019). Las **células NK** protegen frente al parásito por su efecto citotóxico (Hauser & Tsai, 1986). Su activación depende de la producción de IL-12 principalmente por células dendríticas. Además, estas células producirán grandes cantidades de INF-γ (Gigley, 2016). El papel concreto de las células dendríticas y las células NK en la toxoplasmosis ovina se desconoce, con precisón.

Macrófagos: M1 y M2

Los monocitos y macrófagos tienen un papel muy importante en la respuesta inmunitaria innata frente a *T. gondii*, dominando la respuesta inflamatoria local, favoreciendo la eliminación del parásito por un lado y mitigando los efectos adversos de la inflamación por otro (Park & Hunter, 2020).

Cuando los macrófagos son activados por **INF- γ,** antes de que el parásito haya infectado las células, son capaces de limitar su replicación (Sibley et al., 1985) al adquirir un fenotipo de **macrófagos proinflamatorios M1** (Figura 11). La activación de

estos macrófagos se lleva a cabo mediante la vía STAT (Signal Transducer and Activator of Transcription) concretamente STAT1, al haberse demostrado que su eliminación en macrófagos da lugar a cargas parasitarias más altas y menos expresión de iNOS (Gavrilescu et al., 2004; Lieberman et al., 2004). Sin embargo, la presencia exclusiva de INF-γ en la toxoplasmosis no es generalmente suficiente para controlar la infección en macrófagos, aunque depende del aislado, y serían necesarias señales secundarias que consiguieran activar completamente las funciones antiparasitarias de estas células (Park & Hunter, 2020). Estas segundas señales las proporcionan citoquinas como **TNF-α** (factor de necrosis tumoral alfa) que, junto con INF-γ, aumentan la producción de especies reactivas de oxígeno (ROS) (Kondo & Sauder, 1997). Además de ROS, los macrófagos M1 excretan altos niveles NO por la **iNOS** e IDO. Los macrófagos M1 se caracterizan, a su vez, por secretar citoquinas proinflamatorias que, junto con ROS, NO e IDO, favorecerán la eliminación del parásito y una respuesta Th1 de los linfocitos T colaboradores (Mukhopadhyay & Saeij, 2020). En concreto, la producción de IL-12, aunque la mayor cantidad de esta citoquina sea producida por las células dendríticas, favorecería la respuesta inmunitaria frente al parásito (Hunter & Sibley, 2012). Esta citoquina activará a su vez la secreción de INF-γ por parte de las células NK (Park & Hunter, 2020). Se ha demostrado que los macrófagos también pueden producir IL-1, estimulando a su vez la producción de IL-12 (Lima et al., 2018).

Por otro lado, serán las citoquinas **IL-4, IL-10 y IL-13** (Figura 11) las que polaricen los macrófagos a un fenotipo **M2** (Verreck et al., 2006) con propiedades antiinflamatorias que pueden hacer al hospedador sobrevivir a la infección, pero haría a estos macrófagos menos efectivos en la eliminación del parásito (Murray et al., 2014). Esta activación es promovida, en el caso de ratones, por los factores de trascripción STAT6 y PPARγ (Verreck et al., 2006). A su vez los macrófagos M2 aumentarían la producción de citoquinas y quimiocinas antiinflamatorias como **IL-4, IL-10 y TGF-β** (Figura 11)**,** que inhiben la repuesta Th1 y favorecen la respuesta Th2 por parte de los linfocitos (Verreck et al., 2006).

Los macrófagos, por tanto, pese a formar parte de la respuesta inmunitaria innata, pueden modular la respuesta adquirida hacia una reacción inflamatoria de tipo Th1 o antiinflamatoria de tipo Th2, dependiendo del tipo de citoquinas que produzcan como se acaba de describir en los párrafos anteriores. Normalmente existe un balance

entre estas repuestas que varía dependiendo de la virulencia del aislado, la respuesta inmunitaria del hospedador o el estado de gestación (Jensen et al., 2011).

Interacción parásito-macrófago

T. gondii tiene la habilidad de modificar la respuesta de los macrófagos, inhibiendo su activación o utilizando sus actividades migratorias para facilitar su diseminación. Más concretamente, si los macrófagos son infectados antes de que sean expuestos a INF-γ, *T. gondii* podrá secretar proteínas a partir de sus órganos secretores (roptrias y granulos densos) como por ejemplo TgIST (Gay et al., 2016) o ROP5 (Etheridge et al., 2014), de forma que los macrófagos no serán capaces de frenar el crecimiento del parásito. Asimismo, *T. gondii* puede evitar que se desencadene la vía fagolisosomal tras la fagocitosis y, en su lugar, forme una VP (Figura 11) (Dobrowolski & Sibley, 1996; Dubremetz, 1998; Håkansson et al., 2001) que no se fusiona con el lisosoma y de esta forma consige evadir la fagocitosis (Clough & Frickel, 2017). Además de la habilidad de *T. gondii* para evitar las capacidades antimicrobianas de los macrófagos, el parásito los utiliza, junto con las células dendríticas, para diseminarse (Park & Hunter, 2020), haciendo que el parásito pueda persistir y favorecer la diseminación de la enfermedad, estableciendo infecciones crónicas (Muraille et al., 2014; Ólafsson & Barragan, 2020; Park & Hunter, 2020). Se ha observado en estudios *in vitro* que los monocitos infectados con *T. gondii* tienen más facilidad para adherirse a células endoteliales que los no infectados, por expresar una mayor cantidad de integrinas (Harker et al., 2013).

En la respuesta inmunitaria innata, el comportamiento de los macrófagos frente al parásito y su polarización pueden verse **afectados también por el genotipo de *T. gondii*** que está causando la infección. Los aislados tipo II parecen inducir una respuesta M1 de macrófagos en estudios *in vitro,* con un aumento de la producción de IL-12 mientras que los aislados tipo II y III activan macrófagos M2, caracterizados por la expresión de receptores de manosa, una alta actividad de la arginasa y bajos niveles de IL-12 (Jensen et al., 2011). La caracterización genética y fenotípica tiene, por tanto, especial utilidad para entender la virulencia de los aislados de *T. gondii* y cómo pueden modificar las funciones de los macrófagos a los que infectan (Barragan & Sibley, 2002). Además, son necesarios estudios donde se valore el comportamiento de los

macrófagos ovinos frente al parásito y la influencia que tienen los distintos aislados de *T. gondii* en su comportamiento.

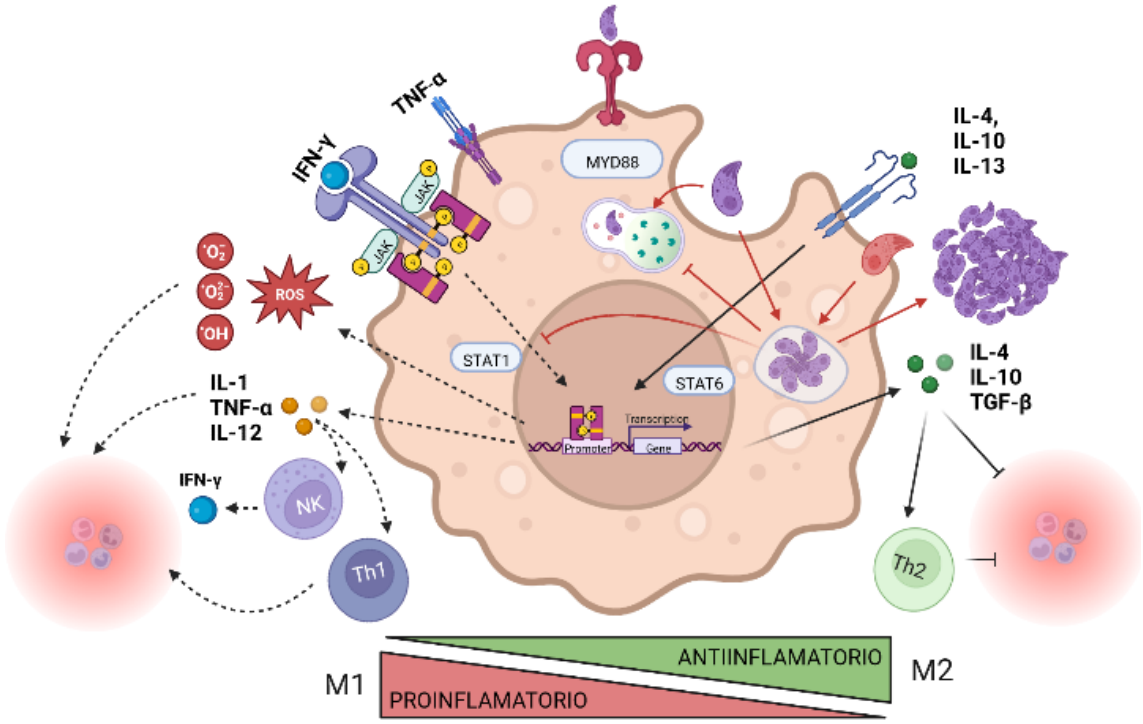

Figura 11. Esquema de los diferentes mecanismos que tienen lugar en macrófagos durante la infección por *Toxoplasma gondii*. Diagrama donde se muestran las vías de señalización que tiene lugar en la polarización de los macrófagos M1 (clásicamente activado) y M2 (alternativamente activado). Creado con Biorrender.com.

4.3.1.2. MECANISMO

Cuando un patógeno produce una infección en un organismo vivo, el hospedador desarrolla una respuesta inmunitaria innata que es el primer mecanismo de defensa y que tiene como funciones prevenir la entrada de patógenos mediante barreras físicas y químicas, evitar su diseminación, eliminar patógenos mediante fagocitosis e inducir el inicio de la respuesta inmunitaria adquirida (Kumar et al., 2021). Las **células dendríticas, macrófagos y monocitos** forman parte de la respuesta inicial frente a *T. gondii*, que reconocen componentes de este parásito llamados PAMPS (Patrones moleculares asociados al patógeno), mediante PRRs (Receptores de reconocimientos de patrones) que pueden estar localizados en la membrana celular, como las lectinas tipo C, o en el interior de las células, como los TLR (Receptores tipo

toll) o los NLR (Receptores tipo NOD) (Sasai & Yamamoto, 2019). Existen hasta 13 **TLR** en mamíferos, aunque los principales, en modelos murinos asociados a la respuesta inmunitaria frente a *T. gondii,* son el TLR 11 and el TLR12, implicados directamente en la resistencia frente al parásito (Yarovinsky & Sher, 2006) y los TLR 2, 4, 7 y 9, de forma secundaria (Andrade et al., 2013). En ratones se ha visto que la profilina reconocida por estos TLR es la mayor estimulante de la producción de IL-12, que inducirá la proliferación de células NK (Plattner et al., 2008). **Sin embargo, las especies ovina, bovina y porcina no presentan TLR11 y 12** en sus genomas (Uenishi & Shinkai, 2009). Se ha demostrado la importancia de los TLR 2 y 4 en infecciones con diferentes agentes infecciosos usando células mononucleares sanguíneas periféricas (PBMCs) de ovejas (Nefefe et al., 2017; Wang et al., 2020b). Lamentablemente, el papel que estos TLR ejercen en la toxoplasmosis ovina aún es desconocido.

La asociación de PAMPS con los TLR activa y recluta la proteína adaptadora MyD88 (Figura 11) (Sasai et al., 2018), que ha demostrado ser esencial para el control del parásito en infecciones en modelos murinos en los que esta proteína no se expresaba (MyD88-/-) (Scanga et al., 2002; Sukhumavasi et al., 2008). MyD88 estimula, en células dendríticas, monocitos, neutrófilos y células NK, la producción de IL-12 (Chacón-Salinas et al., 2005; Gee et al., 2009). Esta citoquina es importante para generación de **células NK** principales productoras de **IFN-γ** durante la fase aguda de la infección (Mahmoudzadeh et al., 2021). La producción temprana de ambas citoquinas (IL-12 y INF-γ) es esencial para restringir el crecimiento y la diseminación de *T. gondii* (Mahmoudzadeh et al., 2021). La citoquina **TNF-α** también interviene en la protección frente al parásito (Ivanova et al., 2019). Es importante destacar que se ha demostrado que los neutrófilos son responsables de la producción temprana de IFN-γ por un mecanismo independiente de la activación de TLR11 en modelos murinos (Sturge et al., 2013), que tiene especial importancia por su posible implicación en la toxoplasmosis ovina, al no poseer estos TLR como se ha descrito previamente.

Otro componente importante para limitar el crecimiento del parásito y su diseminación es el **inflamosoma**, grupo de proteínas del citosol que permite la activación de caspasas proinflamatorias, que por un lado activa la **caspasa-1**, que es una proteasa que a su vez estimulará la producción de citoquinas proinflamatorias como la IL-1 y la IL-18 y puede inducir la piroptosis, un mecanismo de apoptosis de la

célula infectada (Wang et al., 2020c). Estudios recientes han demostrado que macrófagos murinos puede formar estos inflamasomas independientemente de los TLRs (López-Yglesias et al., 2019).

4.3.2. Respuesta inmunitaria adquirida

En la respuesta inmunitaria frente a *T. gondii*, aparte de los mecanismos inmunitarios inespecíficos mencionados previamente, existe una respuesta inmunitaria específica duradera en el tiempo, llamada adquirida o adaptativa. Los linfocitos T y los linfocitos B tienen un papel fundamental en esta respuesta, y conforman la respuesta inmunitaria celular y humoral, respectivamente. Sin embargo, será la respuesta inmunitaria celular la que tenga el papel principal en la defensa contra la toxoplasmosis ovina (Buxton et al., 2007).

Todos los linfocitos T poseen en la membrana celular receptores llamados **TCR**, que permiten dividirlos en linfocitos T cooperadores o Th que poseen el receptor **CD4**, y linfocitos T citotóxicos o Tc, si tienen **CD8** como receptor (Kumar et al., 2021). Estos últimos tienen una importancia clave en el control de la toxoplasmosis crónica (Khan et al., 2019) ya que su actividad citotóxica sobre las células infectadas es el mecanismo de defensa principal en esta fase (Suzuki, 2020). Por otro lado, los linfocitos **T CD4+** son la principal fuente de INF-γ durante la fase aguda de la enfermedad. Se ha demostrado en estudios recientes en ratones en los que la respuesta mediante linfocitos T CD8+ esta inhibida por la eliminación del factor de transcripción Batf3, que son los linfocitos T CD4+ los que desempeñan un papel dominante en la producción del IFN-γ necesario para controlar la infección crónica (Tussiwand et al., 2020) y que en ratones CD4 “knock out” se apreció un aumento significativo del número de quistes en encéfalo, lo que demuestra la importancia de estas células en el control de la multiplicación y diseminación del parásito (Khan & Moretto, 2022). En relación a la **toxoplasmosis ovina** los estudios son mucho más escasos. Mediante la técnica de canulación linfática (Innes & Wastling, 1995), se ha observado un aumento de la citoquina INF-γ desde el día 2 al 9 pi, sobre todo en nódulos linfáticos mesentéricos, con la aparición de células CD4+, que serían el tipo celular mayoritario hasta los 9 dpi, momento a partir del cual predominarían las células CD8+, la aparición de IgG a la vez que el parásito deja de

detectarse (Innes et al., 1995b) y disminuirían las citoquinas INF-γ y IL-12 (Verhelst et al., 2014).

Las **células T CD4+** se pueden dividir en diferentes subpoblaciones dependiendo de con qué citoquinas sean estimuladas: Th1, Th2 y Treg (reguladores). Hay escasos estudios del papel que juegan los **linfocitos T reg** en la toxoplasmosis aguda y crónica, pero se ha observado una disminución de su número durante la enfermedad (Oldenhove et al., 2009). Además, la expresión de t-bet por la población de linfocitos T reg foxp3+ es necesaria para controlar la respuesta proinflamatoria Th1 (Warunek et al., 2021). Se ha demostrado en modelos murinos que, con la ausencia de células CD4+ mediante el uso de ratones CD4 -/-, los niveles de anticuerpos o inmunoglobulinas producidos por las células B disminuirían (Johnson & Sayles, 2002).

La activación de los linfocitos se lleva a cabo por su unión a los **complejos mayores de histocompatibiliad (MHC)** ubicados en las células presentadoras de antígenos como los macrófagos y sus receptores CD4 (que se unirá a MHCII) y CD8 (que se unirá a MHC I). Además, es necesaria la unión de moléculas coestimuladoras (como por ejemplo CD28) para que esta activación tenga lugar (Kumar et al., 2021). La respuesta Th1 típica suele producirse mediante la producción de INF-γ por las células T CD4+ y la producción de células T citotóxicas CD8+. Por ello, el INF-γ, como se ha mencionado en los apartados anteriores, es una citoquina considerada como uno de los principales mediadores de resistencia frente a *T. gondii,* tanto en la toxoplasmosis aguda como en la crónica (Suzuki et al., 1988).

Por otro lado, en cuanto a la **respuesta inmunitaria adquirida humoral** frente a *T. gondii*, los anticuerpos producidos por linfocitos B, como IgA, IgG e IgM, contribuyen al control de la persistencia del parásito al menos en parte, y a la protección inducida por la vacunación (Sasai & Yamamoto, 2019). Hay estudios que han demostrado una menor resistencia a la enfermedad en ratones deficientes de células B (Kang et al., 2000). Estos anticuerpos pueden opsonizar al parásito, facilitando su fagocitosis, o pueden activar el complemento por la vía clásica (Kang et al., 2000; Deshmukh et al., 2021). Las células B reguladoras pueden inhibir la respuesta inmunitaria mediante la producción de IL-10, que se ha relacionado con la formación de quistes parasitarios en el encéfalo y el establecimiento de la fase crónica de la enfermedad (Jeong et al., 2016). En ovejas se observa un aumento de la IgG frente a *T. gondii* en suero entre los

7 y 8 dpi (Wastling et al., 1995). Recientemente se ha observado, también en **ovejas,** una mayor respuesta humoral de IgG frente a antígenos de la pared de los quistes parasitarios que a los taquizoítos (Deshmukh et al., 2021)

Aparte de las células inmunitarias principales mencionadas, casi todas las líneas celulares son capaces de defenderse frente a *T. gondii* mediante un proceso llamado **inmunidad celular autónoma**, que incluye la rotura de la VP, limitación de nutrientes, y otras estrategias antiparasitarias (Randow et al., 2013; Sasai & Yamamoto, 2019; Sanchez & Besteiro, 2021). Por ejemplo, se ha observado en modelos murinos que la producción de GTPasas estimuladas por INF-γ, destruye la membrana de la VP, exponiendo el parásito para su posterior degradación (MacMicking, 2012). También se ha observado que la privación de hierro puede provocar una restricción del crecimiento del parásito en enterocitos murinos (Dimier & Bout, 1998) o en células Hela humanas (Bhushan et al., 2020). Por estos motivos el estudio de *T. gondii* debe realizarse en diferentes tipos de células y diferentes hospedadores, para poder evaluar el comportamiento del parásito en cada una de ellas y determinar si lo que se encuentra en unas células podría extrapolarse a otras.

4.3.3. Respuesta inmunitaria local

La respuesta inmunitaria local es de vital importancia a la hora de estudiar la toxoplasmosis ovina. La placenta es un órgano clave para esta enfermedad, por lo que el estudio de la respuesta inmunitaria que aparece en este órgano es esencial para entender la patogenia del aborto (Pastor-Fernández et al., 2021). Esta respuesta en la placenta ha sido principalmente estudiada en modelos murinos, demostrando que el parásito puede alterar el balance de los mediadores anti y pro-inflamatorios y afectar a las cantidades de las distintas poblaciones de células inmunitarias presentes en este órgano, principalmente células NK, macrófagos y linfocitos (Zhang et al., 2015). Sin embargo, los resultados en este modelo se diferencian de lo observado en **ovejas**, de forma que no hay células NK en su placenta y podrían no juegar un importante papel en la toxoplasmosis ovina en general (Wattegedera et al., 2019; Castaño et al., 2020). Por otro lado, se ha observado que, de forma normal, en la placenta predominarían macrófagos M2 y estos se diferenciarían hacia M1 con la presencia de *T. gondii,* alterando a su vez el balance Th1/Th2, hacia el polo Th1 (proinflamatorio) y Th17. Sin

embargo, en ganado ovino no se ha observado la predominancia de un tipo de respuesta único, sino el incremento tanto de citoquinas proinflamatorias como antinflamatorias (Castaño et al., 2019). Por último, en cuanto al tipo de abortos, los tempranos se asocian con un aumento del número de macrófagos en la carúncula y, por el contrario, en los abortos tardíos hay un aumento de linfocitos T y macrófagos en el cotiledón (Castaño et al., 2019; 2020).

4.4. DIAGNÓSTICO

Ante la sospecha de una infección por *T. gondii* en una explotación ovina, es necesario llevar a cabo diferentes abordajes para poder llegar a un diagnóstico certero. En primer lugar, se debe tener en cuenta el diagnóstico clínico-epidemiológico, basado en la detección de abortos y en la valoración de los factores de riesgo ambientales, como la presencia de gatos y roedores. Posteriormente, se tomará sangre de las ovejas abortadas y de los fetos, así como muestras de tejido de la placenta y fetos, sobre las que realizar técnicas de diagnóstico laboratorial, como las serológicas, las de anatomía patológica y biología molecular, que permitan llevar a cabo un diagnóstico definitivo.

4.4.1. Diagnóstico clínico

El diagnóstico clínico puede ser complicado ya que los signos son inespecíficos en las ovejas no gestantes y las perdidas reproductivas en las ovejas gestantes pueden pasar desapercibidas para el ganadero. Las consecuencias clínicas varían según el momento en el que se produce la infección, como ya se ha señalado. Si ocurre en el **primer tercio,** la muerte fetal es probable que pase desapercibida, puesto que se acompaña de una reabsorción de los fetos que se puede confundir con problemas de fertilidad en la madre (Buxton, 1990). Si sucede en el **segundo tercio** de gestación, la presentación de abortos con fetos momificados sí que pueden hacer sospechar toxoplasmosis (Buxton, 1998). Si tiene lugar en el **último tercio**, el nacimiento de corderos viables, aunque frecuentemente débiles, hace el diagnóstico de toxoplasmosis también difícil (Buxton et al., 2007). Un detalle a tener en cuenta es que los abortos se suelen presentar en forma de **brotes** que afectan a muchos animales en un corto periodo de tiempo, especialmente a individuos jóvenes, que no han tenido contacto previo con *T. gondii*. Esta presentación suele producirse tras la ingesta de

comida o agua contaminada con ooquistes en granjas donde no está circulando el parásito. La presentación de los abortos por *T. gondii* también puede observarse en forma de **casos aislados o por goteo**, cuando la infección se produce en rebaños en los que ya hay ovejas inmunizadas por contacto previo con el parásito o en los que no se sincroniza la gestación, donde hay ovejas gestantes y no gestantes al mismo tiempo (Webster, 2010).

Es importante examinar los fetos abortados y los restos de placenta con el fin de encontrar lesiones macroscópicas compatibles con una infección por protozoos (ver apartado 4.2.3.1) (Ortega-Mora et al., 2007), caracterizadas por la presencia de focos de necrosis en los placentomas, que se observan macroscópicamente como puntos blanquecinos, mientras que la zona intercotiledonaria no muestra alteraciones (Benavides et al., 2017). En el caso de los fetos normalmente no se observan lesiones macroscópicas y, si lo hacen, pueden asociarse también a otros procesos infecciosos (Foster, 2017).

4.4.2. Diagnóstico laboratorial

En relación con el diagnóstico laboratorial, se recomienda remitir al laboratorio el feto completo, en especial el encéfalo, junto con los restos de placenta, con al menos dos cotiledones placentarios, y una muestra de suero materno. Sin embargo, es frecuente que la placenta no se incluya en los casos remitidos para diagnóstico debido a su difícil acceso o mala conservación en muchos casos (Gutierrez et al., 2010). Además, es conveniente realizar varias pruebas diagnósticas simultáneas, ya que un resultado negativo en una de ellas, no permite descartar la infección por *T. gondii* (Aghwan et al., 2021).

4.4.2.1. ESTUDIOS HISTOLÓGICOS E INMUNOHISTOQUÍMICOS

El estudio histológico de muestras de tejido sospechosas de infección por *T. gondii* en el ganado ovino para la detección de lesiones microscópicas, tras su procesado y tinción con H-E, resulta muy útil a la hora de diagnosticar la causa que ha producido el aborto (Buxton & Finlayson 1986). La placenta y el encéfalo del feto son las muestras de elección para el diagnóstico de una infección por *T. gondii* (Partoandazanpoor et al., 2020). Es importante destacar que es en el segundo tercio de gestación, como se ha explicado previamente, donde las lesiones histológicas

explicadas en el apartado 4.2.3.2, serán más extensas y graves. Además, es importante mencionar que, en muchos casos, no se detectan lesiones histológicas compatibles con *T. gondii,* por lo que el uso de técnicas complementarias es necesario (Pereira-Bueno et al., 2004).

Es importante señalar que, en este momento, el diagnóstico de abortos en la fase aguda solo puede hacerse mediante la identificación de lesiones ya que no es posible detectar el parásito ni en el feto ni en la placenta (Owen et al., 1998). Se sospecharía de este tipo de aborto cuando se observen infartos o trombosis en placentomas junto con leucomalacia en el encéfalo del feto, además de demostrar la ausencia de otros agentes infecciosos abortivos (Castaño et al., 2014; Sánchez-Sánchez et al., 2019a). En condiciones naturales de campo, se estima que uno de cada siete abortos producidos por *T. gondii* en ovino es un aborto en fase aguda, pese a que su diagnóstico está infravalorado (Owen et al., 1998).

El diagnóstico histológico resulta complicado en múltiples ocasiones debido a la compatibilidad de lesiones histológicas con otros agentes infecciosos, como *Campylobacter fetus fetus, Chlamydia abortus* o *Listeria* spp. (Buxton & Henderson, 1999) (detallado en el apartado 4.2.3.4), y al estado de conservación que frecuentemente presentan estas muestras, donde la autolisis suele ser frecuente. Por este motivo se pueden emplear técnicas inmunohistoquímicas, con anticuerpos frente a *T. gondii*, que permiten identificar quistes intracelulares o incluso taquizoítos de este parásito en relación con las lesiones y que mediante el empleo de técnicas convencionales son difíciles de detectar (Meixner et al., 2020). Es importante destacar que los anticuerpos que se emplean suelen ofrecer reacción cruzada con *N. caninum* (Meixner et al., 2020). La técnica de hibridación *in situ*, que emplea sondas marcadas que reconocen fragmentos de ADN del parásito, se ha demostrado útil para detectar los diferentes estadios parasitarios, aunque, debido a su baja sensibilidad, debe emplearse junto con otros métodos de diagnóstico (Meixner et al., 2020).

4.4.2.2. PRUEBAS SEROLÓGICAS

Los métodos serológicos son importantes tanto para el diagnóstico de la toxoplasmosis como para realizar estudios de prevalencia de la enfermedad en diferentes poblaciones animales (Liu et al., 2015). Se utiliza suero sanguíneo materno,

fetal o precalostral del cordero, y cuando la sangre no es posible de obtener, fluidos de la cavidad torácica o abdominal, líquido amniótico, líquido cefalorraquídeo o carne triturada (Glor et al., 2013; Borel et al., 2014). Son procedimientos muy empleados, especialmente en el animal vivo, debido a la fácil obtención de la muestra y a su rapidez (Villard et al., 2016). En el caso de los rumiantes, al tratarse de una placenta sinepiteliocorial, los anticuerpos de la madre no pueden atravesar la barrera feto-placentaria, de forma que los fetos obtienen anticuerpos frente al parásito cuando son inmunocompetentes a partir de los 66 días de gestación, por lo que la identificación de estos anticuerpos es una de las pruebas más fiables de la existencia de una infección por *T. gondii* (Silverstein et al., 1963; Sammin et al., 2009; Pastor-Fernández et al., 2021). Sin embargo, debe tenerse en cuenta que un resultado negativo puede deberse a que el sistema inmunitario del feto no está desarrollado en el momento de la infección.

De las diferentes técnicas serológicas disponibles, la más empleada es **la técnica ELISA**, principalmente basada en la detección de IgG frente a *T. gondii* en suero. Existen tests comerciales de ELISA disponibles en el mercado, de los que el "ID Screen® Toxoplasmosis Indirect Multi-species" es uno de los más utilizados (Liyanage et al., 2021), pero la capacidad de adaptar los ELISAS para el desarrollo de ensayos más precisos, ha hecho que los ELISAs "in house" crezcan en los últimos años (Holec-Gasior, 2013; Ferra et al., 2015; Teimouri et al., 2019). Recientemente se ha demostrado que el empleo de combinaciones de antígenos recombinantes y quiméricos en estos ELISAs dan mejores resultados que los antígenos nativos o recombinantes individuales (Liyanage et al., 2021). Aunque la presencia de niveles elevados de IgG es indicativa de que el animal está infectado por *T. gondii*, es importante tener presente que los niveles de IgG pueden ser altos sin necesidad de que se esté produciendo una enfermedad activa o el aborto en ese momento (Lind et al., 1997). Se ha observado que la presencia de IgM, al igual que en humanos, puede detectarse años después de la infección, por lo que no se puede asociar a infección reciente, mientras que su ausencia sí que excluye que ésta haya tenido lugar (Liu et al., 2015; Elsheikha et al., 2020).

El uso de esta técnica ha demostrado sensibilidades del 93.33 % y del 100 %, y especificidades de 96.87 % y 100 % con respecto a otras técnicas de diagnóstico

serológico (Glor et al., 2013). En ocasiones hay infecciones por *T. gondii* en las que no se produce un nivel de anticuerpos suficiente para que sea detectado mediante la técnica ELISA (Nguyen et al., 1996). Por el contrario, puede haber presencia de anticuerpos frente *T. gondii* en ausencia del parásito o falsos positivos por reacciones cruzadas (Nishikawa et al., 2002)

Además, aunque no muy utilizado, se puede detectar la presencia de antígenos en vez de anticuerpos, mediante una prueba de ELISA directo, muy útil a la hora de identificar la cronicidad de la enfermedad (Carvalho et al., 2008; Liu et al., 2015).

Otros métodos serológicos utilizados comúnmente son la técnica **de microaglutinación con antígenos vivos *(*MAT*)* y la técnica de inmunofluorescencia indirecta (IFAT),** que consiste en la incubación del suero a testar con taquizoítos muertos donde, si el suero contiene anticuerpos frente a *T. gondii*, al añadir el anticuerpo secundario unido a un fluorocromo, se podrá observar la presencia de fluorescencia al visualizarlo en el microscopio (Liu et al., 2015).

Las técnicas IFAT y ELISA son las más comúnmente utilizadas (Duarte et al., 2020). En un estudio realizado en 300 sueros, se observó que la técnica MAT tenía una sensibilidad mayor, seguida de la técnica ELISA y por ultimo IFAT (Shaapan et al., 2008). Sin embargo, la especificidad fue más elevada para la técnica IFAT, seguida de MAT y en último lugar la técnica ELISA (Shaapan et al., 2008). Se ha señalado que la técnica ELISA es más útil como método diagnóstico, sobre todo en áreas con una alta prevalencia, mientras que la técnica MAT sería un procedimiento más apropiado para hacer pruebas de cribado (Abdurahaman & Tessema, 2013).

Por último, existen otros test serológicos como el **análisis de liberación de INF-γ** que permite distinguir animales infectados de no infectados, al provocar una fuerte activación de linfocitos ya sensibilizados, después de la estimulación *in vitro* con antígenos parasitarios. Se suele utilizar para el estudio de la inmunidad celular y para diagnósticos tempranos, y se ha señalado que podría llegar a convertirse en una herramienta útil para el diagnóstico de la toxoplasmosis congénita (Chapey et al., 2010; Mahmoudi et al., 2017).

4.4.2.3. TÉCNICAS MOLECULARES

Las técnicas moleculares son métodos de diagnóstico directo que se basan en la detección del ADN de *T. gondii*, mediante la técnica PCR. Se han utilizado tanto en casos naturales (Gutierrez et al., 2012) como experimentales (Esteban-Redondo & Innes, 1998; Castaño et al., 2016) y se puede realizar para detectar tanto taquizoítos en muestras de sangre periférica (infecciones agudas) como para la detección del parásito en diferentes tejidos como músculo y tejido nervioso (infecciones crónicas) (Savva et al., 1990; Garcia et al., 2006). Se basa en la detección de fragmentos genéticos específicos del ADN de *T. gondii* como el elemento repetitivo 529 pares de bases, el gen B1 o el espaciador interno transcrito 1 (Hurtado et al., 2001; Khan & Noordin, 2020).

Con el objetivo de incrementar la sensibilidad, se han puesto a punto técnicas de **PCR anidada** que detectan la presencia de genes multicopia y se utiliza normalmente para la **detección** y diagnóstico de *T. gondii* incluso cuando la carga parasitaria es muy baja (Hurtado et al., 2001; Castaño et al., 2014; Fernández-Escobar et al., 2020a), como en el caso de la sangre (Tavassoli et al., 2013). Por otro lado, la técnica de **PCR a tiempo real o qPCR** se utiliza para la **cuantificación** de la carga parasitaria en diferentes tejidos y se ha empleado principalmente en estudios experimentales, para poder evaluar la progresión de la infección o demostrar la eficacia de un tratamiento (Juránková et al., 2015; Castaño et al., 2016; Santoro et al., 2019; Fernández-Escobar et al., 2021).

Si bien las técnicas de PCR son específicas, un resultado negativo no permite descartar la presencia de ADN parasitario en la muestra por dos razones principales, que el tejido no esté bien conservado o que la carga parasitaria sea tan baja que no pueda ser detectada (Liu et al., 2015). Esta técnica se utiliza como test de referencia para la detección de *T. gondii* al tener una mayor sensibilidad y especificidad que las técnicas serológicas (Shaapan et al., 2008). Sin embargo, la presencia de *T. gondii* en la sangre (parasitemia) es raramente detectada, por lo que no se recomienda su uso como método diagnóstico en esta muestra (Liu et al., 2015).

4.5. CONTROL DE LA TOXOPLASMOSIS OVINA

El **control** de la toxoplasmosis como método para mejorar la sanidad animal, la salud pública y disminuir las pérdidas económicas en el ganado ovino puede realizarse por diferentes vías: **controlando la transmisión**, **tratando** la enfermedad y mediante la **vacunación** de los animales (Buxton, 1998). Debido al escaso desarrollo de las últimas dos medidas en las últimas décadas, una de las principales estrategias es controlar la transmisión *de T. gondii.* Sin embargo, una combinación de las tres estrategias sería lo más recomendable (Dubey et al., 2020a). El control de la transmisión se puede realizar limitando la exposición de las ovejas a los ooquistes en el medioambiente, a los restos de placentas infectadas con el parásito, evitando el contacto con carne infectada de pequeños mamíferos o pájaros, desarrollando protocolos de higiene y buen manejo de los animales o evitando que los gatos tengan acceso a las instalaciones de la granja, a los suministros de agua y comida y estableciendo medidas que eviten el acceso de roedores, evitando de esta forma que el medio se contamine con ooquistes o restos de animales con quistes tisulares (Stelzer et al., 2019).

Existen escasos compuestos comercializados para el **tratamiento** de la toxoplasmosis en animales (Konstantinovic et al., 2019). Hasta el momento, se han realizado múltiples estudios en ovejas para testar compuestos centrados en la **reducción de la mortalidad fetal y la transmisión vertical** como el inhibidor de la quinasa 1294, que protegía a los fetos de la infección por *T. gondii* cuando se administraba durante el periodo de gestación (Sánchez-Sánchez, et al., 2019b), la monensina que tambien ha resultado ser efectiva (Buxton et al., 1988), aunque hay que utilizarla en la fase de parasitemia de la enfermedad y requiere especial cuidado para evitar la intoxicación por este compuesto (Sánchez-Sánchez et al., 2018), el lasalocid que no resulto efectivo en ovejas (Kirkbride et al., 1992) y el decoquinato, entre otros, que retrasaba la aparición de fiebre y reducía las lesiones en placentomas (Buxton et al., 1996). Sin embargo, el tiempo al cual se debe administrar estos compuestos supone un problema, ya que no hay evidencia de que se haya producido la infección hasta que se produce el aborto, momento en el cual sería demasiado tarde para su administración (Sánchez-Sánchez et al., 2018). Otra de las principales desventajas de estos tratamientos es que ninguno es efectivo para la lograr la

desaparición de quistes parasitarios (Kul et al., 2013), algo de gran interés puesto que el consumo de carne libre de quistes de *T. gondii* es de gran relevancia para la salud pública. En un estudio donde se empleó toltrazuril (Kul et al., 2013) sí que se asoció su administración con una reducción de quites tisulares en la musculatura, pero sin lograr su eliminación total.

En cuanto a la **vacunación**, actualmente solo existe una vacuna viva atenuada (aislado S48) de *T. gondii* autorizada para su uso veterinario en oveja, Toxovax™ (MSD) (Wang et al., 2019a). Su atenuación tuvo lugar tras 3000 pases de este aislado en ratones de laboratorio (Buxton & Innes, 1995). Esta vacuna reduce la transmisión congénita de *T. gondii* y la formación de quistes en los tejidos (Katzer et al., 2014) y protege frente al aborto durante al menos 18 meses después de la vacunación (Buxton, 1993). Las bases genéticas de la atenuación de esta vacuna no están exploradas por lo que, además de tener las desventajas de las vacunas vivas en relación a su producción, estabilidad y seguridad (Hasan & Nishikawa, 2022), existe la posibilidad de reversión del parásito a su forma virulenta, razón por la que no se permite su uso en humanos (Barros et al., 2021). Además, pese a ser efectiva, no elimina de forma total el parásito (Barros et al., 2021).

Las limitaciones de esta vacuna atenuada han hecho que se hayan propuesto nuevas formulaciones vacunales, todavía en fase experimental. Se ha analizado la posibilidad de emplear taquizoítos muertos o extractos de estos (Stanley et al., 2004), o el uso de vectores vacunales (Supply et al., 1999) que vehiculizan vacunas basadas en ADN de forma que expresarían ciertos antígenos del parásito (Hiszczyńska-Sawicka et al., 2010; Hiszczyńska-Sawicka et al., 2011; Maraghi et al., 2019), o con vacunas basadas en nanoparticulas que transportan antígenos por la mucosa, simulando una infección natural (Allahyari et al., 2022). De estas dos últimas vacunas existen recientes estudios muy prometedores en cuanto a su eficacia, tanto en ratones inoculados con un cóctel de ADN que expresa gSAG1, TgROP2 y que aumenta el tiempo de supervivencia de estos animales (Sun et al., 2020), como en ganado ovino inmunizado con una nueva vacuna intranasal basada en estas nanopartículas mencionadas, donde se produjo una reducción de la cantidad de quistes parasitarios y de la transmisión a fetos (Ducournau et al., 2020).

JUSTIFICATION AND OBJECTIVES

Toxoplasmosis is a disease of great importance worldwide because it causes high economic losses in livestock due to reproductive failure and multiple concerns in public health, so that it is considered one of the main zoonosis in the European Union (Stelzer et al., 2019). Regarding animal health, and despite the existence of multiple measures for its control, whether based on management, vaccination or treatment, toxoplasmosis is still one of the major health problems in sheep flocks (Sánchez-Sánchez et al., 2018). Although ovine toxoplasmosis has been known for many decades, there are still many gaps in the knowledge of its pathogenesis, especially in relation to abortions and the influence that the genetic variability of *T. gondii* isolates may have on it. Numerous previous *in vitro* and murine studies have shown that different genotypes of *T. gondii* may be associated with different virulence. However, in the recent years, it has been shown that the number of genetic markers analyzed in these models may not be sufficient, as there is a remarkable inter- and intra-genotype variability. Moreover, extrapolation of phenotypic differences observed in one species to another is not straightforward (Taniguchi et al., 2018; Sánchez-Sánchez et al., 2019a). In the light of these results, previous studies have suggested that a much more detailed phenotypic characterization of *T. gondii* isolates, using both *in vitro* and *in vivo* models, in different hosts, would be necessary to understand which characteristics may influence the progression of toxoplasmosis (Calero-Bernal et al., 2022). On the other hand, most of the *in vivo* experiments so far have been carried out in murine models or are observational studies in humans, leaving aside one of the main intermediate hosts, which is the sheep. Besides, recent studies have shown that there is a high genetic variability among the *T. gondii* isolates found in Spanish sheep (Fernández-Escobar et al., 2020a). For all these reasons, it is necessary to investigate how *T. gondii* isolates variability could influence the development of the disease in sheep, but also delve into those mechanisms responsible for abortion in toxoplasmosis, which are not yet fully understood.

This PhD Thesis has been carried out at the “Instituto de Ganadería de Montaña” (IGM), a centre that belongs to the Spanish National Research Council (CSIC) and the University of León (ULE), and at the Animal Health Department of the Faculty of Veterinary Medince of the ULE, more precisely in the "Ruminant Health and Pathology" research group, which is currently a reference in small ruminant pathology,

apart from being an official training centre of the *European* College *of Veterinary Pathologists* (ECVP). On the other hand, the IGM is a centre with a great expertise in intracellular and extracellular parasites, immunology, molecular techniques and research in small ruminant diseases.

The work developed in this PhD Thesis is part of the research line already established in the "Ruminant Health and Pathology" group, focused on the study of the pathogenesis of ovine toxoplasmosis, specifically on the host/pathogen interaction. As a result of previous works, this group has extensive experience in the development of small ruminants' experimental models for the study of intracellular pathogens diseases, mainly paratuberculosis and toxoplasmosis. The latter using a pregnant ovine model, which is essential for the study of the pathogenesis of abortion. In this way, great progress has been made in the last years, such as understanding the influence of the gestation period on the development of the disease, the involvement of mechanisms that have been scarcely studied in the occurrence of early abortions or in the local and peripheral immune responses triggered after infection.

This PhD Thesis is focused on the study of the relation between *T. gondii* and sheep, starting with the analysis of the interaction between ovine macrophages and the parasite, to the study of the consequences of infection in a pregnant ovine model. For the former, an *in vitro* experimental model has been developed, based on ovine macrophages derived from peripheral blood monocytes (OvMØs), with the purpose of studying their response after *T. gondii* isolates infection that have different genotypes or origin. Similarly, in order to analyze how this genetic variability influences host infection, in terms of the development of clinical signs, the appearance of lesions caused and the distribution of the parasite, an *in vivo* experimental model of pregnant sheep was used. The animals were experimentally infected with those isolates that in OvMØs, and in previous studies, showed greater phenotypic differences. In addition, as brain is the most affected organ in the fetuses by this disease, histological lesions were characterized, as well as the changes in the resident cells of the CNS.

Since an essential part of this experimental model is based on the study of the lesions caused by the infection in the placenta and fetus, it is necessary, in both cases to have detailed information on their morphology and normal physiological findings. Both the placenta and the fetus show rapid growth and changes in morphology during

gestation. Due to this condition, it is common to identify histological changes at the microscopic examination whose pathological meaning is unknown. This is why this PhD Thesis also deals with the macroscopic, histological and immunohistochemical characterization of uninfected ovine placenta and fetuses, in order to clarify future doubts that may arise in the assessment of lesions in this ovine experimental model.

This research work arises from a research project funded by the Spanish Ministry of Science and Innovation, which has been developed in coordination with the Saluvet group of the Complutense University of Madrid. This project is aimed to understand the intra- and inter-genotypic variability of *T. gondii* isolates obtained from natural infections, leading to identify virulence factors that would explain their biological variability and to clarify differences between species, as well as to increase knowledge of the host response to the infection.

The **general objective** of this PhD thesis is to study how the *T. gondii* phenotypic and genetic variability influences on the parasite/host interaction in ovine toxoplasmosis and the consequences that this may have on the pregnant sheep.

More specifically, the following **four objectives** have been set:

Objective 1. To study the influence of the genetic variability of *T. gondii* on the immune response of a target cell such as ovine macrophages using an *in vitro* model.

Objective 2. To describe the main macroscopic and microscopic findings in healthy ovine placentas and fetal viscera, as well as the main immune cell populations present in placentomes under physiological conditions.

Objective 3. To investigate the influence of the genetic and phenotypic variability of three *T. gondii* isolates in a pregnant sheep model, by studying the clinical signs and occurrence of reproductive failure, as well as histological lesions and the distribution and burden of the parasite in the placenta, fetuses and neonatal lambs.

Objective 4. To characterize the histological lesions and changes in resident populations in the central nervous system of ovine fetuses following infection with three *T. gondii* isolates with genetic and phenotypic variability.

In relation to these objectives, and following the same order, this manuscript is organized on chapters. Chapter I of this PhD thesis is focused on determining how *T. gondii* interacts with ovine macrophages and the influence that the variability of the different isolates has on their response, corresponding to objective 1. Chapter II is related to the macroscopic and microscopic characterization of ovine placenta and fetal viscera obtained from uninfected animals in order to identify the physiological findings that are normal in the tissues that are most relevant in *T. gondii* ovine pregnant model infection, corresponding to objective 2. Chapter III is focused on determining how the genetic and phenotypic variability of three *T. gondii* isolates influences clinical development, lesions and parasite distribution in an experimental model of ovine toxoplasmosis in pregnant sheep. Finally, Chapter IV is focused on characterizing the histological lesions in the brain of fetuses infected with *T. gondii* and changes on the resident CNS cell populations, corresponding to objective 4.

GENERAL MATERIAL AND METHODS

A brief explanation of the general material and methods of this PhD Thesis will be presented in this section, in order to better understand the experimental procedures and samples taken and analyzed in each chapter of this thesis. A detailed description of the procedures conducted in each study will be included within every chapter.

In order to achieve the general objective of this work, two separate experiments were carried out (Experiment 1 and Experiment 2). The Experiment 1 was based on an *in vitro* assay for the evaluation of how ovine macrophages derived from peripheral blood monocytes (OvMØs) responded after infection by six different *T. gondii* isolates. This experiment was designed to achieve the objective 1 of the thesis and it is presented in the Chapter I of this document. The Experiment 2 consisted on an experimental model of ovine toxoplasmosis based on the oral infection of pregnant sheep with 3 different *T. gondii* isolates, chosen according to their variability in phenotypic and genetic traits. This experiment was carried out in order to achieve objectives 2, 3 and 4 of the PhD thesis and it is further elaborated in Chapters II, III and IV.

Origin of *T. gondii* isolates

The *T. gondii* isolates used in Experiments 1 and 2 are part of a panel of 31 Spanish isolates obtained by the Saluvet Research group (University Complutense of Madrid) within the framework of a coordinated research project (AGL2016-75935-C2-1-R) with our Research group (AGL2016-75935-C2-2-R). The details of the geographical and samples origins and methodology used in their isolation had been already published (Fernández-Escobar et al., 2020a). Briefly, 11 isolates were obtained from placenta or fetal samples from natural cases of abortion and 20 isolates from myocardial samples of adult sheep obtained at two different slaughterhouses.

EXPERIMENT 1

A total of six *T. gondii* isolates was selected from the previously mentioned panel, according to three criteria: *(1)* genetic diversity limited to the three predominant PCR–RFLP genotypes present in Spain (ToxoDB #1, #2 or #3) and archetypal types (type II and III), (2) geographical location within Spain and (3) origin of the clinical sample (abortion-derived tissues or myocardial tissues from chronically

infected adult sheep). Table M&M-1 shows the characteristics of the six selected isolates regarding those criteria. It is important to note that all these isolates have a low and controlled number of lytic cycles in cell culture (from 8 to 12) with the purpose of avoiding their adaptation to *in vitro* cell culture.

Table M&M-1. Panel of the 6 *Toxoplasma gondii* Spanish ovine isolates used in Experiment 1 for the *in vitro* assay in ovine macrophages derived from peripheral blood monocytes (OvMØs).

	Genotype (ToxoDB#)	Type	Geographic origin	Origin of clinical sample	Group
TgShSp1	#3	II	Palencia, central Spain	Ovine fetal brain	PRU II-A
TgShSp2	#1	II	Navarra, northern Spain	Ovine fetal brain	Clonal II-A
TgShSp3	#3	II	Palencia, central Spain	Ovine fetal brain	PRU II-A
TgShSp11	#3	II	Cáceres, western Spain	Myocardium of chronic infected sheep	PRU II-C
TgShSp16	#3	II	Badajoz, western Spain	Myocardium of chronic infected sheep	PRU II-C
TgShSp24	#2	III	Ciudad Real, central Spain	Myocardium of chronic infected sheep	Type III-C

All assays were carried out at three different days. In each day, an independent assay with two duplicates for infection rates and two duplicates for transcript analysis were performed, under the same experimental conditions than the other assays. Thus, a total of six replicates per isolate were obtained for each parameter (i.e. infection rate and transcript analysis). A monolayer of $2x10^5$ OvMØs were seed in each well with a sterile glass slide (Microscope cover glasses, VWR®) and afterwards $6x10^5$ tachyzoites were inoculated at a multiplicity of infection (MOI) of 3. After 6 hours post infection (hpi), the tachyzoites, stained in green, were labelled primarily by a specific antibody against *T. gondii* (220A-15-RUO, Cellmarque®) and subsequently a with a secondary antibody conjugated to Alexa Fluor® 488, the cell cytoplasm stained in orange with Cellmask™ (Thermo Fisher Scientific), a laboratory reactive conjugated with tetramethylrhodamine (TRITC) as fluorocrome, and the nuclei in blue with with 4',6-diamidino-2-phenylindole (DAPI) mounting solution (Thermo Fisher Scientific®). On the other side, at the same time point, macrophages from the other wells without glass

slides were removed and RNA was extracted from the cells in order to evaluate, by quantitative real time PCR (qPCR), the transcription levels of IFN-γ, IL-12, IL-10, IL-4, IL-6, IL-17, TNF-α, TGF-β and IL-1α cytokines, as well as iNOS. Statistical analysis was carried out considering each isolate individually but also comparing between groups according to their origin (A: isolated from the brain of aborted fetuses or C: isolated from the myocardium of chronically infected adult sheep) and genotype (PRU II, clonal type II or type III), thus four groups were established (Table M&M-1).

Specific details of experimental procedures for *in vitro* generation of OvMØs, *T. gondii* cultures, immunofluorescence staining and image analysis, evaluation of OvMØs infection rates, cytokine analysis and iNOS expression and statistical analysis are explained in the material and methods section of Chapter I.

EXPERIMENT 2

Three isolates were selected out of the above-mentioned six isolates of *T. gondii* for further evaluation in an experimental model of pregnant sheep (Table M&M-2). The isolates selected were those showing the highest differences in the phenotypic traits evaluated during Experiment 1. The results from parallel studies carried out under the coordinated project were also taken into account for this selection. This study analyzed the variability of the isolates in an *in vitro* experimental model based in an ovine trophoblasts cell line (AH-1) and an *in vivo* murine model (Fernández-Escobar et al., 2021).

Table M&M-2: *Toxoplasma gondii* Spanish isolates selected for in vivo characterization through an oral infection in a pregnant sheep model.

Isolate ID*	Genotype # (ToxoDB)	Archetypal type	Geographic origin	Origin clinical sample	Ovine trophoblasts (AH1 cell line) (Fernández-Escobar et al., 2021)		*In vivo* murine model (Fernández-Escobar et al., 2021)		
					Tachyzoite yield $_{72h}$ (Zoites/ng of total DNA)	Parasite invasion rate	Cumulative mortality	Parasite burden (30 dpi)	Clinical sings
TgShSp1	#3	Type II PRU variant	Palencia, central Spain	Ovine fetal brain	44.5	Low	0%	Medium	Mild clinical signs (ruffled coat and ascites)
TgShSp16	#3	Type II PRU variant	Badajoz, western Spain	Myocardium of chronic infected sheep	97.9	Medium	20.8%	High	Rounded back Loss of body condition
TgShSp24	#2	Type III	Ciudad Real, central Spain		403.6	High	18.2%	High	

***ID: Identification**

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Briefly, a total of 73 female pregnant sheep seronegative against the main abortifacient agents, were randomly divided into four experimental groups (Figure M&M-1): 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 non infected negative controls (G4, *n*=14).

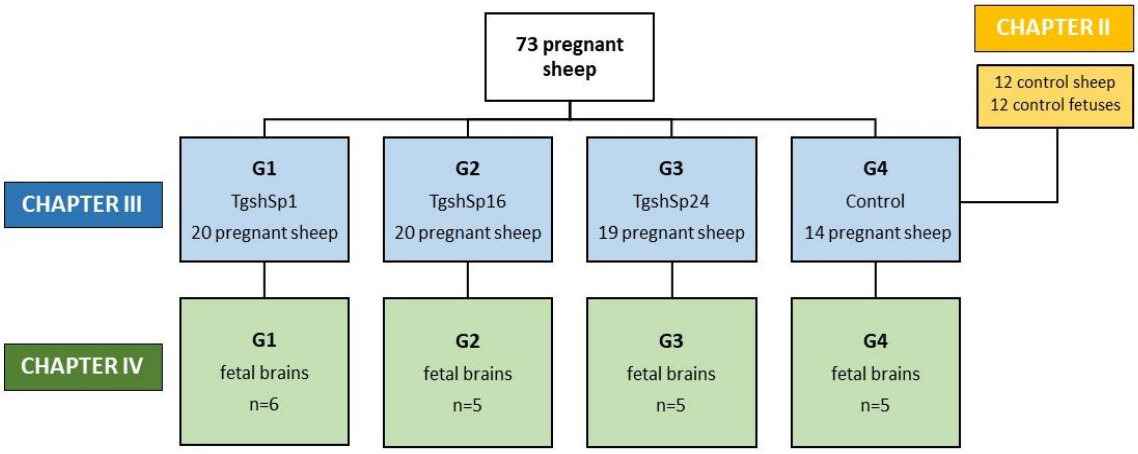


Figure M&M-1. Outline of the Experiment 2 and the correspondence between its different components and the chapters where they are further elaborated

At 90 days of gestation, sheep form G1, G2 and G3 were orally inoculated with 10 oocysts of each *T. gondii* isolate (Figure M&M-2). Rectal temperatures were measured daily and blood samples for IgG antibodies against *T. gondii* evaluation by a commercial ELISA (ID Screen® Toxoplasmosis Indirect Multi-species), were collected at 0, 3, 5, 8, 12, 14, 19, 22 and 28 dpi (Figure M&M-2). In order to characterize the histological lesions and parasite tissue distribution and burden in maternal and fetal tissues, 5 pregnant sheep from each of the four experimental groups were euthanatized at 14 dpi (n=20) and another 5 sheep from each group at 28 dpi (n=20). Then, in order to evaluate the occurrence of reproductive failure, the remaining 33 sheep were kept until early (between 8 and 14 dpi) or late (from 28 to 50 dpi) abortions or delivery at the end of gestation, occurred (Figure M&M-1). In the serial culling, iliofemoral and jejunal mesenteric lymph nodes and 9 placentomes (3 cranial, 3 medial and 3 caudal) were randomly retrieved from each placenta. Tissues collected from early abortions, fetuses sampled in the serial cullings, late abortions, stillbirths and lambs, included the brain, lung, skeletal muscle, liver and heart.

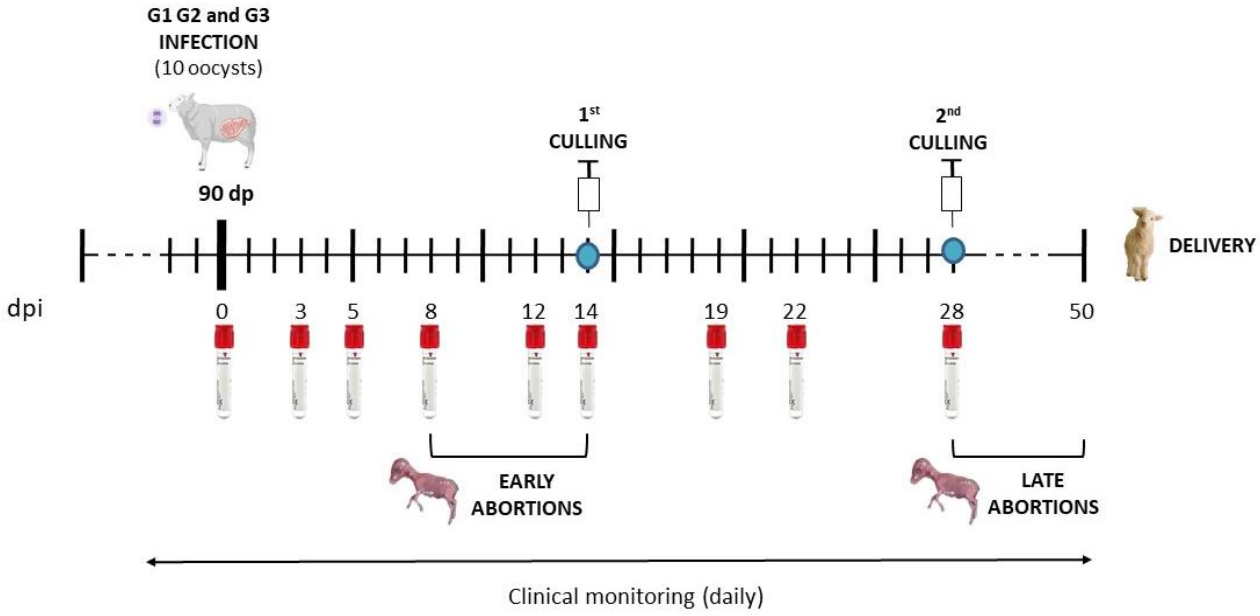


Figure M&M-2. Experimental design and timeline of the Experiment 2. Dpi (days post infection) when blood samples were taken are indicated under the timeline by a red vacutainer. Dg (days of gestation). Rectal temperature was measured every day from -2 to 28 dpi.

Samples from the negative control group (G4) of Experiment 2 were used for conducting the objective 2 (Figure M&M-1), included in Chapter II, in order to evaluate normal findings in sheep placenta and fetuses. First, fetuses (n=12) and the uterus with the placenta (n=12) were macroscopically evaluated and nine placentomes per animal (3 cranial, 3 medial and 3 caudal) were randomly collected (n=108). Fetal tissue samples included brain, tongue, right eye, lung, heart, trachea, esophagus, thymus, thyroid gland, liver, right kidney, small and large intestine, skeletal muscle, mesenteric lymph node and haired skin. All organs were microscopically assessed and special staining techniques were used to further characterize the histological changes. Immunohistochemical labelling of different cellular markers was performed on placentomes. A comprehensive description of the methodology and results from this study is included in the Chapter II of the PhD thesis.

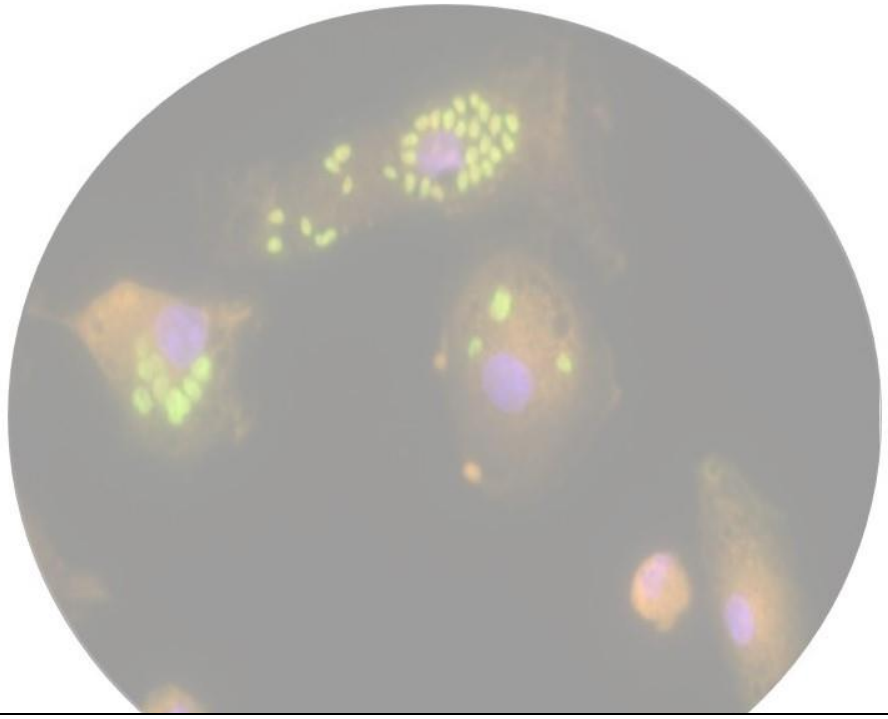
All animals from Experiment 2 were studied in order to achieve the objective 3 of this PhD thesis, regarding the assessment of the influence of *T. gondii* isolate variability on the clinical outcome of the disease, and the results are included in Chapter III. In order to evaluate the occurrence of lesions associated to the infection, all tissue samples were processed for histological evaluation. Genomic DNA was

extracted from placental and fetal or lamb tissues and a nested PCR adapted to a single tube for the internal transcribed spacer (ITS1) region of *T. gondii* was used for the detection of the parasite. Subsequently, the parasite burden of PCR positive placental and fetal samples was estimated by real-time PCR targeting the 529RE fragment.

The analysis of the lesions and changes in resident cell populations of the fetal brains is explained in the Chapter IV, which was performed in collaboration with the Institute of Veterinary Pathology of the University of Zurich (Switzerland) and is aimed to achieve the objective 4 of this PhD thesis. In this study, fetal brains obtained during the Experiment 2 and showing histological lesions consistent with *T. gondii* infection (G1: n=6, G3: n=3) were selected in order to further evaluate and describe the histological characteristics of the lesions. For this purpose, the number of inflammatory foci per square centimeter (No. foci/cm^2) of tissue, the average size of the focus (ASF), and the percentage of brain tissue affected (%LES) was assessed by digital image analysis. Additionally, one section of the brain from 5 fetuses per group, always from the same area of the brain (section C), including midbrain, cerebral hemisphere and lateral ventricles in the same slide, was selected for immunohistochemical evaluation of the cell populations resident in the brain in order to study the possible changes caused by the infection. The cell populations evaluated were astrocytes, macrophages or microglial cells, recently recruited tissue macrophages and neurons. Besides, the occurrence of apoptosis or leukomalacia was also assessed through immunohistochemical labelling using antibodies against caspase-3 and APP (Amyloid Precursor Protein), respectively. The labelling of most of the antigens employed was evaluated through a digitally assisted software (Visiopharm 2020.08.1.8403), although a semiquantitative classification of the frequency of aggregates of microglial cell foci was also performed. This study is further explained in detail in the Chapter IV of the current PhD Thesis.

Ethics statement

Animal handling, blood sampling and euthanasia procedures conducted during this PhD thesis were carried out in accordance with the European Union legislation (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 were authorized by the regional government, Junta de Castilla y León, after a positive report from the ethics committee of the Spanish Research Council (Ref. 100102/ 2018-6 and 1063/2021). All animals were handled by veterinarians in strict accordance with good clinical practices, and all efforts were made to minimize suffering. Animals were monitored for clinical signs of disease throughout the studies.



CHAPTER I

EFFECTS OF OVINE MONOCYTE-DERIVED MACROPHAGE INFECTION BY RECENTLY ISOLATED *Toxoplasma gondii* STRAINS SHOWING DIFFERENT PHENOTYPIC TRAITS.

SUMMARY

Ovine toxoplasmosis is one the most relevant reproductive diseases in sheep. The genetic variability among different *Toxoplasma gondii* isolates is known to be related to different degrees of virulence in mice and humans, but little is known regarding its potential effects in sheep. The aim of this study was to investigate the effect of genetic variability (types II (ToxoDB #1 and #3) and III (#2)) of six recently isolated strains that showed different phenotypic traits both in a normalized mouse model and in ovine trophoblasts, in ovine monocyte-derived macrophages and the subsequent transcript expression of cytokines and iNOS (inducible nitric oxide synthase). The type III isolate (TgShSp24) showed the highest rate of internalization, followed by the type II clonal isolate (TgShSp2), while the type II PRU isolates (TgShSp1, TgShSp3, TgShSp11 and TgShSp16) showed the lowest rates. The type II PRU strains, isolated from abortions, exhibited higher levels of anti-inflammatory cytokines and iNOS than those obtained from the myocardium of chronically infected sheep (type II PRU strains and type III), which had higher levels of pro-inflammatory cytokines. The present results show the existence of significant intra- and inter-genotypic differences in the parasite-macrophage relationship that need to be confirmed in *in vivo* experiments.

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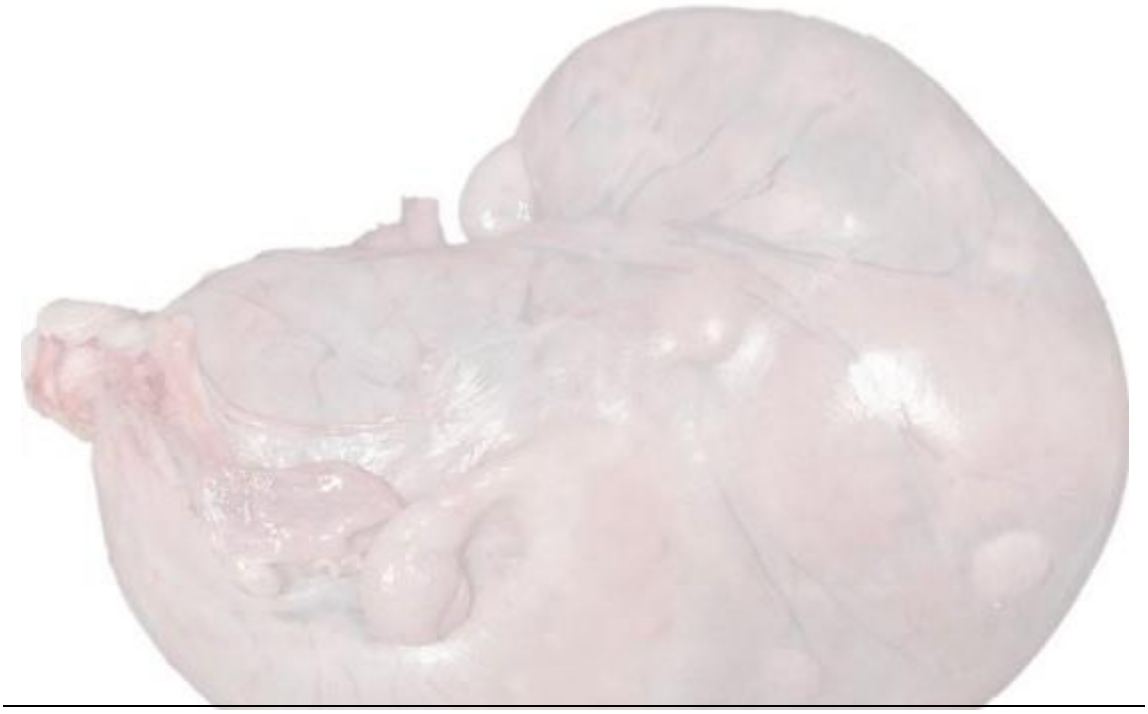


CHAPTER II

OVINE FETAL PLACENTA AND FETUSES. MACROSCOPIC AND HISTOLOGIC PHYSIOLOGICAL FINDINGS AND IMMUNOHISTOCHEMICAL CHARACTERIZATION OF IMMUNE CELL POPULATIONS IN THE PLACENTOMES.

SUMMARY

There is a scarcity of studies addressing normal gross or microscopic findings in ovine placentas and/or fetuses. A detailed knowledge of the structural and physiological complexity of the ovine placenta and its cell populations is essential to better understand the pathogenesis of abortifacient infections. This study is aimed to determine normal morphological findings in placentomes (N=108) and fetuses (N=12) from 12 control ewes from Experiment 2, during second half of gestation through macroscopic and histological examination. In addition, immunohistochemical labelling for macrophages (Iba1, calprotectin), B (CD20) and T cells (CD3), Natural killer (NK) cells and caspase-3 was also evaluated in placentomes. Macroscopically, amniotic plaques (11/12, 92%), allantoic calculus (10/12, 83%) and placentomes with different shapes and sizes (12/12, 100%) were noticed. Histological analysis of the placentomes showed areas of mineralization (39/108, 36%) and scattered foci of cellular debris (25/108, 23%), extravasation of proteinaceous material around vessels (29/108, 27%), hemorrhages (33/108, 31%), thickening of maternal blood vessel walls (108/108,100%) and hyalinization of the terminal portion of maternal crypta (108/108, 100%), among other changes. Immunohistochemical characterization of cell populations showed positive cells to CD20, CD3, Iba1 and calprotectin with a mean of 0.6, 18, 25.4 and 7.3 positive cells per mm^2, respectively, in the interdigitating zone of the placenta, indicating that the infiltration of a moderate number of macrophages and T cells is a normal finding in the placentome. Cellular debris or areas of hyalinization were not immunostained for caspase-3. No Natural killer (NK) cells were found. Regarding the fetuses, hematopoiesis was present in liver and lymph nodes. Lymphocyte infiltrates were present in various organs without association with tissue damage. Our results evinced that placenta and fetuses can show various morphological changes under physiological conditions, which must not be misdiagnosed as lesions.

INTRODUCTION

The placenta has been defined as an apposition or fusion of the fetal membranes to the uterine mucosa for physiological exchange (Mossman, 1991), that can be divided into two components: a fetal part and a maternal part (Burton &

Jauniaux, 2015). This single organ has plenty of functions: respiratory, nutritive, excretory and endocrine (Donnelly & Campling, 2014), besides modulate the local immune response in order to tolerate the “fetal allograft” by the mother, so that pregnancy can be successful (Male, 2021). These particularities, not seen in other organs, the great morphological changes that occur during gestation and the variety of placental types between mammals makes it a difficult organ to study (Wooding & Burton, 2008).

Ovine placenta has been classified as villous and cotiledonary (Pastor-Fernández et al., 2021) which means that the contact surface area is increased by multiple placentomes, composed of a caruncle (maternal part) and a cotyledon (villous projections of the fetal part), forming a round to oval structure (placentome) with concave shape, although occasionally a convex shape can be present, resembling bovine placentomes (Sammin et al., 2009; Morgan-Ortiz et al., 2015). The cotyledon is formed by the fusion of avascular chorion and vascular allantois, with chorioallantoic blood vessels entering the placentome through a depression (the hilus) located in the center of the placentome (Sammin et al., 2009). This hilus on cross section is seen as a red-brow area, named as arcade hematomata, hematomata zone or hemophagous areas, which consists of an accumulation of maternal blood that is thought to have the function of transferring iron to the fetus (Wimsatt, 1950; Burton, 1982). Placentomes weight 9 g on average with a size of 10 mm to more than 50 mm in diameter (Sammin et al., 2009). Normally, there are around 60 to 100 placentomes per sheep placenta that reach their maximum development around day 90 of gestation until delivery (Makowski, 1968; Heasman et al., 1998).

According to the number of layers, ovine placentation is classified as synepitheliochorial, because it is composed of all layers (epitheliochorial) in the intercotyledonary placenta, including endothelium from maternal vessels, maternal connective tissue, maternal epithelial layer, endothelium from fetal vessels, fetal connective tissue and fetal epithelium or trophoblasts (Wimsatt, 1950; Burton et al., 2006). However, in the placentomes, a feto-maternal syncytial layer frequently replaces the maternal epithelial layer (Wooding, 1982). The fetal epithelium or trophoblast layer consists of uninucleate cuboidal cells with occasional binucleated cells that migrate to the maternal component fusing with maternal epithelial cells and

forming feto-maternal hybrid cells, giving rise to the previously mentioned syncytial cells (Wooding, 1984). Moreover, chorioallantoic vessels and binucleated cells from the fetal part increase in size and number as pregnancy advances (Majeed et al., 2012). On the other hand, maternal epithelium or endometrial epithelium extends up to the hilus of each placentome and it consists of columnar uninucleated cells, where occasional lymphocytes are present (intraepithelial lymphocytes). This infiltration of lymphocytes is not found inside the placentome (Segerson et al., 1991).

Reproductive failure is a major global problem for sheep industry affecting both the economy and animal health, caused by a variety of factors, including inadequate nutrition, genetic, toxicity, metabolic, physical or infectious problems (Schlafer et al., 2016). Among them, abortive infectious agents, affecting in multiple occasions only the placenta, are the most commonly diagnosed (Kirkbride, 1993). *Chlamydia abortus*, *Campylobacter sp, Toxoplasma gondii, Listeria monocytogenes, Brucella spp, Coxiella burnetii, Salmonella spp* and Border disease virus (Buxton & Henderson, 1999; Menzies, 2011; Clune et al., 2021; Righi et al., 2021) are the major agents that cause abortions in sheep, including some zoonotic pathogens. These infectious agents cause histological lesions on placentas and fetuses, where they induce degenerative and inflammatory changes (Borel et al., 2014; Meixner et al., 2020; Brom et al., 2021). Pathology is the cornerstone for diagnosing abortions in ruminants. As recently evinced in bovine, physiological findings of the placenta during pregnancy can mimic lesions induced by infections (Botta et al., 2019). Accurate understanding of the macroscopic and histologic features of ovine placenta and fetuses is crucial to properly assess the etiological diagnosis and avoid misdiagnosis. Besides, this knowledge is necessary when experimental studies on the pathogenesis of infectious abortifacient agents are being carried out.

Despite the relevance of the fetal placenta and fetus development in the ovine pregnant model during gestation, there are multiple gaps in the knowledge of their morphological and physiological complexity as few and not recently works can be found in the literature. Similarly, little is known regarding the presence and distribution of the main immune cell populations, so there are still many gaps in the knowledge of the structural and physiological complexity of ovine placenta. For these reasons, the objective of this study is to describe the normal macroscopic and

histological physiological findings in ovine placenta and fetuses in the second half of pregnancy and to characterize the physiological distribution of immune cells and presence of apoptosis within the placentome.

MATERIAL AND METHODS

Animals and Experimental design

Twelve, 14 to 24 months-old age, primiparous pregnant Rasa Aragonesa sheep were selected from a commercial flock without history of any abortive infectious agents, and seronegative for *T. gondii, N. caninum, border disease virus, Schmallenberg virus, Coxiella burnetii*, and *Chlamydia abortus*. The estrus was synchronized, and sheep were mated for 2 days. One month and a half after mating, pregnancy and fetal viability were confirmed by ultrasound scanning housed in the facilities of "Instituto de Ganadería de Montaña" (IGM) (CSIC-University of León), and euthanized at last third of gestation. Sheep were euthanized by intravenous administration of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain) right after sedation by intramuscular administration of xylazine (Bayer, Mannheim, Germany) (Experiment 2).

Collection of samples

A systematic necropsy was performed and all the gross findings were recorded. The uterus was separated from the carcass by coronal section at the cervix. Fetuses were separated from the placenta after longitudinal section of the uterus wall at the horn where the fetus was located and nine placentomes per animal (3 cranial, 3 medial and 3 caudal) were randomly collected from each placenta (n=108). Fetal (n=12) samples included: brain (frontal lobe, corpus callosum, midbrain and cerebellum), tongue, right eye, lung (left dorsal caudal lobe), heart (apex), trachea, esophagus, thymus, thyroid gland, liver (right lobe), right kidney, small (jejunum) and large (colon and caecum) intestine, skeletal muscle (left semitendinous muscle), mesenteric lymph node and haired skin. Tissues were fixed by immersion in 10% buffered formalin for histopathological processing.

Histology and immunohistochemistry

Following formalin fixation for at least 24 hours and no more than 7 days, tissues were trimmed and embedded in paraffin wax. Four µm thick sections were cut, mounted on glass microscope slides and stained with hematoxylin and eosin (H-E). Histochemical special techniques were used in selected slides in order to analyze the presence of ferric pigment (Perls´ Prusian blue), the presence of calcium deposits (Von Kossa) and the type of mucins (Alcian blue and PAS) (Suvarna et al., 2019). Histological slides were studied under an optical microscope by 2 ECVP board certified veterinary pathologists (RV and VP). Color digital images were obtained of representative histological findings (Leica DM2000 LED microscope).

Immunolabeling for T cells (CD3), B cells (CD20), macrophages (CD163, calprotectin and Iba1), NK (NKR1) cells and caspase-3 for apoptosis detection, was performed in 4 µm thick sections of 24 placentomes, mounted on electro charged adhesive gelatin-coated microscope slides (Superfrost^{TM}Plus Adhesion Microcope Slide, Braunschweig, Germany). Data for antibody specificity, dilution and unmasking protocols employed are shown in Table II-1. Briefly, deparaffination, rehydration and epitope retrieval were accomplished in Tris-based solution (PT-Link System, Agilent technologies, Santa Clara, United States). After this, slides were immersed in a 3% H_2O_2 in methanol solution for 30 minutes in darkness at room temperature and then incubated overnight at 4 ^{0}C with the primary antibody diluted in a phosphate-buffered saline in a humidified chamber. Next day, after washing, immunolabeling was performed using a ready-to-use kit EnVision System (Agilent Technologies) where slides were incubated for 40 minutes with the appropriate horseradish peroxidase–conjugated polymer. Finally, the slides were incubated at room temperature with 3,3-diaminobenzidine (DAB; Agilent Technologies), rinsed in tap water, and counterstained with Mayer's hematoxylin for 10 seconds. Positively immunolabelled cells were identified by the presence of brown signal. One investigator (RV) evaluated the presence and distribution of these cell populations and apoptosis in the whole placenta and two investigators (RV and MS) counted the number of clearly labeled cells in 20 randomly selected 200x fields in the interdigitating zone of the placentome. All the measurements were expressed in cells per square millimeter (cells/mm^2).

Table II-1. Details of the antibodies and procedures used for the immunohistochemical examination.

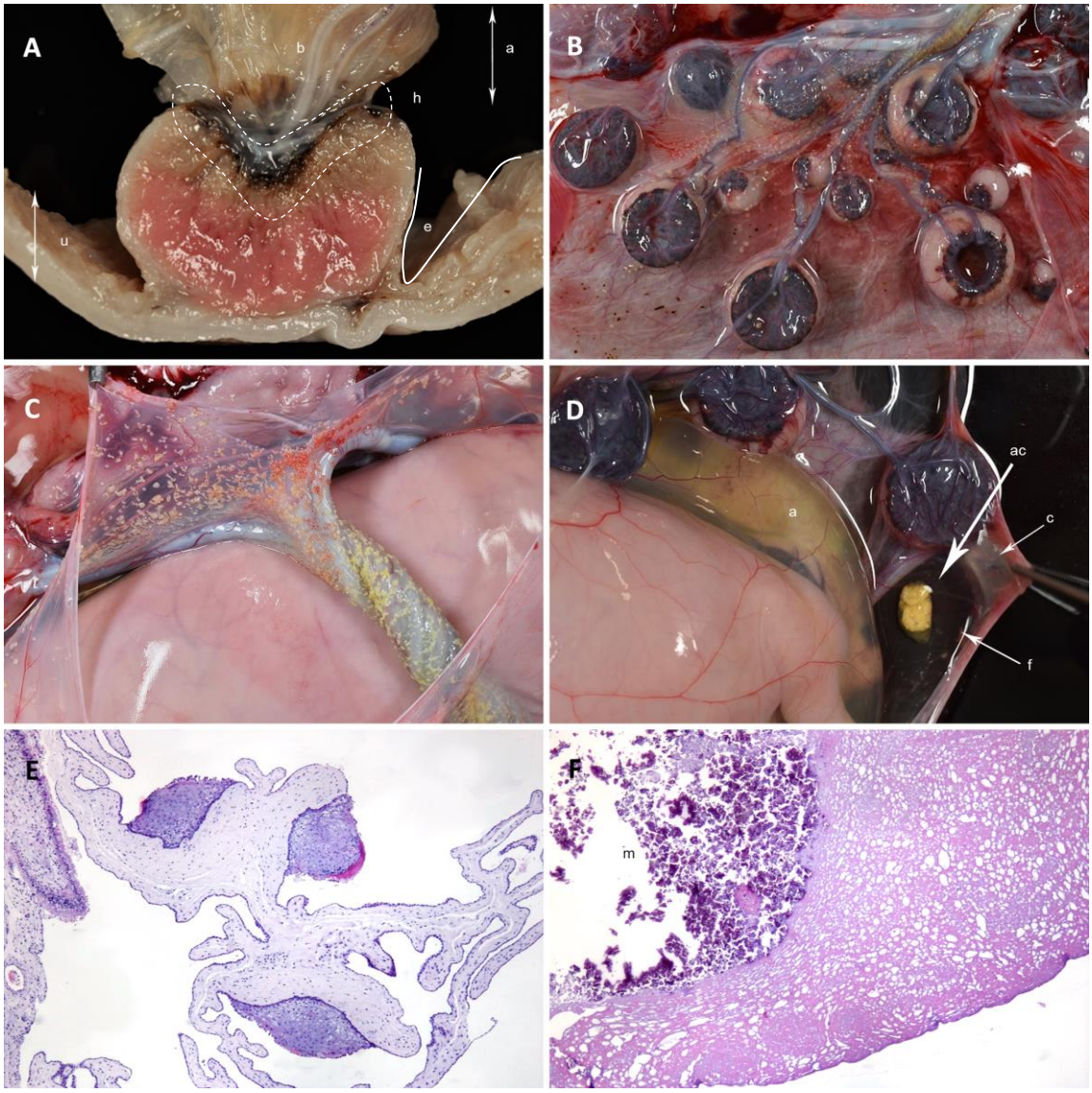
Antibody	Cell populations labelled	Monoclonal/ polyclonal	Antigen retrieval	Primary Antibody/ Dilution	Source
CD3	T cells	Policlonal	HIER, pH6	1:300	Dako
CD20	B cells	Policlonal	-	1:200	Thermo
Calprotectin	Recently recruited tissue macrophages	Monoclonal	HIER, pH9	1:500	Clone MAC387 Genetex
Iba-1	Macrophages	Policlonal	HIER, pH6	1:2000	Wako
Caspase-3	Apoptosis	Policlonal	HIER , pH6	1:250	ABCAM
NKR1	Natural Killer cells	Policlonal	HIER, pH6	1:100	Bioss

HIER:Heat-induced antigen retrieval with ciytrate buffer

RESULTS

Macroscopic findings in ovine placenta

Macroscopically, chorioallantoic membrane from the fetal part and endometrium from the marternal part were juxtaposed and easily separated in the intercotyledonary placenta. In placentomes, chorioallantois was entering the endometrium by the hilus of the caruncula and was firmly attached (Figure II-1A). In all the examined placentas (12/12, 100%), placentomes were asymmetric with different sizes and shapes (Figure II-1B). Indeed, they range among 1 to 4 cm in size and were mostly concave but occasionally flat or convex in shape.



Figures II-1. Normal placenta. Sheep. A. Cross section of a normal placentomes where chorioallantoic fetal membranes (a) with chorioallantoic blood vessels (b) are entering the hilus. Clearly demarcated arcade hematomata (h). The urterus (u) is clearly seen in the intercotyledonary placenta with the endometrium in the inner part that surrounds the plecentome in its external area (e). Formol fixed tissue. **B.** Multiple placentomes viewed from the fetal side with marked different shape (convex and concave) and sizes. Chorioallantoic blood vessels going inside placentomes are also present. **C and E**. Amniotic plaques, fetal membranes, second half of pregancy. Sheep. **C.** Macroscopic view of the amnion from the inside, were amniotic plaques are usually present mainly adjacent to the umbilical cord. **D.** Microscopic view of three amniotic plaques, where the normal unicellular epithelium of the amnions is replaced by a keratinized or non-keratinized squamous epithelium. 40x. H-E. **D and F.** Allantoic calculus, fetal membranes. second half of pregnancy. Sheep. **D.** Macrocopic view of an allantoic calculus (ac) in the allantoic fluid (f), located between the amnios (a) and the chorioallantoic (c) membranes. **F.** Microcopic view of an allantoic calculus with a mineralized basophilic center (m). 100x. H-E.

Fetal membranes were differentiated and evaluated in each placenta, with the chorioallantoic membrane in the external part and the amnion or amniotic membrane in direct contact with the fetus. Amniotic plaques were found in the inner part of the amniotic membranes in 92% (11/12) of the examined placentas (Figure II-1C). They were characterized by yellow hairy flocculent material, moderately hard and were found mainly adjacent to the umbilical cord but also in the rest of the membrane surface. In 83% of the placentas (10/12) there was a mucoproteinaceus yellow material, consistent with allantoic calculus with hard (Figure II-1D) or gelatinous consistence, floating in the allantoic fluid, which is filling the allantoic cavity between the amniotic and the chorioallantoic membranes, or attached to the chorioallantoic fetal membrane. To a greater or lesser extent, in all the placentas the amniotic fluid filling the amniotic cavity and in direct contact with the fetus had floccules of meconium of brown to green color, granular appearance and different sizes. When it was abundant, it gave the skin and hair of fetuses a yellowish discoloration. Regarding ovine fetuses, no significant macroscopic findings were observed.

Histological findings in ovine placenta

1. Placental membranes and intercotyledonary zone.

Amniotic plaques were characterized by multifocal areas of keratinized or non-keratinized squamous epithelium (Figure II-1E), where the normal cuboidal to flattened epithelium of the amnion showed squamous metaplasia. Allantoic calculi were composed of an eosinophilic homogeneous mucoproteinaceus material which frequently had a basophilic central area of mineralization, consistent with the greater consistency found at gross examination (Figure II-1F).

In the intercotiledonary placenta, no remarkable findings were observed. Maternal and fetal placenta were not attached and the separation between both was clearly seen.

2. Placentomes.

In the evaluation of histologic sections of placentomes taken from pregnant ewes, a typical synepitheliochorial placental structure, highly vascularized, was present. Regarding the hematomata arcade (Figure II-2A), where pool of extravasated maternal blood was present, two main findings were observed in all placentomes (108/108, 100%). First, caruncular septa distal terminal tips, with loss of contact with the base of fetal villi and in contact with blood from the arcade hematomata, showed loss of cellular architecture replaced by a brightly eosinophilic (hyalinized) material admixed with abundant hemorrhages and pyknotic and karyorrhectic cells (Figure II-2B). On the other hand, in all placentas (108/108, 100%), the cytoplasm of trophoblasts in direct contact with the extravasated blood in the arcade hematoma, was filled and expanded by erythrocytes, eosinophilic globular proteins and aggregates of golden-brown granular to globular pigment (Figure II-2C). This pigment was negative to Perls´ Prusian blue stain and produced a non-specific stain in every immunohistochemistry performed (Figure II-2D).

Regarding the interdigitating zone (Figure II-2A), the mother and fetal component were separated by a clear space, probably an artifact due to the histoprocessing technique. Multifocal mineralization, without any damage or inflammatory infiltration in relation to these it, was found in 36% (39/108) of the placentomes, affecting maternal and fetal parts indistinctly and ranging from very small basophilic foci to large multifocal foci of mineralization occupying large part of the tissue (Figure II-2E). In those sheep showing this change, mineralization usually appeared in several placentomes, affecting from one up to the nine placentomes studied. The presence of calcium salts was confirmed with Von Kossa stain (Figure II-2E-inset). Protein extravasation was seen in 27% (29/108) of placentomes, characterized by the presence of globular eosinophilic protein droplets in the cytoplasm of trophoblasts, frequently near the vessels (Figure II-2F) but also without association to visible vascular structures (Figure II-2G). Thickening of the blood vessels wall at maternal part was observed in all placentomes (108/108, 100%) (Figure II-2F and 2H), affecting a large proportion of these in each placentome. Extravasation of erythrocytes (hemorrhages), mainly between the fetal and maternal villi in the

interdigitating zone, was present in 31% (33/108) of placentomes with erytrophagocytosis in nearby trophoblasts. In 23% (25/108) of placentomes, small foci of cellular debris were observed (Figure II-2I), not associated with inflammatory cells.

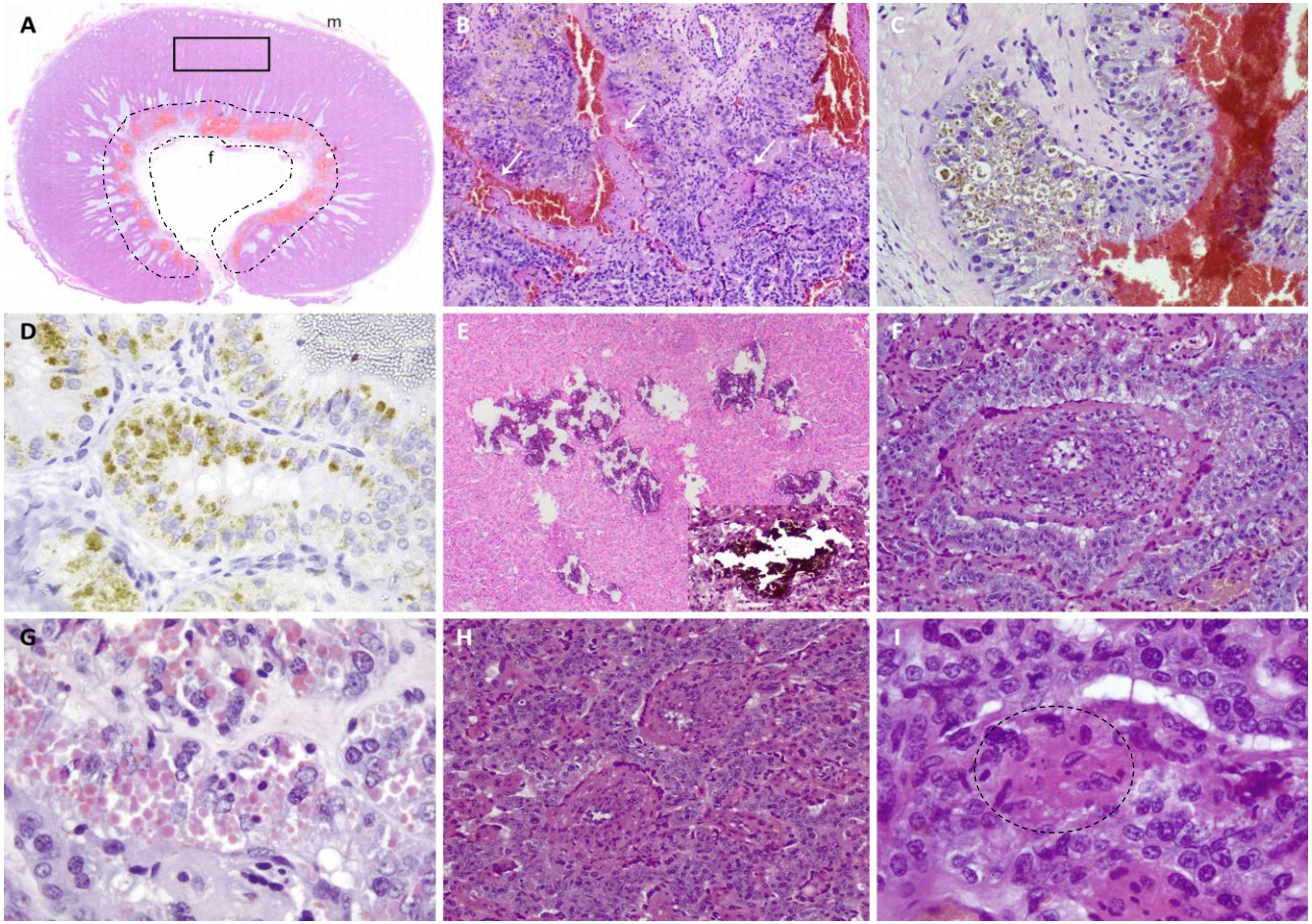


Figure II-2. Normal placentome. Sheep. **A.** Section of a normal placentome where the hematomata arcade or hematophagous area is surrounded by a dotted line, characterized by the presence of a pool of blood form the mother. Fetal (f) and maternal (m) sides of the placentome. Interdigitating zone (inside de square). Whole slide image. H-E. **B-D.** Microscopic normal findings in the arcade hematomata. Placentome. Sheep. Second half of pregnancy. **B.** Caruncular septa with hyalinized ends and nuclear pyknosis and karyorrhexis (white arrows). 100x. H-E. **C.** Presence of erythrocytes and aggregates of golden-brown granular to globular pigment of different sizes in the cytoplasm of trophoblasts. 200x. H-E. **D.** Presence of pigment in the trophoblasts of the hematomata arcade. 400x. CD3 IHC. **E-I.** Microscopic normal findings in the interdigitating zone. Placentome. Sheep. Second half of pregnancy. **E.** Mineralization. Areas of multifocal basophilic mineralization, affecting maternal and fetal parts indistinctly. 40x. H-E. Inset: Positive von Kossa staining (black areas). **F.** Maternal blood vessel with a thickened wall surrounded by extravasation of proteins inside fetal trophoblasts. 400x. H-E. **G.** Protein extravasation, characterized by the presence of globular eosinophilic proteins in the cytoplasm of trophoblasts (arrow heads). 600x. H-E. **H.** Thickening of the wall of two maternal blood vessels. 400x. H-E. **I.** Small foci of cellular debris. 600x. H-E.

Immune cell populations in the placentome and presence of apoptosis

Immunostaining of the placentomes showed a high number of lymphocytes positively immunolabelled for CD3 antibody (T cells) among the epithelial cells of in the endometrium and the endometrial glands (Figure II-3A). Scattered T cells, never grouped in aggregates, were found in the interdigitating zone (Mean of 17.96 positively immunostained cells per mm²). Contrarily, round cells positively immunolabelled for CD20 antibody (B cells) were nearly absent in the whole placetome including the interdigitation zone, with only 0.60 positively immunolabelled cells per mm² at the latter location. Regarding macrophages, Iba1 positive cells were more numerous than lymphocytes in all areas of the placentome. There were 25.36 cells per mm² positive for Iba1 in the interdigitating zone. Iba1 positive macrophages were most commonly seen at the base of the placentome (hilus) infiltrating the fetal chorioallantoic connective tissue, both scattered or in aggregates. In the caruncula there was also abundant numbers of positive macrophages intermixed with the connective tissue. Interestingly, all trophoblasts presented a membranous positive immunolabeling for Iba1 (Figure II-3B). The number of cells expressing calprotectin (i.e. positive labelling for MAC387) was lower than those Iba1+, with a count of 7.34 cells per mm². The entire placentome lacked any NK cell positive labeling. Moreover, no caspase-3 positive labelling was detected in the interdigitating zone or fetal chorioallantoic connective tissue. Only epithelial cells in the maternal side of the placentome (endometrium) was seen immunolabelled with this antibody.

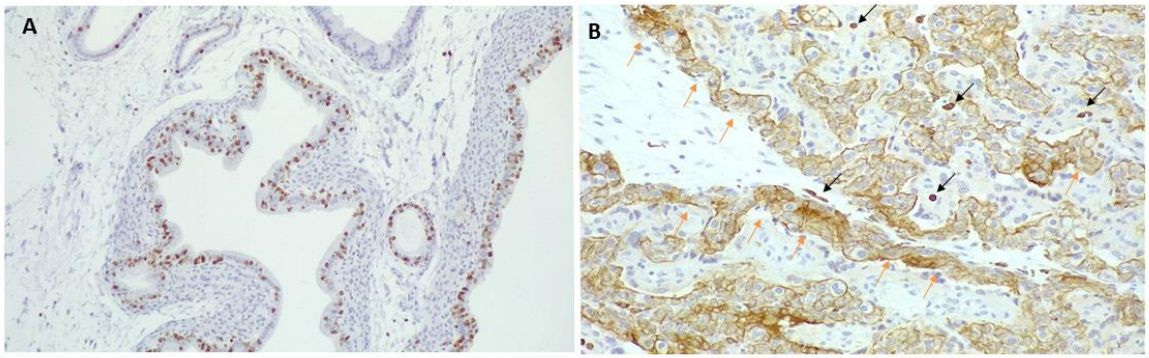


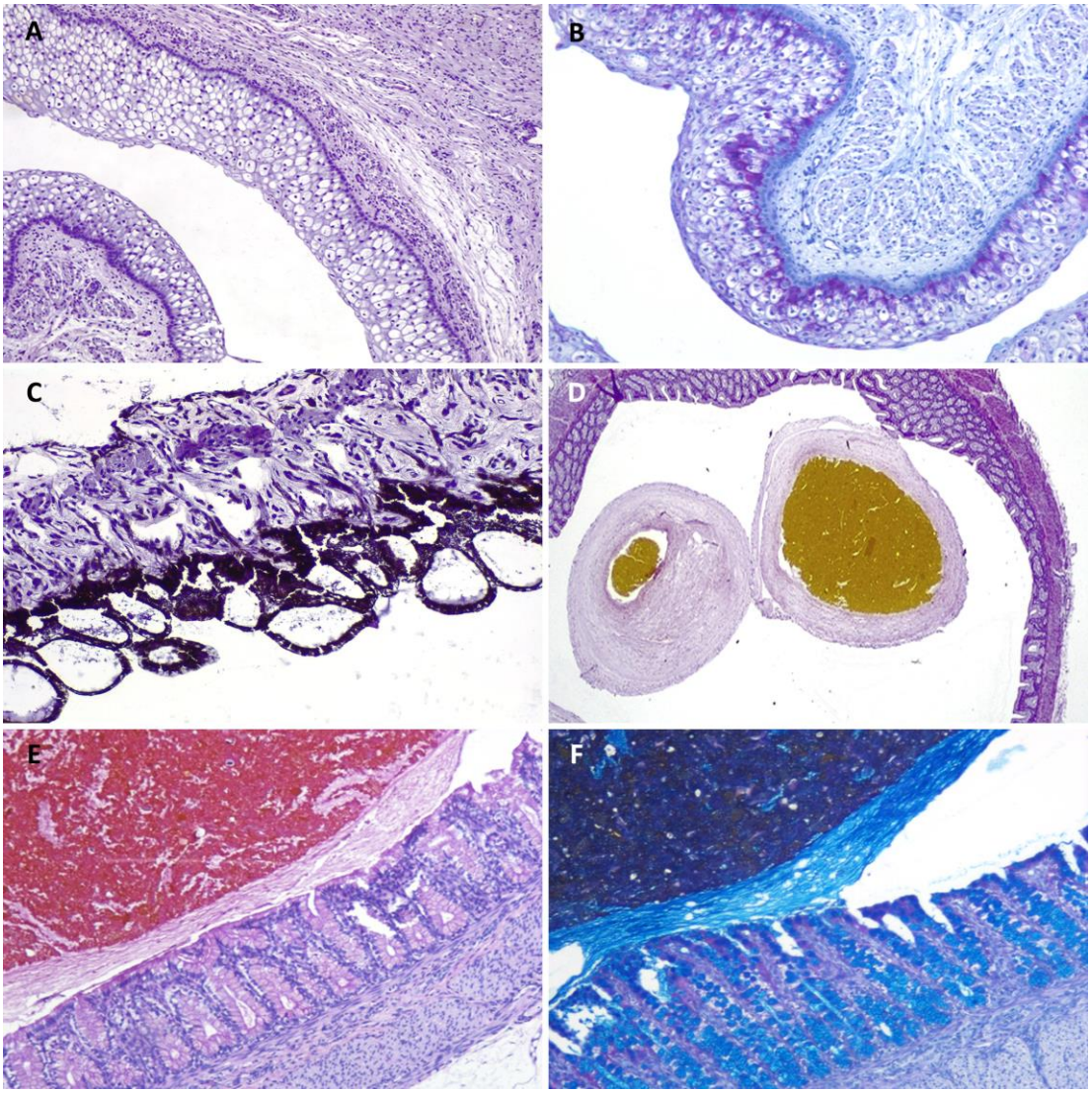
Figure II-3. Ovine placentome, normal pregnancy. Immunohistochemistry. **A.** Maternal endometrium. Small number of CD3 (T cells) immunolabeled cells in the maternal connective tissue and high numbers of intraepithelial immunolabeled cells in the endometrial epithelium and endometrial glands.100x. CD3 IHC **B.** Membranous and cytoplasmic intense immunolabeling for Iba1 in macrophages (black arrows) and membranous moderate inmmunolabeling for Iba1 in trophoblasts (orange arrows). 200x. Iba1 IHC.

Histological normal findings in ovine fetuses

All fetuses (12/12, 100%) showed marked hematopoiesis in the liver, seen as the presence of high number of erythroid precursors, moderate number of myeloid cells and scarce megakaryocytes, located in the sinusoids. Hematopoiesis was also observed in the medullar area of the examined lymph node in a 33% of cases (4/12). Cytoplasmic vacuolation of epithelial cells was observed in all fetuses (12/12, 100%) maily affecting the corneal endothelium of the eye, esophageal mucosa (Figure II-4A), upper half of the small intestine epithelium, urothelium and epithelial cells of the kidney (mostly in collecting and proximal convoluted tubules). Most of these cells show no staining with Periodic Acid-Schiff, except for the presence of scant purple granules detected in the cytoplasm of epithelial cells near the basal membrane of the esophagus (Figure II-4B) and urothelium. Regarding the eye, one quarter of the fetuses (3/12) showed multiple cysts in the pigmented epithelium of the iris (Figure II-4C) of different shapes and sizes, lined by a pigmented cuboidal to flattened epithelium. These cysts were empty or with a granular content formed by cellular debris. Luminal content in large intestine of all fetuses (12/12, 100%) was characterized by a central green-brown part that correspond to biliary pigments with a frequently eosinophilic proteinaceous material surrounding it, admixed with cellular debris and scattered red blood cells (Figure II-4D), which corresponds to the gelatinous appearance of this content at macroscopic examination. This material was PAS negative (Figure II-4F) and Alcian blue positive (Figure II-4G). Interestingly, all goblet cells in the large intestine presented an intense blue staining positive to Alcian blue (Figure II-4G). In the 8% (1/12) of the fetuses, intestinal crypt ectasia was found. Finally, small foci of mononuclear cells, consistent also with lymphocytes, were found in multiple organs (Table II-2): in the connective tissue below the epithelium of the tongue mainly near circumvallate or fungiform papillae, in the connective tissue of the thyroid gland between follicles, in the liver mainly next to portal areas, between cardiomyocytes or myocytes in the endomysium or perimysium of the cardiac and skeletal muscle and in the tracheal epithelium.

Table II-2. Percentage of fetuses and lambs in which the presence of small foci of mononuclear cells has been observed in different organs.

Foci of mononuclear cells	Fetuses
Tongue	92%
Liver	25%
Thyroid gland	42%
Cardiac muscle	8%
Skeletal muscle	25%
Tracheal epithelium	100%



Figures II-4. Microscopic normal findings in ovine fetal tissues. A. Esophagus with vacuolation of the epithelium. 40x. H-E. **B.** Esophagus section presenting purple positive granules only in the cytoplasm of epithelial cells near the basal membrane. 100x. PAS. **C.** Eye, multiple cysts in the pigmented epithelium of the iris. 400x. H-E. **D.** Large intestine with intraluminal content characterized by a brown-green central area (biliary pigment) with a pale eosinophilic proteinaceous material surrounding it. 40x. H-E. **E.** Large intestine. Intraluminal material exterior part is negative to PAS stain. 200x. PAS. **F.** Intraluminal material exterior part is positive to Alcian blue stain. 200x. Alcian blue-PAS.

DISCUSSION

Since the first morphological description of sheep placenta, studies investigating the normal histology, the presence of immune cell populations or the assessment of apoptosis in this organ have been scarce (Wimsatt, 1950; Boshier, 1969; Sammin et al., 2009), despite the importance that reproductive disorders affecting the placenta have in the ovine species. Therefore, to differentiate what is a normal macroscopical or histological finding in ovine placenta and fetuses can be of great utility when investigating cases of abortion or developing experimental studies on abortive agents. It has been stated that ovine placenta reaches its maximum development around 90 days of gestation due to the expansion of fetal villi and capillary vessels (Sammin et al., 2009). Consequently, as the sheep from Experiment 2 selected to carry out this study had more than 90 days of gesation all the placentomes were fully grown to optimally assess all the placentome characteristics.

Regarding macroscopic changes in ovine placenta, one of the main findings was the observation of allantoic calculi. They can be compared to hippomanes, also found in the allantoic fluid of pregnant horses and zebra mainly after 85 days post gestation (King, 1967; Ginther, 2022). Histological appearance of hippomanes is similar to what we found in our study, an eosinophilic amorphous material. Nevertheless, in the center of these structures, it has been reported that in hippomanes mineralization is not present and only a mass of cells admixed with ghost cells can be observed (King, 1967), while in sheep, central mineralization was frequently found. A possible origin of this mineralization could be a dystrophic calcification over death tissue coming from desquamation. Moreover, allantoic calculi in sheep are smaller than hippomanes similar to what was described by King in 1967, as white flecks of tissue debris forming a sediment (early allantoic fluid sediment) that appear in horses before hippomanes are properly formed. A possible origin of this structures could be the desquamation from fetal membranes, which provides a nucleus of tissue debris and subsequent formation of allantoic calculi by concentric deposition of this material, as it has previously been stated in horses (Dickerson et al., 1967). This type of structure has also been reported in lemurs but in the yolk sac (Hamlett, 1935), in impalas (Wilsher et al., 2020), in cows and in (Nasr & Moustafa, 1988).

On the other hand, the amniotic plaques frequently detected in sheep placentas of this study, have an unknown function and are commonly reported in ruminants (Pourlis et al., 2008; Simões & Stilwell, 2021) where they have also been called amnnotic papillae (Turner & Garlick, 1978). This finding has been also described in deer (Sinha et al., 1970), umbilical cord of horses (Da Silva et al., 2021a) and other herbivorous species (Foster, 2017). Amniotic plaques were much more frequent around umbilical cord, which could let us think that this squamous metaplasia occurs due to the friction of the amnion with the fetus transforming a monolayered epithelium into a stratified one. The high frequency of occurrence of amniotic plaques in the placentas of this study, makes them a useful tool for the identification of the amnion during the necropsy of sheep. It is important not to misdiagnose these macroscopic findings with macroscopic lesions, such us deposition of fibrin, necrosis or proliferative lesions, caused by abortifacient agents (Caspe et al., 2020). For instance, a recent study in water buffalos stated that squamous papillomas caused by papillomavirus should be differentiated from amniotic plaques in the amnion. Macroscopic lesions caused by papillomavirus are larger with filiform shapes and microscopically supported by a thin fibrovascular connective tissue which is absent in the amniotic plaques (Russo et al., 2020). Meconium appears when ovine fetuses defecate and it has been associated with fetal stress in other ruminants (Schlafer et al., 2000) or fetal compromise in humans (Khalil et al., 2015; Steer et al., 2022), where it can give rise to meconium aspiration syndrome, a serious fetal condition in humans (Mitchell & Chandraharan, 2018). Finally, the changes in placentomes shape and size of this study where previously reported in sheep, not by necropsy evaluation but by magnetic resonance imaging (Flouri et al., 2021). Changes in shape from concave to convex, the typical shape in bovine placentomes (Carter, 2019), have been associated in sheep with functional adaptation in order to maintain normal nutrient delivery to fetuses during maternal nutrient restriction (Vonnahme et al., 2006; Longo, 2018), indicating also an increase in placentome size and capillarity but a reduction in the number of placentomes in sheep at high altitudes with restricted oxygen supply (Parraguez et al., 2006).

Regarding histological evaluation of placentas, changes were only detected in placentomes, and not in the intercotyledonary placenta, probably because this is the

place where maternal and fetal components interdigitate and where the exchange of nutrients and gases takes place. The arcade hematomata, characterized by the presence of extravasated blood, is thought to be a mechanism of iron absorption by extravasation of maternal blood and consequent phagocytosis by trophoblasts in cattle, sheep, goats and even dogs and cats (Oliveira et al., 2012). Several authors suggest that this covers part of the iron needs that fetuses have in the placenta (Lawn et al., 1969; Myagkaya et al., 1984), but do not contribute significantly to maternal-fetal exchange of nutrients (Oymans et al., 2020). In sheep, as it has been shown in this study, extravasated maternal blood occupies extensive areas, whereas in other ruminants, maternal erythrocytes are immediately phagocytized by adjacent trophoblasts and accumulated inside their cytoplasm (Oliveira et al., 2012). The existence of this type of trophoblasts, showing intracytoplasmatic erythrocytes, was also a common finding in all the placentomas of this study, in agreement with what was found in dwarf goats from 45 to 140 days of gestation (Igwebuike & Ezeasor, 2012) and also in other ruminants, in which case they have been called “phagocytic trophoblast cells” (Schlafer et al., 2000). These type of cells have also been found in the chorioallantois overlaying endometrial glands of the intercotiledonary areas of the cow placenta (Schlafer et al., 2000), but not in our study.

All placentomes in this study presented an intracytoplasmatic golden-brown pigment in the trophoblasts located in this area that is supposed to be a product of hemoglobin metabolism after erythrocyte phagocytosis. When Prussian Blue staining was applied, a negative result was obtained proving that iron was not present in this pigment. Accumulation of non-ferruginous pigment inside the cytoplasm of trophoblasts in arcade hematoma was previously described in other ruminants (Oliveira et al., 2012), including buffalos (Pereira et al., 2010), while only one study in buffalos showed that those granules were positive for hemosiderin (Ranjan et al., 2012). However, there are not reports in small ruminants were this pigment was Prussian Blue positive, except for one study in caprine trophoblasts that confirmed the presence of hemosiderin (Santos et al., 1996). A previous study in sheep, using electron microscopy, demonstrated that the pigment was formed by hemoglobin digestion lysosomes containing hemoglobin-derived pigments and highly electron-dense, acid phosphatase positive, residual bodies (Myagkaya & Schellens, 1981).

Considering this fact, and the absence of positive reaction to Prussian Blue staining in this study and in many others (Sammin et al., 2009), a possible explanation for this phenomenon could be that, in this study, placentomas were examined at a late gestation period, and so the iron from the phagocyted erythrocytes could be liberated from hemoglobin in a ferrous form that cannot be detected with ferrocyanide methods (Myagkaya et al., 1984). The presence of large amounts of throphoblasts containing this brown intracytoplasmatic pigment has led to the fact that the arcade hematomata is a very difficult area to be evaluated using routine immunohistochemical techniques, due to the background brown stating that can be mistaken with positive immunolabelling specially when diaminobenzidine is used as substrate for horseradish peroxidase. Therefore, the area normally used for the assessment of lesions and immune cell populations in placentomes is the interdigitating zone (Figure II-2A) (Castaño et al., 2019). As an alternative, other chromogen substrate, leading to colors different to brown-goldenish, may be used to develop the immunolabelling.

The hyalinization of the distal ends of caruncular septa found in this study was previously reported in ovine placentomes (Sammin et al., 2009). These eosinophilic areas with pyknotic and karyorrhectic nuclei could be cause by the presence of death cells. This cell death is most probable not apoptosis as the morphology is not compatible with apoptotic cells and it has been proven in this study to be negative to caspase-3. This death cells could be originated by degeneration of cells by necrosis induced by vascular changes, probably causing hypoxia, occurring only in the terminal part of maternal septa that is in direct contact with the extravasated blood of the arcade hematomata, as it is believed that blood in this zone is resultant from the maternal capillaries opening to the surface (Jorkman, 1965), and it may be arising from erosion of these maternal distal ends. It is important not to mistake this hyalinization of maternal septa for necrosis caused by infectious diseases (Palmer et al., 1996), as this hyalinization is focal, small and only located in maternal ends, free of adjacent fetal tissue, whereas infectious agents produce variable sized necrotic foci, randomly located and usually admixed with inflammatory cells.

Hemorrhages present in the interdigitating zone of placentomes in our study were not described before in normal sheep placenta and they have only been related to fetal death in ovine clones (Fletcher et al., 2007). It is important to differentiate

them from those hemorrhages associated with necrosis of trophoblastic epithelial cells or with infiltrates of inflammatory cells, that have been reported in different studies caused by infectious agents such as bluetongue virus (Van Der Sluijs et al., 2011), rift valley fever virus (Oymans et al., 2020) or border disease virus (Barlow, 1972). In the same way, the presence of small foci of cellular debris seen in a 25% of the ovine placentomes in this study, which had been previously reported in bovine placentomes (Botta et al., 2019) but not in sheep, should not be mistaken for small foci of necrosis, mainly in experimental studies with low infective doses of abortive agents, like in *T. gondii,* where foci of necrosis could be mild (Chapter III). The cellular debris detected in this study was negative to caspase-3, contrary to what was seen in placentomes of cows, were this cellular debris was positive to caspase 3 and cleaved laminin A (Botta et al., 2019). This could mean that cellular debris in ovine placentomes has not the same origin as in cows and it could be produced by other forms of cell death that do not imply caspase-3 in their process, such us necrosis (Santagostino et al., 2021), or that apoptosis is in a stage negative to caspase-3, although could be positive for initiator caspases, such as caspase-9. Further investigation needs to be done to state where this cell debris in ovine placentomes comes from. Thickening of maternal part blood vessels and extravasation of protein globules had not been reported before in ruminant placentas, only thickening of fetal chorioalantoic vessels in sheep placentomes has been described (Sammin et al., 2009). Furthermore, mineralization of placentomes was also described in an exhaustive study in cows showing similar percentage of affected placentomes (35%) (Botta et al., 2019) similarly to the frequency of this finding in our study in sheep (i.e. 36%). Where these foci were formed by calcium salts deposits, without evidence of dead or degenerated cells, although dystrophic calcification seems to be the most likely mechanisms explaining this finding.

Regarding the immunohistochemistochemical characterization of the placentomes, Iba1 positive cells were the most frequently found in the interdigitating zone and witin the mesenchymal tissue of the fetus. They are most probably resident macrophages as only a few of these cells were positive to calprotetin which stains recently recruited macrophages (Chilosi et al., 1990; Soulas et al., 2011). Placental macrophages in humans have been reported to be composed of two different

populations named as decidual macrophages and hofbauer cells (Mezouar et al., 2021). These macrophages would be important in the defense against placental pathogens but may also play an important role in the transmission of infectious agents (Thomas et al., 2021). An expansion of this cell population (Iba1+ calprotectin-) within the fetal mesenchyme has been observed after infection with *T. gondii* in pregnant sheep (Castaño et al., 2020), which support their relevance in the local immune response against abortofacent pathogens. It has been stated that in bovine placenta at parturition, macrophages mainly shown a pro-inflammatory M1 polarization (Hooshmandabbasi et al., 2021). The fact that macrophages found in this study are M1 or M2, requires further investigation. T cells were much more common in the epithelium of the endometrium and endometrial glands of the placenta than in the interdigitating zone. Besides, B cells were almost inexistent compared to T cells. A plausible explanation for this could be that T cells are, together with macrophages, the main cell populations against frequently found abortive intracellular agents (Castaño et al., 2020). These T cells could be also associated with the immunosuppressive environment in the placenta for the mother to tolerate the semi-allogenic fetus, as T cells have been associated with restriction of allorecognition (Erkers et al., 2017). Even though ovine placenta as a non-invasive synepitheliochorial placentation, it has been stated to require a less intense immunoregulation compared to humans and rodents (Wattegedera et al., 2019). Absence of NK cells in sheep placenta, is a key finding also reported in ovine uterine wall, placental membranes and placentomes at term (Wattegedera et al., 2019). NK cells are one of the most important cell populations in the placenta of human and rodents for the regulation of angiogenesis and immune response (Parham & Moffett, 2013) and for the control of abortive agents (Shmeleva & Colucci, 2021), being the most prevalent leukocyte population during early pregnancy (Renaud et al., 2017). However, NK are seem not to be involved in ovine pregnancy. This statement is supported by the lack of NK cells in *T. gondii* infected ovine placentomes whereas in other species NK are of great importance for its pathogenesis (Castaño et al., 2019). This underlines the difference between placental types and the need for caution when extrapolating results across species. Apoptosis has been reported in the endometrium of humans by electron microscopy and caspase-3 evaluation (Wood & Levison, 1976) and it has been reported to be more common in

the third trimester of pregnancy (Halperin et al., 2000). In our study caspase-3 positive cells were present only in the endometrium surrounding the ovine placentomes at second half of pregnancy. This finding seem to be due to the continuous remodelling and renewal of this tissue, as apoptosis has been reported to be essential for the normal physiology of pregnancy in humans (Meresman et al., 2010) and ruminants (Martins et al., 2004). However, in other ruminants, in contrast to our study, apoptotic cells were also present in fetal villous trophoblasts detected by TUNEL (JiangFeng et al., 2011). Other techniques for the detection of apoptosis should be performed in order to understand which is the role of apoptosis in sheep placenta.

Regarding histological findings in fetuses, vacuolation of epithelial cells seen in corneal, kidney, intestine and esophagus epithelium of fetuses had not been described previously to the authors' knowledge. Only in the case of the small intestine, neonatal pigs have shown vacuolation in the upper half of the small intestine where these cells were called "vacuolated fetal-type enterocytes", which gradually disappeared 21 days after birth (Moon, 1972; Skrzypek et al., 2007). Fetuses from rats (Mathan et al., 1976) and cows showed also this vacuolated cells, that were replaced by normal epithelial cells one week after birth (Asari et al., 1987). While the cytoplasm of these vacuolated cells in the small intestine has been reported to give a positive reaction with the PAS method, in our study this only occurred in the basal layers of the esophagus, but not in the rest of the organs, suggesting that the amount of polysaccharides (probably glycogen) is low, or that they may contain another substance that could not be identified by the histochemical methods employed and would need further investigation. Anyway, this vacuolation should be distinguished from degenerative changes in these cells.

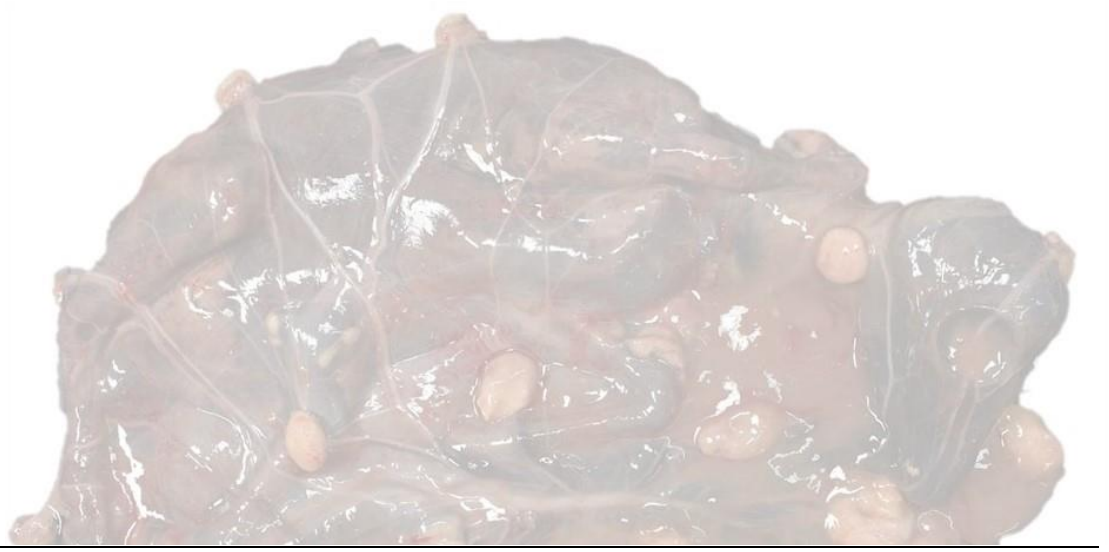
Another interesting finding was that all the globet cells from the large intestine were positive to Alcian blue stain, indicating that they contain acidic mucins. Normally in the large intestine of sheep fetuses, there is a high amount of acid mucins but also a low amount of neutral mucins (Özbek et al., 2018). However, in this study basic mucins were not present. A recent study in sheep fetuses, stated that acidic mucins may contribute to the strength of the intestinal barrier against pathogens and digestive enzymes, and described that large intestine was mainly composed of acidic mucins that consisted predominantly of acidic sulphomucins before the crypts structure was

formed and acid sialomucins after the crypts were developed (Özbek et al., 2018). Moreover, the characteristic features of the large intestinal content present in the lumen, composed of biliary pigments in the center and an external eosinophilic Alcian blue positive material, is consistent with meconium surrounded by acid mucins. This intestinal content was classified as organic content of 95% of dry weight in previous studies in ovine fetuses (Sherman et al., 1996). The external eosinophilic acidic proteinaceous material should not be mistaken with a fibrinous exudate in the fetal intestine.

Regarding foci of cells morphologically consistent with lymphocytes, found in different fetal organs, they should not be mistaken with an inflammatory infiltrate induced by an infectious agent. Fetuses in this work are becoming immunocompetent (Buxton & Henderson, 1999), which means their immune system is increasing the number of circulating lymphocytes (Sammin et al., 2009), that could be accumulated in different organs, probably acquiring protective functions against unknown antigens to the fetal immune system or just as tertiary lymphoid tissues.

CONCLUSION

In conclusion, this work describes the physiological changes in the ovine placenta and fetuses at second half of gestation, which must not be misdiagnosed with mild lesions such as small foci of necrosis, presence of fibrin or mild inflammation. Mineralization of the placentome and squamous metaplasia of the amnion are physiological changes. Infiltration with a moderate number of macrophages and T; minimal numbers of B cells and absence of NK cells is normal in the interdigitating zone of the healthy placentome. In fetuses, the existence of hematopoiesis, epithelial vacuolation and small foci of inflammatory cells are normal common findings. A complete understanding of the physiological changes that could be found in non-infected placenta or fetuses is crucial for the diagnosis and research of abortifacient conditions in sheep.



CHAPTER III

EXPERIMENTAL INFECTION OF SHEEP AT MID-PREGNANCY WITH ARCHETYPAL TYPE II AND TYPE III *Toxoplasma gondii* ISOLATES EXHIBITED DIFFERENT PHENOTYPIC TRAITS.

SUMMARY

Toxoplasma gondii is a major cause of reproductive failure in small ruminants. Genotypic diversity of *T. gondii* isolates 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 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, fetal dead at the time of euthanasia or stillbirth were similar among infected groups (50%, 40% and 47%, respectively). Histological lesions in placentomes and fetal 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 fetuses 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 isolates variability could influence the outcome of the infection and mechanisms responsible for it.

INTRODUCTION

Toxoplasmosis, caused by *T. 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 described in experimental infections and might be underdiagnosed in the field as, contrary to late abortions, neither parasite in the placenta or fetal 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). *T. gondii* isolates have been traditionally classified into three different clonal lineages according to their cumulative mortality in murine models [highly virulent (type I), intermediate virulent (type II) and non-virulent (type III)] (Howe & 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., 2020; 2021; Largo-de la Torre et al., 2022; Vallejo et al., 2022 or Chapter I). 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., 2019a).

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.

MATERIALS AND METHODS

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., 2020). 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 III-1 (Fernández-Escobar, et al., 2021; Vallejo et al., 2022 or Chapter I).

Table III-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 fetal brain	Adult myocardium of chronic infected sheep	Adult myocardium of chronic infected sheep
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

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 (Müller 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.

Experimental design

A total of 73, 14 to 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 fetal 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., 2019a). Two different approaches were conducted within the same study aiming at studying the outcome of the infection as well as characterising the lesions and the parasite tissue distribution and burden in maternal and fetal 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.

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 at the day of 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, fetuses were separated from the placenta, and 9 placentomes (3 cranial, 3 medial and 3 caudal) were randomly retrieved from each placenta. Placentomes showing whitish discoloration and generalised autolysis adjacent to mummified fetuses, 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 −20ºC for *T. gondii* DNA detection (Castaño et al., 2016).

Fetal 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, also the heart). Fetuses were considered as infected if at least one out of the three analyzed organs (brain, liver and heart) tested positive by PCR. Fetal brains were first fixed in 90% ethanol and 10% formaldehyde, that gave them a firmer consistency, and after 48 hours in 10% neutral buffered formalin solution for another 48 hours (Gutiérrez-Expósito et al., 2020a). In those fetuses 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 fetuses either early or late abortions.

Histological processing and APP immunohistochemistry

After formalin fixation, placentomes and fetal tissues were trimmed and conventionally processed for embedding in paraffin wax (Castaño et al., 2014; Sánchez-Sánchez et al., 2019a). Specifically, from each fetal brain, 4 different sections

were processed (frontal lobe, corpus callosum, midbrain and cerebellum) as described previously (Gutiérrez-Expósito et al., 2020a). Histological sections were stained with hematoxylin 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. Amyloid precursor protein (APP) monoclonal antibody (clone 2cc11; Millipore) was used for labelling necrotic areas in the white matter (i.e., leukomalacia) as previously described (Gutiérrez-Expósito et al., 2020a). 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 minutes 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 minutes and with 3,3-diaminobenzidine (DAB; Agilent Technologies) for 5 minutes was performed, producing brown signal. Finally, slides were rinsed in tap water and counterstained with Mayer's hematoxylin for 10 seconds.

DNA extraction and PCR procedures for parasite detection and quantification

T. gondii detection was studied in 9 placentomes of each sheep, lymph nodes and fetal (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 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.

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 fetal 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 fetal fluids was considered a positive reaction.

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 fetuses, 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 analyzed 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 $p<0.05$.

RESULTS

Clinical observations

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 (Figure III-1A). 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) (Figure III-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.

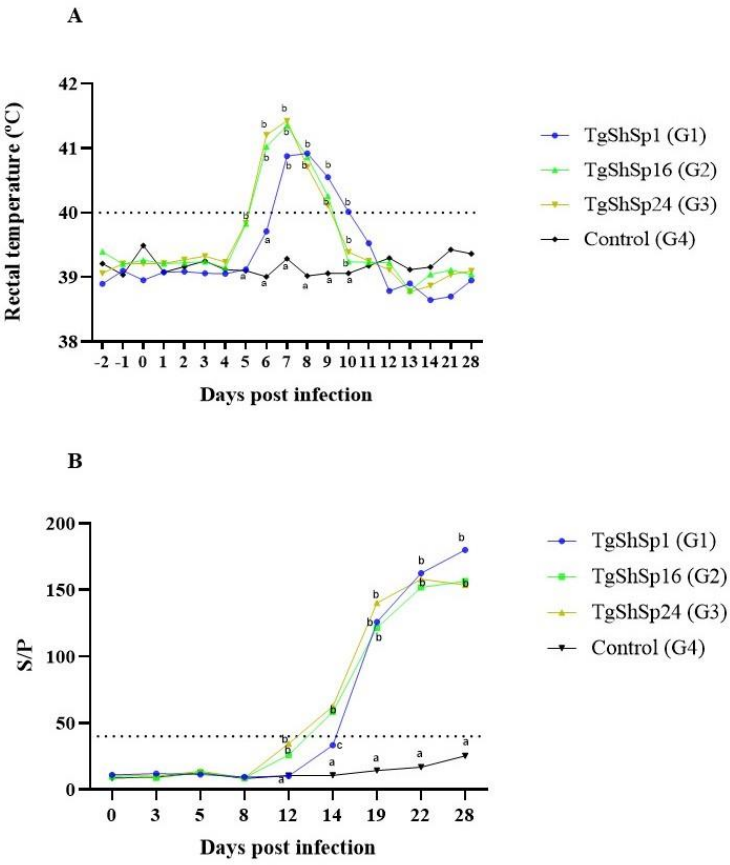


Fig 1. Clinical observations in G1 (TgShSp1), G2 (TgShSp16), G3 (TgShSp24) and G4 (control) groups following *Toxoplasma gondii* orally infection with 10 sporulated oocysts of each isolate. **A.** Mean rectal temperature. **B.** Dynamics of antibody levels. Each point represents the mean value at the 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) ($p \leq 0.05$).

2. Reproductive losses.

Outcome for the pregnancy has been summarised in Table III-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).

Table III-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 eutanasia*		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

*Number of sheep with at last one dead fetus/total euthanized sheep

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

fetuses 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 fetuses 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 fetuses at 14 dpi and 2 sheep had dead fetuses at 28 dpi) (Table III-2).

Anti-*T. 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) (Figure III-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 fetuses from the serial euthanasia, only one fetus from a sheep of G1 euthanized at 14 dpi was seropositive by IFAT with a titer of 1:64, whereas all fetuses of G2, G3 and G4 were seronegative. At 28 dpi three out of eight fetuses from two sheep of G1 (38%) were seropositive with IFAT titers that ranged from 1:64 to 1:128 and seven out of nine fetuses from the five sheep of G3 (78%) were seropositive with IFAT titers ranging from 1:64 to 1:512. All fetuses of G2 and G4 were seronegative at 28 dpi (Table III-3).

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

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

Proportions (n/N): number of placentomes, lymph nodes or fetal tissues showing lesions or *Toxplasma gondii*-DNA positive samples among the total number of tested samples; [a] All fetal tissues are included. [b] Two fetuses 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.

Pathology and lesions

1. Gross lesions.

Early and lately aborted fetuses 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 (Figure III-S1A).

Regarding the findings during serial euthanasia, three fetuses from G2 and two fetuses from G3 were found dead at 14 dpi, and two fetuses from each group were dead at 28 dpi. These dead fetuses showed different macroscopic changes, from subcutaneous oedema, suggesting a recent death, to those with advanced autolysis and mummification. Two fetuses from G1 and one fetus 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 fetus at the time of euthanasia showed macroscopic changes, denoted by multifocal white pinpoint foci in the interdigitate area of the placentome (Figure III-S1B). No lesions were found in the non-infected control animals.

1. Microscopic lesions.

a. 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 fetal 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) (Figure III-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 fetal connective tissue (Figure III-2B).

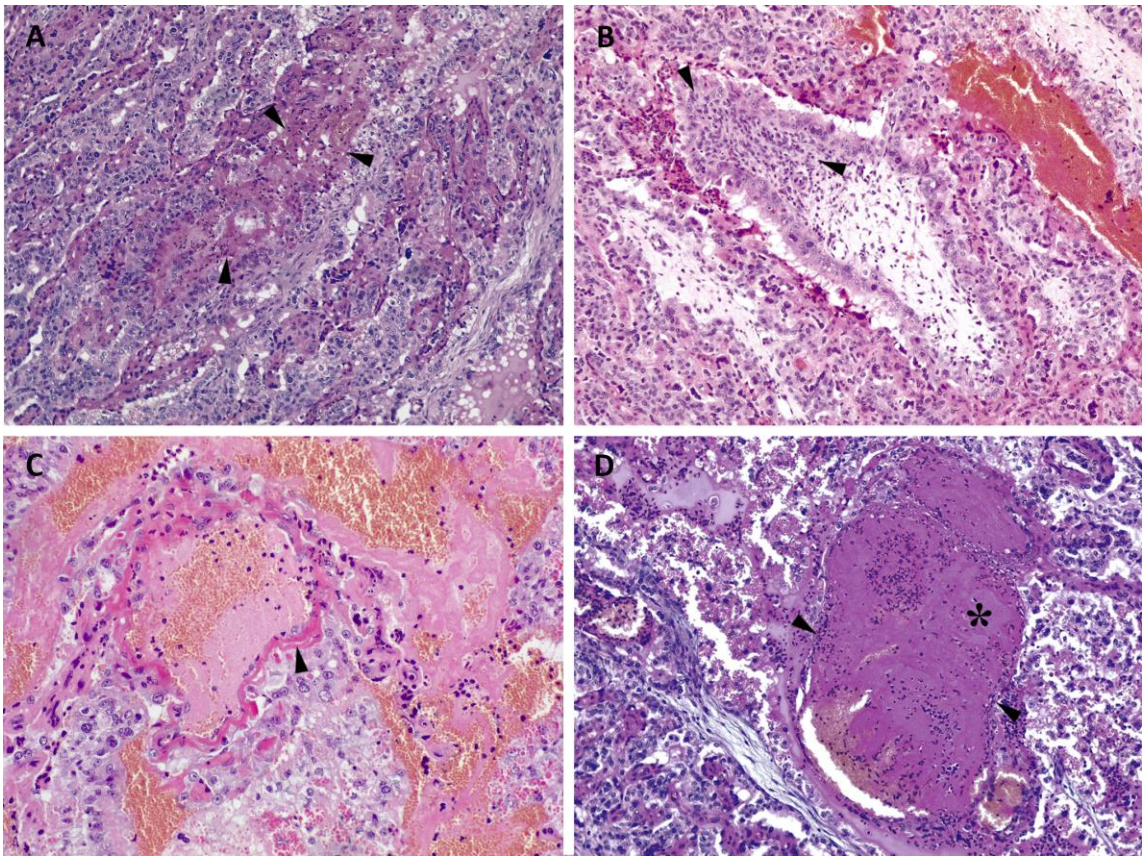


Figure III-2. Microscopic lesions in the placenta of sheep at 28 days post infection. A. Foci of necrosis at the interdigitate area of the placentome (necrotizing placentitis) from a sheep infected with TgShSp1 isolate characterized by homogeneous eosinophilic material admixed with numerous degenerated cells and karyorrhectic debris (arrowheads). 100x. H-E. **B.** Hypercellularity at the mesenchymal tissue of a fetal 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. 200x. H-E. **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 hemorrhages and eosinophilic globular proteinaceous material. 400x. H-E. **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). 200x. H-E.

A placentome was considered damaged by *T. gondii* when any of above types of lesions were found. Microscopic lesions are summarized in Table III-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 fetuses 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 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) (Figure III-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) (Figure II-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 iliofemoral 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).

b. Fetuses and lambs

Samples of the fetuses from early abortion showed variable degree of autolysis and oedema. APP accumulation at the white matter denoting leukomalacia (Figure III-3A) was detected in all fetuses from G1 (11/11, 100%), in seven out of eight of G2 (87.5%) and all fetuses 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).

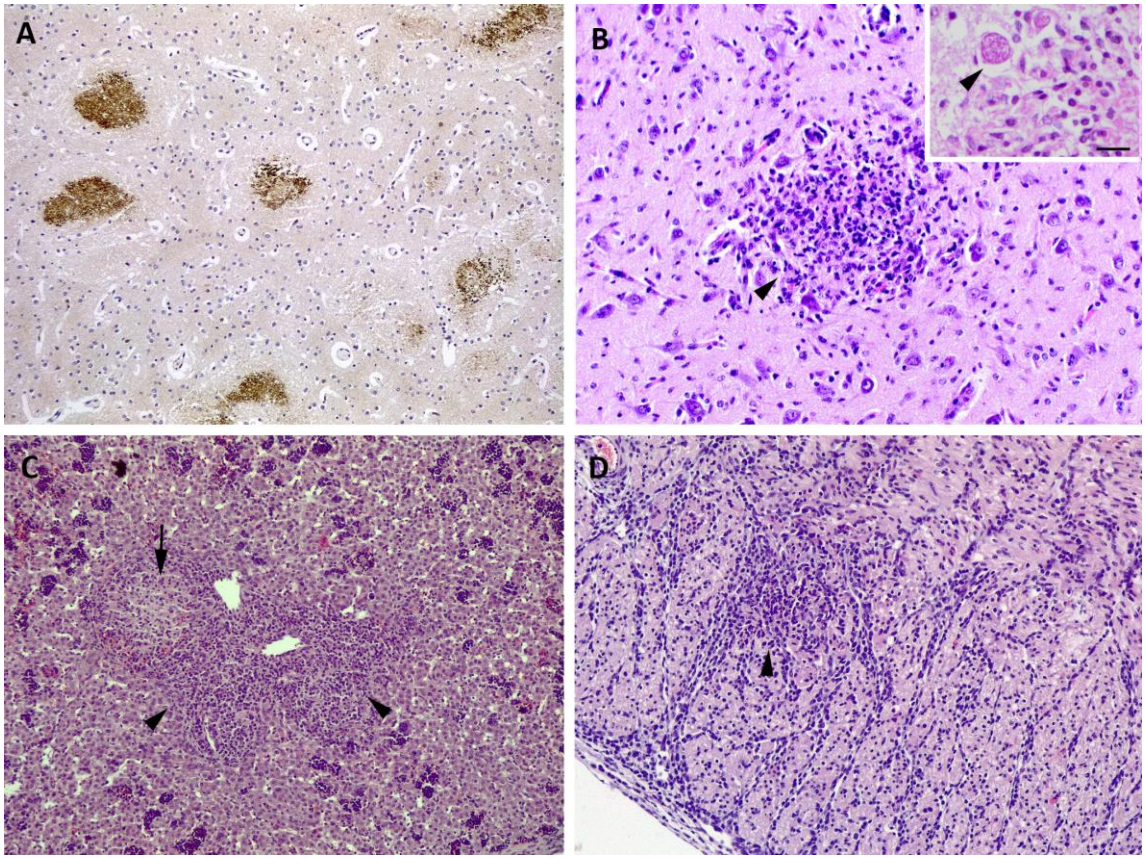


Figure III-3. Microscopic lesions found in infected fetuses. A. Fetal brain from an early abortion from G1. Multiple areas of white matter necrosis (leukomalacia) labelled in brown signal. 100x. APP IHC. H-E. B-D: Microscopic lesions in fetuses from sheep euthanized at 28 days post infection (G1). **B.** Brain. Foci of gliosis. Notice the absence of necrosis. H-E. 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). 400x. H-E. **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 hematopoiesis is present throughout the organ. 100x. H-E. **D.** Heart. Mononuclear inflammatory cells (arrowheads) are infiltrating, separating and effacing cardiomyocytes (myocarditis) affecting the pericardium (pericarditis). 100x. H-E.

Regarding the fetuses from the serial euthanasia at 14 and 28 dpi, those collected at 14 dpi showed no lesions. The degree of autolysis of two fetuses from G1 and one fetus from G3 at 28 dpi, all of them with gross lesions suggesting fetal death before euthanasia, hampered a proper histological and PCR evaluation. In the rest of the fetuses, the lesions were characterised by multifocal foci of gliosis (Figure III-3B), some of them with central area of necrosis. Tissue cysts were occasionally observed in the brain with crescent shaped basophilic bradyzoites (Figure III-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) (Figure III-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 (Figure III-3D).

Fetuses 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 fetuses with lesions (6/9; 67%) (Table III-3); moreover, lesions were more severe, in terms of frequency, in G1 compared with G2 and G3 (p<0.01). In the brain, six fetuses from four sheep of G1 (6/8, 75%) showed multifocal encephalitis. In contrast, in G3 only three fetuses from the same sheep had lesions in the brain (3/9, 33%) (Table III-3). Scant tissue cysts containing bradyzoite-like structures were seen in the brain of one fetus from G1 and other from G3. Neither microscopic lesions nor tissue cysts were found in any of fetal brain of G2. Again, G1 had more severe lesions in the liver than G2 or G3 (p<0.05), as all fetuses of G1 had lesions in the liver (8/8; 100%), whereas in G3 they were found in four fetuses belonging to four different sheep (4/9, 44.4%) and two fetuses from G2 had lesions (2/10, 20%). Similarly, there were significant differences regarding the lesions in the heart, since seven out of 10 fetuses (70%) of G1 presented inflammatory cells infiltrating the myocardium and, occasionally, the pericardium (7/8, 87.5%) and no lesions were seen in fetuses 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 fetuses from G1 (4/8, 50%) and

two from G3 (2/9, 22%) showed non-purulent myositis. Finally, the lungs of four fetuses 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 fetuses were found in the same sheep (i.e. siblings), none of the fetuses 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 fetuses 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 III-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.

Parasite detection and quantification

All placentomes and lymph nodes of the control group as well as all tissue samples from early aborted fetuses 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 III-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 all iliofemoral lymph nodes were negative. All samples from fetuses of infected sheep (G1, G2 and G3) euthanized at 14 dpi resulted negative including controls animals (Table III-3).

In the second serial euthanasia at 28 dpi, *T. gondii*-DNA was detected in placentomes and fetuses 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 III-3). In contrast, three out of five 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 III-3). Regarding parasite detection in fetuses, all fetuses in G1 (8/8; 100%) and eight out of nine in G3 (87%) resulted positive. Moreover, 75% of fetuses from G1 and 55% from G3 had more than one positive tissue sample. In contrast, only two out of 10 fetuses, 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 fetuses 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 fetuses from G1 (6/8, 75%), five fetuses from G3 (5/9, 55%) and no *T. gondii*-DNA was detected in fetal livers from G2 (p<0.05). Finally, cardiac muscle resulted positive in six fetuses 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) (Figure III-4). Regarding fetal organs, G1 had the highest parasite burden in all three organs analyzed whereas G3 had lower parasite burdens having significant differences in the brain with G1 (Figure III-4) (p<0.05). Brain samples of fetuses from G2 showed the lowest parasite burden (11.9 tachyzoites/mg of DNA) (Fig 4).

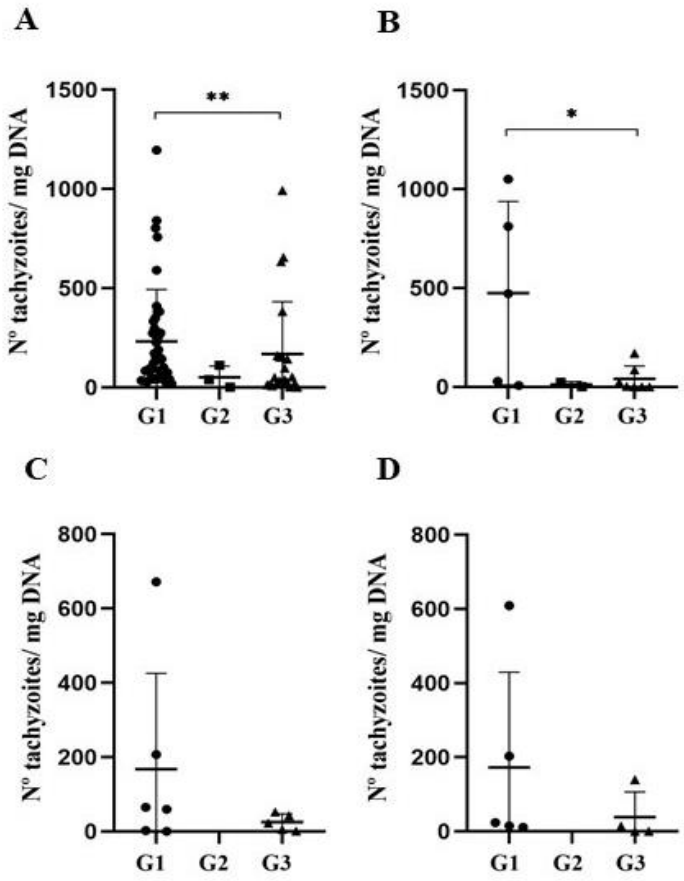


Figure III-4. Dot-plot graphs of *Toxoplasma gondii* burdens at 28 days post-infection in placental and fetal tissues from sheep infected with *Toxoplasma gondii* oocysts of each isolate. A. Placentomes. **B.** Fetal brain. **C.** Fetal liver. **D.** Fetal heart. Each dot represents individual values 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.

Regarding parasite detection in fetuses aborted later than 28 dpi, stillbirths or lambs DNA-*T. gondii* was detected in all fetuses 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 III-4). The frequency of parasite detection in G2 was significantly lower than in G1 and G3 (p<0.001) (Table III-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 III-4), with statistically significant differences between G1 and G2 (p<0.05). These fetuses 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 III-4), with statistically significant differences between G1 and G2 (p<0.0001).

Table III-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 *71.4%*	14/14 *100%*	4/14 *28.6%*	14/14 *100%*	9/14 *64.2%*	4/14 *28.6%*	4/14 *28.6%*
TgShSp16 (G2)	3/9 *33.3%*	5/9 *55.5%*	4/9 *44.4%*	2/9 *22.2%*	2/9 *22.2%*	5/9 *55.5%*	0/9 *0%*
TgShSp24 (G3)	2/3 *66.7%*	2/3 *66.7%*	0/3 *0%*	2/3 *66.7%*	0/3 *0.0%*	2/3 *66.7%*	0/3 *0%*
Control (G4)	0/10 *0%*	0/10 *0%*	0/10 *0%*	0/10 *0%*	0/10 *0%*	0/10 *0%*	0/10 *0%*

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

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 & 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 et al., 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., 2019a). 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 fetal 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., 2019a). 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 (Harmer et al., 1996; Howe et al., 1996; Jerome et al., 1998; Sánchez-Sánchez et al., 2019a). The influence of the number of passages might explain why microscopic lesions found in the 14 and 28 dpi placentas and fetuses 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., 2019a). 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., 2020a).

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., 2022a). 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., 2019a). 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 fetuses 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. (2020) 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 (Dubey et al., 2020a). 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 & Innes, 1998; Castaño et al., 2019; Sánchez-Sánchez et al., 2019a). 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 3-10 dpi in sheep (Dubey & Sharma, 1980; Esteban-Redondo et al., 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-fetal 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 fetuses 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 fetuses (Rassouli et al., 2013; Nunes et al., 2017), was the organ that showed the higher differences between isolates, where lesions were found in most fetuses 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., 2019a). 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., 2022 or Chapter I). 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 fetal 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 fetuses; or even preventing it from occurring as there were sheep and fetuses 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 fetal 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.

CONCLUSION

This study shows that *T. gondii* infection of sheep at 90 days of gestation 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, ToxoDB#3) was more pathogenic if frequency of lesions and dissemination and parasite loads are considered, followed by TgShSp24

isolate (type III, ToxoDB#2). By contrast, TgShSp16 isolate (type II, ToxoDB#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.

SUPLEMENTARY INFORMATION

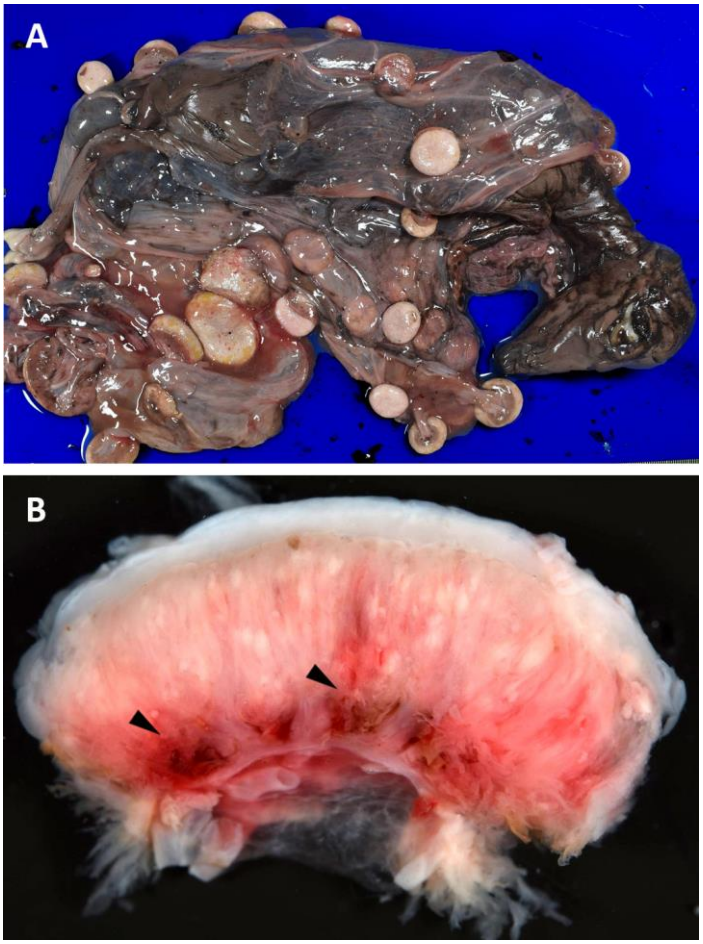


Figure III-S1. Macroscopic lesions of the fetuses and the placenta. A. Aborted fetus showing an advanced degree of mummification with edematous placenta and necrotic/autolytic placentomes. **B.** Multifocal whitish foci of necrosis throughout the placentome. The red areas located at the lower side of the placentome (arrowheads) corresponds to blood accumulation at the physiological hematomata arcade.

CHAPTER IV

CHARACTERIZATION OF HISTOLOGICAL CHANGES IN OVINE FETAL BRAINS AFTER INFECTION WITH DIFFERENT *Toxoplasma gondii* ISOLATES.

SUMMARY

Toxoplasma gondii infection is one of the main causes of abortion in small ruminants, triggering important economic losses in this industry. When infecting the fetus, it can produce necrotic and inflammatory lesions in a wide variety of locations, where the brain is the main, and most frequently affected organ. However, the pathogenesis of this lesions is still poorly understood. Experimental studies in laboratory animals have also described that *T. gondii* infection could modify the resident cell populations of the brain, but how these results could be extrapolated to sheep is not known. The aim of the current study was to characterize histological lesions in the brain of vertically infected ovine fetuses, and also the changes cell populations resident in the brain. Besides, studies in murine experimental models have shown that the characteristics of these lesions could be different depending on the variability of *T. gondii* isolates. Thus, this study also evaluates the influence of variation in the phenotypic traits of *T.* gondii isolates over the lesions and resident cells in fetal brains. For this, three different isolates were used as infectious inoculum, TgShSp1 (genotype II-PRU, ToxoDB#3), TgShSp16 (genotype II-PRUToxoDB#3) and TgShSp24 (genotype III, ToxoDB#2).

Pregnant sheep were orally infected with 10 oocysts of each isolate at 90 days of gestation and other group was left non infected as negative controls. Those fetal brains showing histological lesions consistent with ovine toxoplasmosis were further characterized by digital analysis of hematoxylin/eosin stained sections. Immunohistochemical labelling of neurons, astrocytes, microglial cells/macrophages and recently recruited macrophages, together with the detection of caspase-3-mediated apoptosis and axonal damage, was also carried out in the midbrain level of the brain of five fetuses per group.

The histological examination showed more severe lesions (larger and higher number of glial foci), in those fetuses infected with TgShSp1 than on those infected with TgShSp24, while the ones infected with TgShSp16 did not shown any lesion. Furthermore, the fetuses infected with TgShSp1, showed astrocytosis and microgliosis together with fewer neurons and more apoptotic events than fetuses infected with TgShSp16 and negative controls. These results showed that the phenotypic variations

between in *T. gondii* isolates could influence the severity of the lesions and histological changes in fetal brains. Besides, microgliosis and, to a lesser, extent astrogliosis, were the main findings in affected brains.

INTRODUCTION

Ovine toxoplasmosis is a zoonotic disease of sheep caused by the protozoan parasite *T. gondii*, giving rise to reproductive failure, including fetal absorption, abortions or birth of weak lambs; causing important economic losses worldwide (Stelzer et al., 2019). Abortions have two clinical presentations: early abortion, when they occur between 7 to 14 dpi (Owen et al., 1998; Benavides et al., 2017) or late abortion, from 28 dpi onwards (Buxton et al., 2007). Vertical transmission to the fetus during pregnancy causes characteristic histopathological lesions in fetal brains, which vary depending on the clinical presentation: in early abortions leukomalacia is the only histopathological lesion present (Castaño et al., 2014), whereas in late abortions, fetal brains show multifocal foci of gliosis with or without central necrosis (Buxton et al., 2007). Additionally, non-suppurative leptomeningitis extending into the submeningeal parenchyma or parasite tissue cysts with bradyzoites inside can also be found (O´Donovan et al., 2012). This damage to the brain could be sensed by the peripheral immune response, that overcomes the blood brain barrier (BBB) and leads to the infiltration of the neuroparenchyma by inflammatory cells (Sasai & Yamamoto, 2019). Besides, the resident cells in the brain, including astrocytes, are essential in the control of the BBB and synapse homeostasis (Blackburn et al., 2009), and microglial cells, with phagocytic functions as well as production of cytokines (Wake et al., 2011), also reply to this aggression. Both cells populations have been shown to exert protective functions against *T. gondii* infection in human and mice (Chao et al., 1994; Still et al., 2020).

Previous studies have found that infection with different isolates of *T. gondii* could be associated with variations in the histological lesions of the host and these variations could be used as a phenotypic marker to characterize isolates (Calero-Bernal et al., 2022). Traditionally, the virulence of the three main genotypic groups of isolates of *T. gondii* had been characterized regarding cumulative mortality in mice as: highly virulent for type I, moderately virulent for type II and nonvirulent for type III (Hunter et

al., 1995; Dumètre et al., 2006). However, this virulence characterization is controversial, as nowadays there are differences between isolates of the same genotype (Taniguchi et al., 2018; Sánchez-Sánchez et al., 2019a; Fernández Escobar, 2021; Vallejo et al., 2022; Chapter III). Furthermore, there are many other genetic and phenotypic markers studied in *in vitro* or *in vivo* models that can be used to characterize isolates virulence (Calero-Bernal et al., 2022).

There is scant information regarding the cell populations associated to nervous lesions in ovine toxoplasmosis, especially regarding the role of resident cells of the central nervous system (CNS). Although these populations have been evaluated in other experimental models, such as mice, the extrapolation of results between species is not always possible (Taniguchi et al., 2018; Sánchez-Sánchez et al., 2019a). Moreover, there are still many knowledge gaps on the pathogenesis of toxoplasmosis and the relation between the genetic background of the infecting isolate and histological lesions it causes.

For these reasons, the aim of the present study was to better characterize the histological lesions and changes in the resident cells of the brains of ovine fetuses vertically infected with three recently obtained *T. gondii* isolates showing phenotypic variations. This would contribute to the better understanding of the pathogenesis of ovine toxoplasmosis and how inter- and intra-genotypic variability of *T. gondii* isolates could influence on it.

MATERIAL AND METHODS

Origin of samples

All samples from the current study were obtained from the archive generated in a previous study focused on the clinical outcome of ovine toxoplasmosis after infection with three different isolates of *T. gondii* (Experiment 2). Briefly, the experimental design of that study was as follows.

The isolates selected were: TgShSp1 (genotype II PRU, ToxoDB#3); TgShSp16 (genotype II PRU, ToxoDB#3) and TgShSp24 (genotype III, TodoDB#2). These isolates have been characterized in previous studies carried out in ovine trophoblasts and mice (Fernández-Escobar et al., 2021), in piglets (Largo-de la Torre et al., 2022), in ovine

macropaghes (Vallejo et al., 2022 or Chapter I) and pregnnat sheep (Chapter III). The summarized results are as follows: TgShSp1 showed a mortality rate of 0% and mild clinical sings in murine models, whereas in pregnant sheep model was the isolate with the highest frequency of histological lesions in placenta and fetuses, parasite detection and parasite burden, compared with TgShSp16 that showed the lower frequency of histological lesions and parasite detection. TgShSp16 and TgShSp24 isolates showed mortality rates around 20% in murine models, together with increased clinical sings and higher parasite burden. Besides, TgShSp24 in piglets had higher parasite burdens than TgShSp1. In swine and sheep, TgShSp24 showed an earlier dissemination and higher percentage of early abortions in the pregnant ovine model. In spite of all these differences in sheep, clinical outcome was similar among the three isolates regarding total fetal mortality.

Regarding the infection of the fetuses analyzed in the current study, 10 sporulated oocyts of each isolate were orally inoculated to pregnant sheep of the Rasa aragonesa breed at day 90 of gestation. The ewes were between 12 and 24 months old and were selected from a flock without previous history of toxoplasmosis and seronegative for *T. gondii* or other abortifacient agents. The sheep were grouped according to the isolate used for the inoculation: Group 1, 20 animals (G1: infected with TgShSp1 (Type II, ToxoDB#3), Group 2, 20 animals (G2) infected with TgShSp16 (Type II, ToxoDB#3), Group 3, 19 animals (G3) infected with TgShSp24 (Type III, ToxoDB#4) and Group 4, 14 animals (G4) of non-infected control sheep. From each group, 5 ewes were euthanized at 14 dpi and another 5 at 28 dpi, the remaining sheep were left as control of gestation. Expanded description of the experimental design is detailed in General material and methods and Chapter III.

Fetal brains

Fetal brains from those sheep were first fixed in 90% alcohol and 10% buffered formaldehyde for 48 hours, in order to obtain a firmer consistency of the organ, and afterwards in 10% neutral buffered formalin solution for another 48 hours. Histological examination of the brain samples showed lesions consistent with *T. gondii* infection only in fetuses euthanized at 28 dpi, but not all the fetuses showed lesions: six fetuses from G1 (6/8), none from G2 (0/10) and three from G3 (3/9). Only fetal brains with

lesions were chosen for further histological evaluation in hematoxylin and eosin (H-E) in this study. It should be noted, as it has influence on the results from the current study, specifically in the histological characterization of the lesions, that in G3, one of the three studied fetuses showed macroscopic changes suggesting a recent death at the time of euthanasia.

In addition, one section of the brain from five fetuses per group (G1 n=5, G2 n=5, G3 n=5, G4 n=5) was selected for immunohistochemical characterization. The same area at the level of the mesencephalon was taken from all the cases in order to homogenized the comparisons. For this selection, firstly the brains showing lesions consistent with toxoplasmosis were chosen (n=5 in G1 and n=3 in G3), then, as the number of selected samples per group samples was below 5 in G3 and G2, fetal brains with no lesions were selected in order to complete 5 samples per group (n=5 in G2 and n=2 in G3). In G3, the fetus that showed macroscopic changes suggesting recent death before euthanasia of the mother was not considered for immunohistochemical labelling of slides. Individual data of these fetuses are shown in Table IV-S1.

Histological processing and digitally-assisted evaluation of histological lesions

For the digital analysis of the histological lesions, as has been previously stated four areas from each brain were selected in those 9 fetuses that showed encephalitis (n=6 in G1 and n=3 in G3) at the histological examination during the previous study (Chapter III). These areas included frontal lobe (sample labelled as A), carpus callosum (B), midbrain (C) and cerebellum (D) as it is shown in Figure IV-S1. Samples were processed for paraffin-embedded tissue blocks and orientated into the cassette so the rostral face of the sample was exposed in the paraffin block. Four micrometer (µm) sections from these blocks were cut, mounted in glass slides and stained with H-E. None of the collected brains showed macroscopic lesions.

Scanning of the slides was performed in an Olympus BX51 microscope (Tokyo, Japan) with an Olympus XC10 digital camera (Tokyo, Japan) using an image software VS-AWS 2.8 (Olympus, Tokyo, Japan). Qupath v0.3.2 programme (Bankhead et al., 2017) was use for digital image analysis by one veterinary pathologist (RV). The parameters analyzed were as follows: the number of foci per square centimetre (No. Foci/cm^2), the size of foci of gliosis with and without necrosis, named as the average

size of focus (ASF), and the percentage of brain tissue affected (%LES). In the digital scanned slides, the perimeter of each focus was traced manually and areas were measured automatically by the software. The total area of lesion (TAL) was calculated adding up the area of each individual focus found in the four sections from each brain. Total area studied (TAS) was calculated adding up the total area of the section. The percentage of brain tissue affected by lesion (%LES) was calculated by the coefficient between TAL and TAS. In order to compare between groups, the average of these measurements (i.e. foci/cm^2, ASF and %LES) was calculated for each section (A, B, C or D), then for each brain, and afterwards for each group. In addition to these measurements, the occurrence of other lesions such as necrosis and/or calcification within glial foci, perivasculitis, meningitis or tissue cysts was also recorded per tissue section.

Immunohistochemistry and digital evaluation of positive labelling

In order to study the occurrence of caspase-mediated apoptosis, axonal damage and also to characterize the cell populations in the CNS, the sample C, which included midbrain (caudal colliculus), telecephalon (cerebral hemisphere and lateral ventricles) was taken for immunohistochemical analysis (Figure IV-S2). This sample was chosen as it was the one that most frequently showed histological lesions. The following antigens were labelled: glial fibrillary acidic protein (GFAP) for the detection of astrocytes, ionized calcium binding adaptor molecule I (Iba1) for macrophages or microglial cells, calprotectin (MAC387) for labelling tissue macrophages recently recruited, caspase-3 for the identification of apoptotic cells, neuronal nuclei (NeuN) for identification of neurons and amyloid precursor protein (APP) in order to find disturbances of the fast axonal transport indicating acutely damaged neuronal axon (Table IV-1). Immunohistochemistry was executed using an AutostainerLink48 (Dako Agilent Technologies, Inc) or the Discovery XT autostainer (Ventana Medical System, Inc). Briefly, after samples were dewaxed overnight at 37°C followed by hydration though progressively decrease alcohols. Antigen retrieval was performed with DKB basic buffer (pH 9) for APP, Iba1, GFAP or with DKC acidic buffer (pH 6) for APP, Iba1 and GFAP, at 98 °C for 20 minutes. Samples were incubated with hydrogen peroxide solution (peroxidase block, Agilent Dako) for 10 minutes for endogenous peroxidase

activity blocking. The dilution of the primary antibody and conditions or incubation are listed in Table IV-2. Sections were washed with phosphate buffered saline (pH 8) between each phase. Afterwards, polymer detection systems conjugated with horseradish enzyme were incubated with the slides (Table IV-2). DAB substrate buffer (Agilent) was used as detection chromogen and counterstained with hematoxylin for 40 seconds and mounted.

Table IV-1. Details of the antibodies and procedures used for the immunohistochemical examination.

Target	Specificity	Monoclonal/ polyclonal	Primary Antibody: Dilution / Incubation	Detection system
GFAP	Astrocytes	Polyclonal	1:600 / 10 min room temperature (Dako)	MACH4 kit (Biocare)
Iba1	Macrophages/ microglial cells	Polyclonal	1:1000 / 1 h room temperature (Wako)	Envision rabbit (Dako)
Calprotectin	Tissue macrophages recently recruited	Monoclonal	1:100 / 30 min room temperature (MAC387 clone. Dako)	Envision mouse (Dako)
Caspase-3	Apoptotic cells	Polyclonal	1:400 / overnight 4°C (Dako)	Envision rabbit (Dako)
NeuN	Neurons	Mononuclear	1:4000 / 1 h room temperature (Clone A60, Merk Milipore)	MACH4 kit (Biocare)
APP	Disturbances of the fast axonal transport indicating acutely damaged neuronal axon	Monoclonal	1:6000 / 1 h room temperature (Clone 22C11, Merck Millipore)	Envision mouse (Dako)

Slides were scanned with an automated slide scanner (NanoZoomer-XR C12000; Hamamatsu) and analyzed for DAB labelling with the digital image analysis software Visiopharm Integrator System, Version 2020.08.1.8403 (Visiopharm, Horsholm, Denmark). Regions of interest (ROI) were manually introduced in the total surface area of the midbrain (Figure IV-S3B) for GFAP, Iba1, Calprotectin, Casase-3 labelling or 20 square-shaped ROIs, that measured 16 µm, were stablished in a random manner in the cortex (Figure IV-S3A) and midbrain for evaluation of NeuN labelling. Subsequently, the software detected the positive labelling and the number of immunopositive-labelled cells per mm^2 was counted for NeuN, Calprotectin and Iba1 labelling. In addition, on those slides labelled for Iba1, the presence of microglial foci was also evaluated and the slides were subjectively categorized as: (*) 1-5 foci with

really low number of Iba1 positive cells, (**) 5-10 foci with moderate number of Iba1 positive cells and (***) 10-40 foci with high number of Iba1 positive cells.

Regarding GFAP labelling, it was not possible to identify individual cells due to their ramified morphology and the intertwining of labelled structures, which made it impossible to differentiate individual cells from each other (Figure IV-S3C). For this reason, instead of counting the number of labelled cells, the percentage of positive labelled area regarding the total studied area per slide was calculated. As caspase-3 is a marker for apoptotic cells (Holubec et al., 2005), the pyknotic nucleus of the cell and cytoplasmic fragments (i.e. apoptotic bodies) were considered as a unit for the purpose of counting (Figure IV-S3D). For this, the selection parameters of the software were adjusted so that contiguous immunolabeled areas were measured as a single element. The number of units per mm^2 of tissue was evaluated.

Statistical analysis

Normality distribution for all the results was analyzed using Shapiro-wilk test and Kolmogorov-Smirnov test. Regarding histological scoring, for No. Foci per cm^2 and %LES, non-parametric Mann–Whitney test was used in order to compare between groups and for ASF parametric unpaired t test with Welch´s correction was performed instead. Comparison between groups for the presence of calcification, perivasculitis, focal meningitis and tissue cysts was performed using Chi-squared test. Regarding immunohistochemistry results, one-way-ANOVA was carry out for NeuN (No. Neurons/mm^2), GFAP (% of + Area), Iba1 (No. + cells/mm^2) and caspase-3 (+ units/mm^2) results followed by a tukey´s multiple comparison test. In a second step, pairwise differences between specific groups were analyze by a t test with Welch´s correction.

RESULTS

Microscopic lesions scoring

Regarding the digital quantification of histopathological lesions, the mean values of foci/cm^2, ASF and %LES from the fetal brains belonging to each group, in addition to the percentage of foci with necrosis and fetal brains with calcification, perivasculitis, focal meningitis and tissue cysts are summarized in Table IV-2. Individual values for each studied sample are detailed in Table IV-S2. As stated in the previous study (Chapter III), histological lesions, characterized by foci of mononuclear cells, were only found in fetuses from G1 and G3 at day 28 post infection. In both groups, lesions were randomly localized within each section, whereas when comparing among sections, they were more common at the level of the midbrain (section C) with one focus to 30 foci per section; and less common at the level of the cerebellum, that was only found in one 1 out of 6 fetuses studied in G1 and two fetuses out of three in G3 with only one to two foci per section regardless the group. In G1, 20% (19 foci with necrosis out of 96 total foci found in this group) of the lesions showed central areas of necrosis while in G3 this feature was found in more foci (p<0.050), specifically in 49% (18 foci with necrosis out of 37 total foci found in this group); however, the necrotic foci of G3 were only found in the one fetus that showed macroscopic signs of recent death at the necropsy at 28 dpi. In G1, foci were more numerous with 1,5 foci per cm^2 compared with G3 that had 1 focus per cm^2. The fetus with the higher number of foci was from G1, with a total of 54 foci in the sections examined. Foci were slightly bigger in G1 with an ASF of 45453 μm^2 in G1 and of 30287 μm^2 in G3. Resulting in a slightly higher percentage of damage area (% LES) in G1 (0.012%) when compared to G3 (0.009%). However, none of this differences were statistically significant. Foci with mineralization was present in one fetus (17%, 1/6) of G1, with 24 foci affected out of 54 (44%), and none form G3. Perivasculitis characterized by two to ten layers of mononuclear cells expanding the Virchow-Robbin space was present in 17% (1/6) of fetuses from G1 and 67% (2/3) of fetuses from G3. This perivasculitis was located only in the brain stem, frequently next to foci of gliosis, affecting 1 to 4 vessels in the whole tissue section regardless the groups. Focal meningitis, composed of mononuclear cells always surrounding blood vessels of the leptomeninges, minimally affecting the

adjacent brain parenchyma and frequently with a necrotic focus nearby in the surrounding area, was found in 50% (3/6) of fetuses from G1 and 33% (1/3) of fetuses form G3. When present, this meningitis was located in the midbrain level (section C) except in one case from G1 that was also located at the periphery of the cerebral cortex. Tissue cysts up to 100 µm containing multiple crescent shaped basophilic bradyzoites near foci of gliosis were present in one fetus of each group. Regarding these last four lesions (i.e. mineralization, perivasculitis, focal meningitis and tissue cysts), the statistical analysis showed that there were no differences between groups. None of these lesions were present in negative controls or G2.

Table IV-2. Average of histological scoring, presence of mineralization, perivasculitis, focal meningitis and tissue cysts in ovine fetal brains infected with different isolates of *Toxoplasma gondii* TgShSp1 (G1), TgShSp16 (G2) and TgShSp24 (G3).

Group	No. Foci/cm^2	ASF[a] (µm^2)	%LES[b]	No. Foci with necrosis	Mineralization*	Perivasculitis*	Focal meningitis*	Tissue cysts*
G1	1,455	45453	0,012	19,792	17%	17%	50%	17%
G2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
G3	1,011	30287	0,009	48,649	0%	67%	33%	33%
Control	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

* % of fetuses with each lesion. [a] ASF: average size of focus. [b] LES%: Percentage of brain tissue affected by lesion. n/a: Not available

Immunohistochemistry

All data from each immunomarker was normally distributed for all the groups when analyzed by Shapiro–Wilk test. Regarding positive NeuN labeled cells, fetuses from G1 showed a fewer (p<0.008) neurons per mm^2 in the midbrain (158.0) compared with G2 (203.4); there was also fewer (p<0.050) neurons at the cortex (537.4) of G1 than in G2 (676.9). Whereas G3 only showed a decrease in cortex neurons (558.0), although these differences were significant both with G2 (p<0.0038) and negative controls (634.5) (p<0.024) (Figures IV-1A and IV-1B).

In GFAP immunohistochemistry, fetuses from G1 (3/5) and G3 (1/5) had increase astrocytes compared with controls. All brains with astrocytosis had histological lesions on H-E, whereas not all brains with lesions had astrocytosis. These astrocytes were located scattered throughout the midbrain and also accumulated around blood vessels with their ramifications extending into the Virchow-Robbins space (Figure IV-2A). In G2 and negative controls, astrocytes were not surrounding vessels (Figure IV-2B). GFAP labelling had not association with foci of necrosis or gliosis

seen in H-E. Regarding the percentage of labelled area, there were differences among groups (p<0.05). In pairwise comparisons, fetuses from G1 showed greater percentage of positive area per mm^2 (3.043), showing significant differences with G2 (0.611) (p<0.0208) and negative controls (0.970) (p<0.0258) (Figure IV-1C).

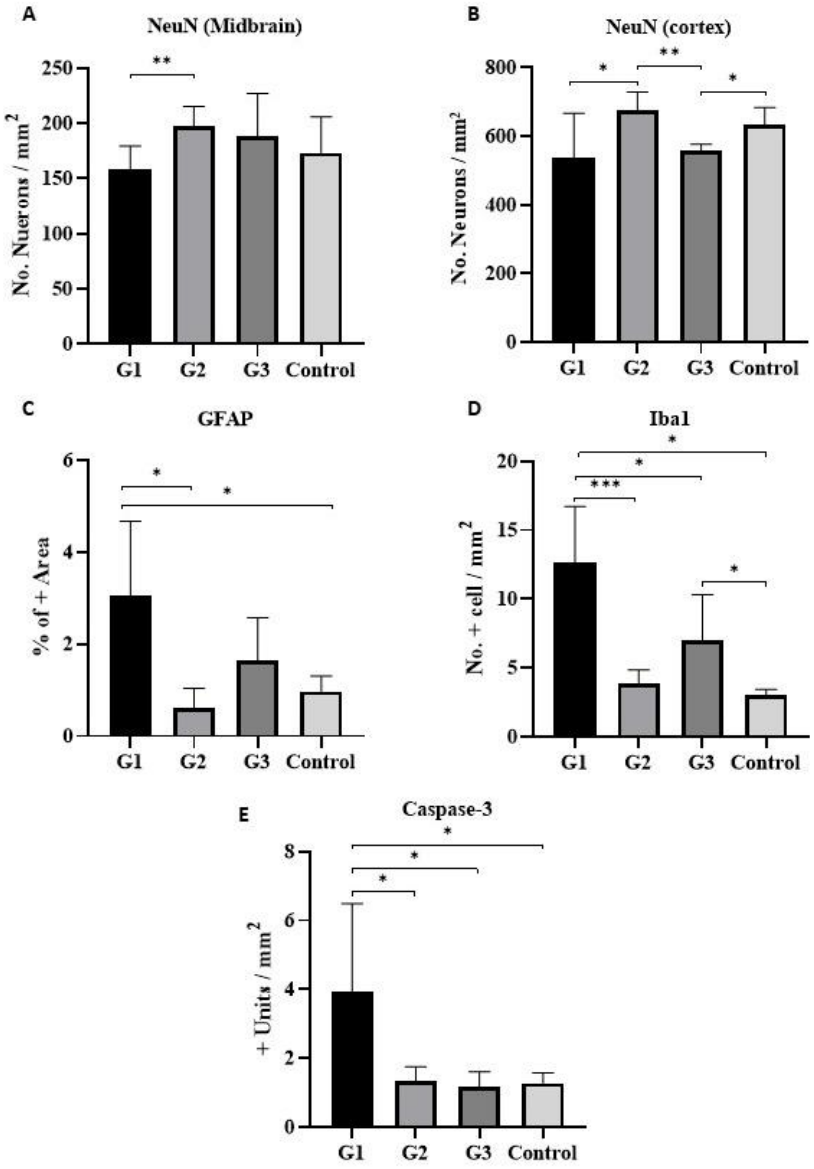


Figure IV-1: Results of the immunohistochemical characterization of resident cell populations and apoptotic events in negative controls and the three infected groups: G1 (TgShSp1), G2 (TgShSp16) and G3 (TgShSp24). A-B. The number of positive cells (neurons) for NeuN per mm^2 in the midbrain and cortex, respectively. **C.** Percentage of positive area in the midbrain for GFAP. **D.** The number of positive cells (microglial cells) for Iba1 per mm^2 in the midbrain. **E.** The number of apoptotic units (nuclei and cytoplasmic ramifications) per mm^2 in the midbrain. Significant differences between infected groups are indicated with asterisk where *, ** and *** note p <0.05, p<0.01 and p<0.001, respectively.

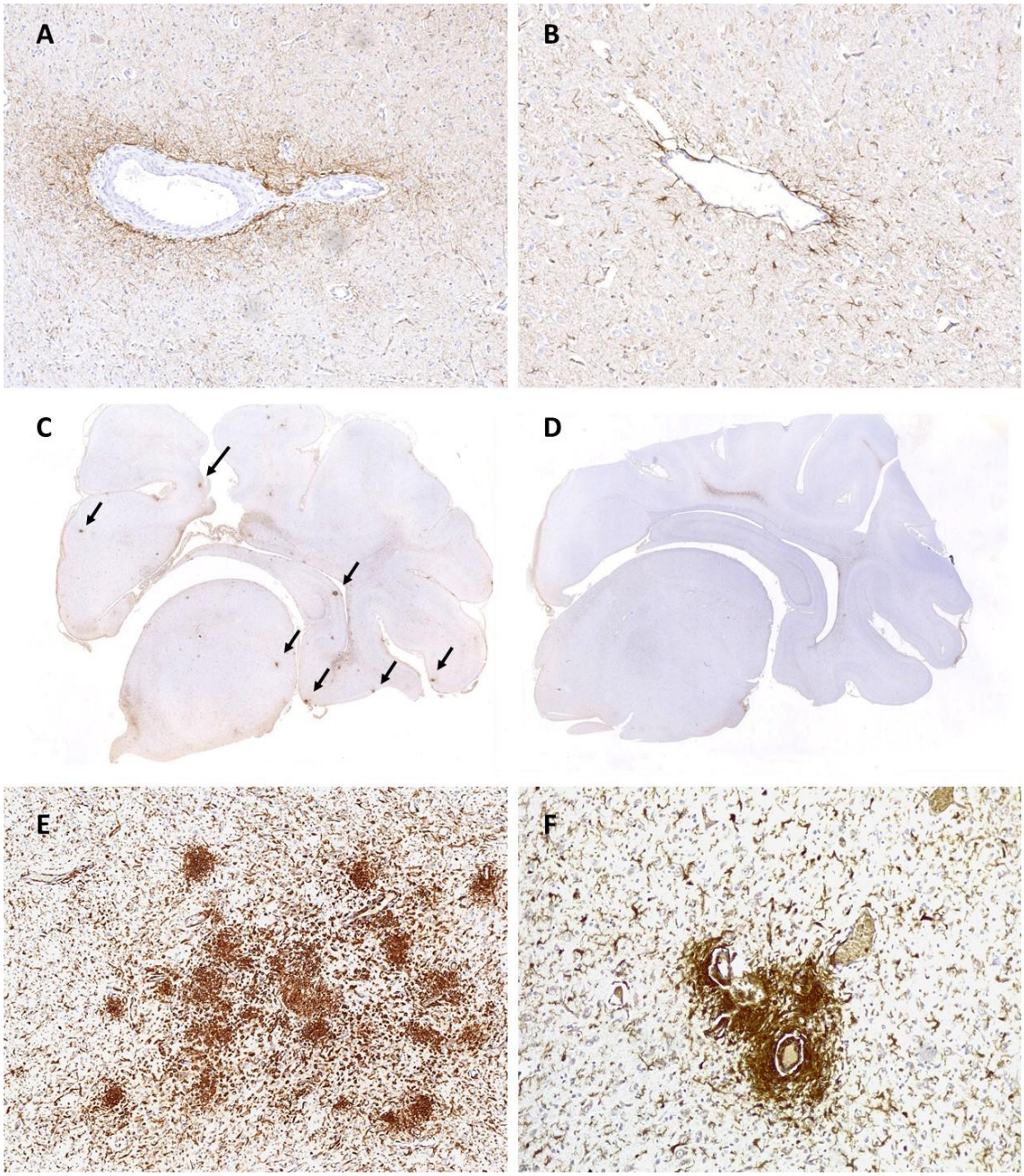


Figure IV-2: Immunohistochemical labelling of different cell populations in fetal brains infected with different isolates of *Toxoplasma gondii* and negative controls at 28 dpi. A. Increase number of astrocytes around blood vessels in the midbrain of a fetus from G1 (TgShSp1) infected group. 200x. GFAP IHC. **B.** Brain blood vessels without increase in the number of astrocytes around it from a fetus of G2 (TgShSp16). 200x. GFAP IHC. **C.** Foci of Iba1 positive cells in the midbrain and cortex of a whole tissue brain section of a fetus from group G3 (TgShSp24). Whole slide image. Iba1 IHC. **D.** Section from a negative control fetus brain without Iba1 positive cells forming foci. Whole slide image. Iba1 IHC. **E.** Multifocal glial foci in a G1 fetus with stained cells positive to Iba1. 100x. Iba1 IHC. F: Brain blood vessels with perivasculitis. Cells are positive stained with Iba1. 200x. Iba1 IHC.

The number of Iba1 stained cells varied between groups (p<0.05), the higher number of positive cells was observed in G1 (12.670) (Figure IV-1D) showing significant differences with the rest of the groups: negative control group (3.029) (p<0.0002), G2 (3.916) (p<0.005) and G3 (6.975) (p<0.0184). The two fetuses from G1 with the lower number of Iba1 positive cells, corresponded to the ones with the lower percentage of GFAP+ area and with low number of total glial foci. It is worth highlighting that even when microglial aggregation was not evident on histological examination of H-E stained slides, the immunohistochemical labelling of Iba1 unveiled the presence of such aggregates in G3, and this occurred when the aggregates were subjectively classified as mild. Actually, all of fetuses from G1 (100%) showed them, three moderate (**) and two severe (***) as semiquantitatively evaluated (Figure IV-2E). In this group microglial aggregates frequently surrounding foci of necrosis and were mainly found in the midbrain but also affecting the cortex (Figure IV-2C). Interestingly, 5/5 (100%) fetuses from G3 showed foci of Iba1 positive cells, in three of the fetuses the foci were considered as mild (*), one moderate (**) and in one fetus, severe (***). No foci of positive cells were seen in brain samples from G2 (Figure IV-2D) or negative controls. Foci of gliosis (Figure IV-2E), perivasculitis (Figure IV-2F) and focal meningitis seen in H-E were mainly compound of these iba1 + cells (Figure IV-2E and IV-2F). There was not positive labeling for Calprotectin in any of the samples studied, regardless the group.

Caspase-3 positive labelling was located strongly in the nuclei and moderately in cytoplasmic ramifications (apoptotic blebs) of scattered and randomly distributed positive cells throughout the tissue section, not associated with gliosis or necrosis. There were significant differences between groups by one-way-ANOVA (p<0.05). In multiple comparisons (Figure IV-1E), G1 had the highest number of caspase-3 positive units per mm^2 (3.992), with significant differences compared to negative control (1.278) (p<0.0286), G2 (1.336) (p<0,0414) and G3 (1.179) (p<0.0293). In spite of this, variability of data was high because two fetuses from G1 had low number of positive units compared with the other three of the same group, corresponding to the ones with the highest number iba1 positive cells, higher astrocyte positive area and higher number of glial foci in their brain. Axonal damage seen by APP labelling was not found in any of the studied samples, regardless the group.

DISCUSSION

After *T. gondii* infection of fetuses, regardless the species, brain is usually one of the organs, if not the organ, with highest number of lesions and parasite burdens (Dubey, 2009). Despite this, there are still many gaps in the understanding of toxoplasma neuropathology, at least in sheep, a species where toxoplasmosis had a major welfare and economic impact. A previous study on the distribution of lesions in the ovine fetal brain has shown that they are more numerous at the optic tract, the rostral margin of the pons and caudal to the ansate sulcus levels (O'Donovan et al., 2012), but there was scant information regarding their histological characteristics and which impact *T. gondii* infection had on the resident cells of the CNS. Besides, it is still little known if the variability of *T. gondii* isolates could influence these parameters.

The lesions found at the CNS in the current study were those characteristic of ovine toxoplasmosis in fetuses, where glial foci are the main finding (Buxton, 1998). Central area of necrosis in these foci was also present, although not as a common feature. Actually, the higher number of necrotic foci was found in a fetus from G3 (i.e. infected with TgShSp24 isolate), which was the one non-viable at the time of culling. This might suggest that the occurrence of necrosis is closely related to the compromised viability of the fetus. Previous studies analyzing the histopathological findings in fetuses due to *T. gondii* infection had described the occurrence of necrosis as a major a common finding, although it should be noted that the majority of these investigations have studied aborted fetuses (Buxton, 1998; Pereira-Bueno et al., 2004) while the current study is based on euthanized fetuses at a fixed time point. Similarly, to the findings in the current study, a frequent occurrence of necrosis in fetal tissues had been previously associated with fetuses found already dead at serial culling (Buxton & Finlayson, 1986). To assess whether the occurrence of necrosis could cause a compromise of the fetal viability, or it is a consequence of it, needs further research. Besides that, mineralization of the necrotic area, which is occasionally present in the glial foci characteristic for ovine toxoplasmosis (Buxton, 1990), was only present in the G1 (i.e. infected with TgShSp1 isolate) fetus with the highest number of glial foci in this study. Probably caused by a dystrophic calcification of already death cells. However, it has been stated that the incidence of dystrophic calcification is not associated with the

number of foci in murine brains (Ferguson et al., 1991). This suggest that there must be other factors modulating this calcification.

When looking at the distribution of the lesions, similarly to previous descriptions (O'Donovan et al 2012), the cerebellum was the location least affected and the higher number of foci were located at the level on the midbrain/rostral pons. Moreover, there were no differences in the distribution of the lesions regarding the isolates causing the infections. There is scant information regarding objective measurements of fetal lesions in ovine toxoplasmosis. However, a previous study developed with a similar experimental design and day of gestation when the animals were infected (Castaño et al., 2016), described more severe lesions in the brain than those found in the current study. They were characterized by a higher percentage of affected area and a higher number of foci per cm^2, while the ASF was similar in both studies. The main differences between both studies was based on the infectious inoculate, as the one used in Castaño et al., 2016 had a higher dose of oocysts, 50 instead of 10, and the isolate was also different, M4 instead TgShSp1, TgShS16 or TgShS24. While the influence of a higher dose should be obviously considered, as it is probable that higher doses caused more severe lesions. Dose-lesions correlations that have been previously observe in other infectious diseases (McDougald & Fuller, 2005; Kimblin et al., 2008; Ryan et al., 2021). It is also worth highlighting that isolates with a high number of cell culture passages, as the one used in Castaño et al 2016, could show an increased virulence due to their adaptation to cell culture lines (Frenkel & Ambroise-Thomas, 1997; Khan et al., 2009; Sánchez-Sánchez et al., 2019b) and they could cause more severe lesions than those isolates recently obtained, as the ones used in this work, when used in experimental models. This finding should be considered when designing experimental studies using *in vivo* animal models.

When analyzing the number of neurons labelled in the fetuses, there was a significant decrease at the midbrain and the cortex for G1 and slightly in the cortex for G3 when compared between the groups. There is little information regarding the effect of *T. gondii* infection on the neuronal population of ovine fetal brain. However, toxoplasmosis in chronically infected mice have been shown to decrease the dendritic arbor and spice density of neurons in the cortex and hippocampus, even if these were not directly infected by the parasite (Parlog et al., 2014). Besides, infected mice in

experimental models had a downregulation in genes that were involve in neurological functions, while genes involve in cell activation were increase, together with an increase in glial cells (Tanaka et al. 2013). In this way inflammatory cytokines and NO produced by glial cells may have damaged neurons, as inflammatory cells and activated immune resident cells could interact with neurons in *T. gondii* mice infection (Kettenmann et al., 2013; Parlog et al., 2014). On the other hand, neural death can be also triggered by *T. gondii* after direct infection of the neurons (Haroon et al., 2012). Furthermore, changes in the neuron cell populations have been related to behavioral changes in mice after *T. gondii* infection (Berdoy et al., 1995; 2000; Haroon et al., 2012). They have also been associated to the neurological and psychiatric disorders in humans infected by the parasite (Pedersen et al., 2012; Beste et al., 2014). It is not clear however whether this reduction in the number of neurons would have any consequence in infected lambs.

The increase in Iba1 positive cells was also more evident in G1, and then, to a lesser extent, in G3. Iba1 is a marker for microglia cells of the brain (Ito et al., 1998) and macrophages in general (Imai et al., 1996). Considering this finding together with the absence of labelling for calprotetin (i.e. MAC387 antiobody) it suggests that most of the Iba1 labelling corresponds to resident microglial cells, as calprotectin would have been expressed in those cells recently arrived from the blood stream, such as monocytes (Chilosi et al., 1990). An increase of microglial cells was also observed in ovine fetuses aborted early after infection (Gutiérrez-Expósito et al., 2020a). As well as in J20 mice infected with type II and II isolates and C57BL/6J mice infected with type II isolates (Cabral et al., 2017; Carrillo et al., 2020). Furthermore, glial foci, perivasculitis and focal meningitis were mainly composed of these Iba1 positive cells. These same finding was observed in adult goat and aborted fetuses infected with *Neospora caninum,* a closely related protozoan parasite*,* where perivascular cuffs and glial foci were marked with RCA1+ positive macrophages and rare T and B lymphocytes were found (Costa et al., 2014). Although further immunhistochemical analysis would be needed in order to study the presence of other populations of cells, such as lymphocytes, the findings from this study suggest that microglial cells have an important role in ovine toxoplasmosis encephalitis and resident macrophages cells in the meningitis. Microglia can be activated and produce INF-γ or can be infected by *T.*

gondii, and be a niche for parasite replication (Dellacasa-Lindberg et al., 2011). For this later case, it has been stated that infected microglial cells could act as trojan horse and show a migratory profile after infection, promoting in that way the dissemination of the parasite through the parenchyma (Bhandage et al., 2019). Further phenotypic characterization of microglial cells on these lesions would be useful for a better understanding of their role in toxoplasmosis, as they can show a proinflammatory (M1) phenotype or an alternative activated (M2) one. A remarkable finding from this study was that immunohistochemical labelling of Iba1 allowed for the identification of several mild foci that had been gone unnoticed on H-E stained slides. Besides showing the relevance of microglial reaction in this disease, it also means that such reaction could be underdiagnosed in toxoplasmosis, or other conditions affecting the brain, when only H-E staining is used.

The increase apoptotic units present in the brain of G1 fetuses could be an explanation of the reduction of neurons. It has been demonstrated that *T. gondii* infection can led to apoptosis of host cells (Nishikawa et al., 2007; Xu et al., 2012; L. Zhang et al., 2015) including brain cells (Dincel & Atmaca, 2016). Specifically, neuronal apoptosis had been detected in chronic infected mice (Wang et al., 2019b). In the current study, caspase-3, considered the major moderator of apoptosis in the brain cell populations (Etewa et al., 2021), was significantly increased in G1 compared with the other groups. It seems that its expression level could be correlated with the severity of the lesions caused by infection, as G1 was also the group showing the most severe lesions. This increase in caspase-3 expression has also been described in CNS of mice intraperitoneally infected with 20 *T. gondi* cysts at 15 and 30 dpi (Dincel & Atmaca, 2016). However, they also found immunopositivity in endothelial cells, meninges and glial foci, that was not present in the ovine fetal brains from the current study. It is relevant to notice that the highest increase of labelling in caspase-3 was found in G1, where there was also the highest increase in labelling for Iba1. It might be that the decrease in neurons found in this group could be related with the microgliosis, as activated microglia has been associated with neuronal apoptosis in a murine model of toxoplasma reactivated encephalitis and *in vitro* studies (Zhang et al., 2014). Actually, the inhibition of microglial activation has been shown to reduce neuronal apoptosis. This finding suggests that an excessive activation of microglial cells could

release secondary signals that induce apoptosis (Wang et al., 2019b). On the other hand, it could be the direct infection of the neurons by the parasite (Cabral et al., 2016), or other brain cells, what could trigger this apoptosis. In order to elucidate the role of apoptosis in ovine toxoplasmosis, it would be also interesting to explore other mediators of this process, such as caspase-9, marker for the intrinsic apoptotic pathway (Santagostino et al., 2021). This mediator has also been shown to play a key role in the induction of cellular apoptosis at the brain in murine models of toxoplasmosis (Dincel & Atmaca, 2016).

Astrocytes were also significantly affected in the groups showing histological lesions, especially in G1 while there was only a slight increase of these cells in G3. An increase in the labelling of astrocytes was also observed in *T. gondii* infected ovine fetuses that aborted at an early stage of the disease, within the first two weeks after infection (Gutiérrez-Expósito et al., 2020a). This is relevant because so early after infection, the parasite has not even reached the fetus (Benavides et al., 2017), so it seems that the astrocytic reaction in the brain does not always require the actual invasion of the neuroprenchyma by the parasite and could be triggered by other mediators such us activated microglia/macrophages, presence of damaged tissue, or certain cytokines (Sofroniew & Vinters, 2010; Still et al., 2020). On the other hand, astrocytes have been shown to play a key role in the CNS defense against infections (Shulyatnikova & Verkhratsky, 2020) and, specifically, have a crucial function in *T. gondii* infection (Drögemüller et al., 2008). Astrocytes could be activated as soon as 10 dpi, in response to the invasion of tachyzoites into the brain of orally infected mice (Hunter et al., 1992). Besides, they are one of the main target cells infected by *T. gondii* and, frequently, where parasite tissue cysts are formed (Hulinska et al., 1990). Whether the increase of astrocytes is due to the direct presence of *T. gondii* in the fetal ovine brains or due to other mediators such us active microglia or tissue damage, requires further investigation. Interestingly, in fetuses from G1 there was a clear increase in labelling around blood vessels, which might be associated with the invasion of the brain through the vessels or also as an attempt to maintain brain homeostasis after invasion, restricting damage to the CNS (Shulyatnikova & Verkhratsky, 2020). When astrocytes are activated they could produce cytokines to help control the infection, such as Il-6 and INF-γ (Halonen et al., 1998; Wilson & Hunter, 2004).

Axonal damage, in the form of leukomalacia, has been shown to be a key finding in the brains of ovine fetuses suffering early abortions (Castaño et al., 2014; Gutiérrez-Expósito et al., 2020a). Previous studies have also showed hypereosinophilic necrotic foci of leukomalacia in low numbers of infected fetuses culled at 35 dpi (O'Donovan et al., 2012) and in aborted ones (Dorsch et al., 2022). However, in the current study there was no positive labelling of axonal damage in any of the studied fetuses. This lack of labelling is not probable a consequence of differences in the experimental designs (i.e. dose, isolate, time points of study…) of these studies, as leukomalacia is closely associated with early abortion, regardless the dose or isolate used in the inoculum (Benavides et al., 2017). Thus, the lack of axonal damage found in this study may suggest that the mechanisms behind damage to fetal brain at day 28 pi on this study were not severe enough to cause axonal damage, which have been found in early abortions linked to extensive vascular damage of the placenta (Castaño et al., 2014; Gutiérrez-Expósito et al., 2020a), or when the infecting dose was higher, fetuses came from abortions or had been more time infected (O'Donovan et al., 2012; Dorsch et al., 2022).

While fetuses from G1 and G3 showed similar lesions, although with differences in frequency and severity, fetuses from G2, infected with isolate TgShSp16, show no histological lesions in the CNS. Besides, there were also variations regarding the findings on immunohistochemical characterization of resident cells in the brain or the occurrence of apoptosis among groups. These results show that there are differences between isolates in the lesions caused at the CNS. Differences in histological lesions had already been associated to isolates variability (Dubey et al., 2016; Fernández-escobar et al., 2021). Whether this is because the isolates had differed dynamic in spreading to the fetus or because their virulence once reached the CNS is different, remains to be further investigated. Variations in histological lesions and brain cell populations could be influenced by the genetic diversity of the three different *T. gondii* isolates used in this study. Besides, these results demonstrate that the isolate TgShsp1 (G1), that has been considered as a low virulence isolate in *in vitro* and *in vivo* murine models studies (Sánchez-Sánchez et al., 2019a; Fernández-Escobar et al., 2021), gave rise to a greater number of lesions, together with a decrease in the number of neurons and an increase in the number of astrocytes and microglia, as well

as apoptosis in comparison with the other two isolates. This finding is in accordance with previous studies suggesting that the extrapolation of results between experimental infections in different species might not be always directly extrapolated (Taniguchi et al., 2018; Sánchez-Sánchez et al., 2019a). On the other hand, fetuses from G2 showed no evident lesions or differences with the control group. Whether the absence of histological changes of brain cell population compared to control animals in G2 is due to a less virulence of TgShSp16 isolate or due to the isolate being less neurotropic requires further investigation.

CONCLUSION

These results contribute to a better understanding of the pathogenesis of fetal nervous lesions in ovine toxoplasmosis and the influence that inter- and intra-genotype variation of *T. gondii* isolates might have on the changes in the ovine fetal brain after infection. Larger lesions in *T. gondii* were directly related with an increase in microglial cells, astrocytes and apoptotic events, as well as a reduction in the number of neurons. The microglial cells played a relevant role in the pathogenesis of these lesions as they were the predominant cell type associated to them.

SUPPLEMENTARY INFORMATION

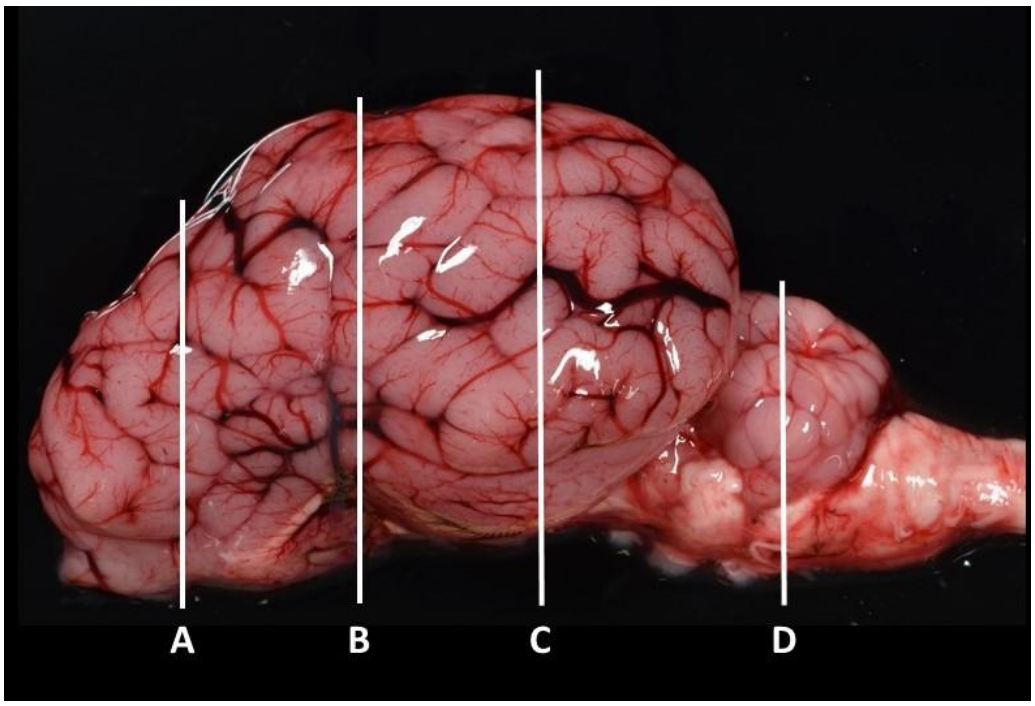


Figure IV-S1. Ovine fetal brain. Lateral surface, gross. The four sites of coronal slicing included: Frontal lobe (A), corpus callosum (B), midbrain (C) and cerebellum (D).

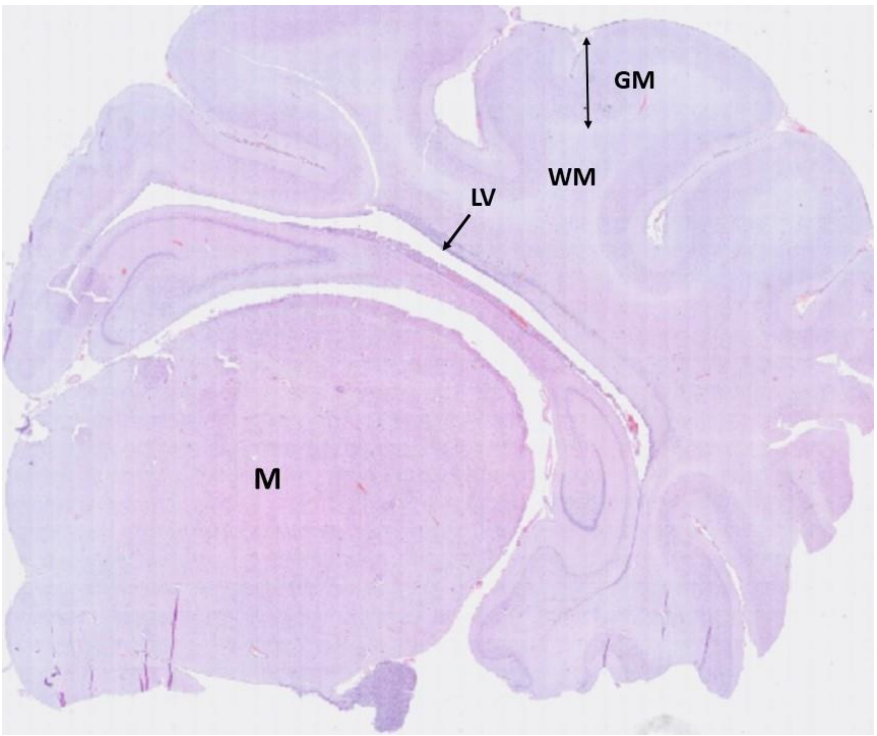


Figure IV-S2. Ovine fetus. Brain section selected for immunohistochemistry and digital evaluation. Non-infected control group. Representative histological section C including midbrain (M), lateral ventricles (LV) and cerebral hemisphere: white matter (WM) and grey matter (GM). Whole slide image. H-E.

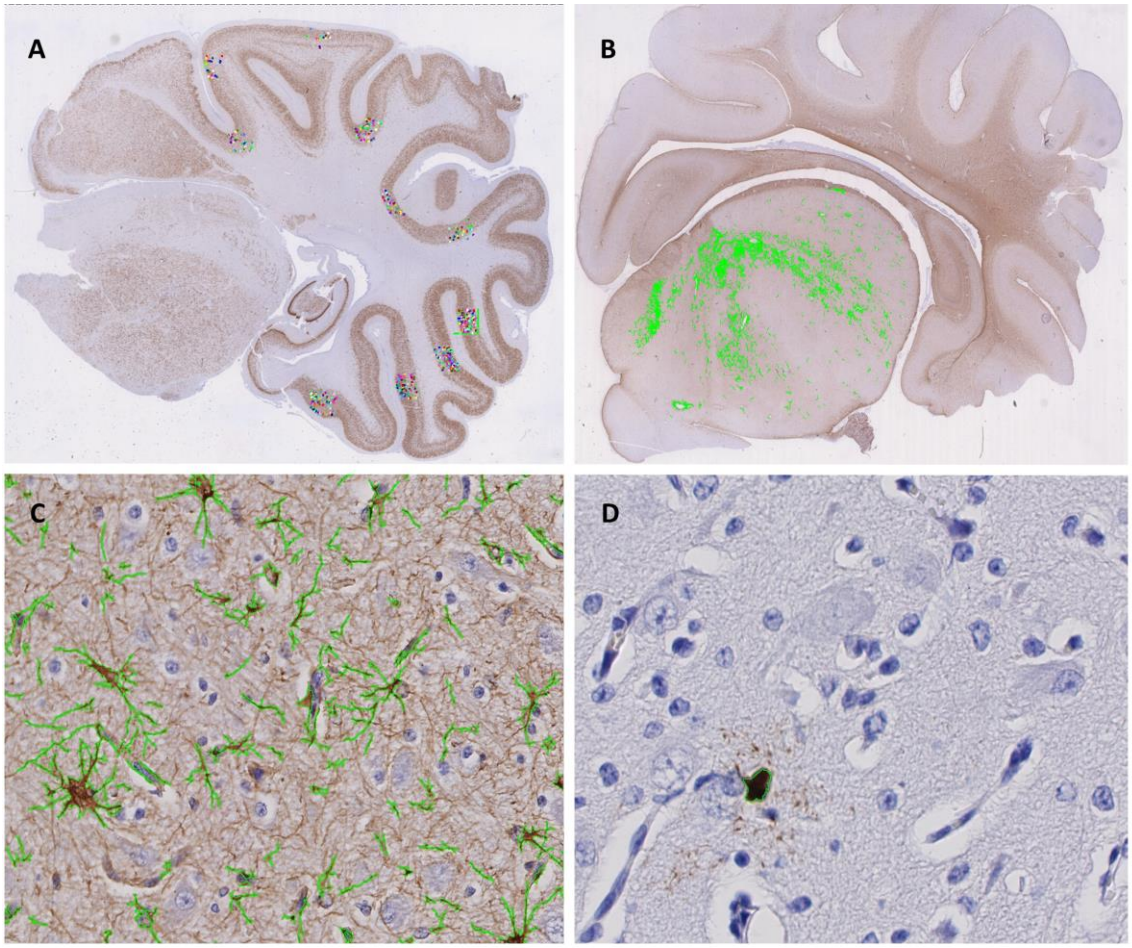


Figure IV-S3. Ovine fetal brain immunohistochemistry and digital evaluation. **A.** Ten square shaped ramdomly selected ROIs (16x16µm) in cerebral cortex where positive neurons were measured. ROI: Region of interest. Whole slide image. NeuN IHC. **B.** Midbrain GFAP posive area is highlighted in green. Whole slide image. GFAP IHC. **C.** Astrocytes with ramified morphology. The positive stained area is surrounded by a green line. 400x. GFAP IHC. **D.** Apoptotic cell nucleus is marked by a green line, surrounded by positive stained cytoplasmic fragments. Both structures as a whole were considered an apoptotic unit for evaluation. 600x. Caspase-3 IHC.

Table IV-S1. Individual data of the fetal brain samples used in the present study culled at 28 days post infection with isolate TgShSp1 (G1), TgShSp16 (G2) and TgShSp24 (G3).

Group	Fetus reference	Histological Lesion	Chosen for immunohistochemical characterization
G1	1 F1	L	Yes
	2 F1	L	Yes
	3 F1	L	-
	3 F2	L	Yes
	4 F1	L	Yes
	4 F2	L	Yes
G2	5 F1	NL	Yes
	6 F1	NL	Yes
	7 F1	NL	Yes
	8 F1	NL	Yes
	9 F1	NL	Yes
G3	10 F1	NL	Yes
	11 F1*	L	-
	11 F2	L	Yes
	12 F1	NL	Yes
	12 F2	L	Yes
	13 F1	NL	Yes
Control	14 F1	NL	Yes
	15 F1	NL	Yes
	16 F1	NL	Yes
	17 F1	NL	Yes
	18 F1	NL	Yes

* Non-viable at the time of culling. NL: no lesion, L: Lesion

Table IV-S2. Individual quantification of lesions in fetal brains.

Group	Fetus reference		No. Foci per cm²	ASF (µm²)	%LES	Total No. of foci	No. Foci with necrosis	Calcification	Perivasculitis	Focal meningitis	Tissue cysts
G1	1	F1	0,482	38608,45	0,004	5	1				
	2	F1	1,284	21879,81	0,010	16	2			Yes	
	3	F1	0,677	40699,97	0,008	8	3			Yes	
	3	F2	0,298	98382,58	0,005	4	1				
	4	F1	0,885	46272,79	0,010	9	4		Yes	Yes	Yes
	4	F2	5,101	26354,47	0,037	54	8	Yes			
G3	11	F1	2,102	32613,33	0,023	27	18				Yes
	11	F2	0,429	43147,75	0,003	3	NL			Yes	
	12	F2	0,501	15100,34	0,002	7	NL		Yes		

Table IV-S3. Individual quantification of immunohistochemestry in fetal brains.

Group	Fetus reference		NeuN (Midbrain) No. Neurons/mm2	NeuN (Cortex) No. Neurons/mm2	GFAP % + Area	Iba1 No. + cell/mm2	Caspase-3 +Units/mm2
G1	1	F1	195,463	514,340	3,535	14,670	4,096
	2	F1	149,349	474,109	5,010	18,140	4,374
	3	F2	148,970	601,255	1,411	8,662	2,033
	4	F1	153,651	718,976	1,303	8,758	1,250
	4	F2	145,427	378,149	3,955	13,110	7,858
G2	5	F1	177,498	661,539	0,623	4,530	1,404
	6	F1	214,377	737,067	0,451	2,309	1,038
	7	F1	217,730	601,445	1,340	4,358	1,992
	8	F1	187,050	711,448	0,224	4,456	0,950
	9	F1	191,473	672,833	0,418	3,928	1,298
G3	10	F1	222,031	575,889	0,733	3,178	0,856
	11	F2	198,218	556,056	1,564	6,963	1,873
	12	F1	194,008	544,008	1,473	9,882	1,083
	12	F2	121,706	537,049	3,217	4,168	0,799
	13	F1	206,090	577,028	1,223	10,684	1,284
Control	14	F1	191,541	639,778	0,733	2,728	1,390
	15	F1	136,951	701,960	0,979	2,633	1,708
	16	F1	138,848	567,476	1,532	3,675	0,900
	17	F1	200,679	613,717	0,935	2,926	1,209
	18	F1	198,657	649,576	0,672	3,181	1,184

GLOBAL DISCUSSION AND FUTURE PERSPECTIVES

Toxoplasmosis is an infectious disease caused by the protozoan parasite *Toxoplasma gondii* (Innes et al., 2009). It is a worldwide animal and public health problem that entails a significant threat to the animal welfare and large economic losses by direct impact on reproductive failure in farm animals (Stelzer et al., 2019). Currently, several methods for the control of this disease have been proposed in livestock, from farm management procedures to a variety of treatments but, among them, vaccination is the most effective one (Dubey et al., 2020a). However, the only vaccine available in the market is based in a life attenuated isolate, and its production and commercialization has several drawbacks, together to the threat of reversion to virulence that is inherent to attenuated vaccines (Innes et al., 2011). Protection conferred by the vaccine is not absolute and moreover, the mechanisms that operate in vaccinated animals have not been fully understood, probably due to the complexity of the interaction between the pathogen and the host that takes place in toxoplasmosis (Wang et al., 2019a). Thus, and despite the great importance of ovine toxoplasmosis, there are still many gaps in the knowledge of its pathogenesis, especially regarding the occurrence of abortion (Benavides et al., 2017) that makes necessary to continue investigating in this disease, especially now when the sustainability of the ovine farms is severely compromised.

There are several variables that could participate into the pathogenesis of toxoplasmosis. Among them, the role that the variability of the parasite virulence could play is still greatly unknown. *T. gondii* isolates have been traditionally classified in three archetypal types which also reflects their virulence to mice, evaluated as the cumulative mortality rate in outbred laboratory mice as follows: type I virulent isolates (100% lethal to mice, regardless the dose), type II intermediate virulent isolates (99-30% lethality) and type III non virulent isolates (<30% lethality) (Sibley & Boothroyd, 1992; Su et al., 2002), with type II being predominant in Europe (Fernández-Escobar, 2022a). However, thanks to the development of new markers for PCR-RFLP and MS molecular techniques, it has been proved that the genetic variability of the isolates is much more diverse than previously thought (Fernández-Escobar, 2022a). Moreover, there are atypical isolates located mainly in South America (Ajzenberg et al., 2004) that show a higher virulence than the classically described isolates (Grigg & Suzuki, 2003; Calero-Bernal et al., 2022). Recent studies delving into the genetic and phenotypic

variability of recently obtained isolates have shown that there might be additional phenotypic traits or genetic markers that could add a lot information to the characterization of the isolates (Fernández-Escobar et al., 2022a). Besides, the necessary reduction and refinement of experimental *in vivo* models is promoting the development of new *in vitro* models and other parameters have started to be used in those *in vivo* studies addressing the virulence of *T. gondii* isolates (Calero-Bernal et al., 2022). As examples, several criteria such as the parasite burden, cyst loads, histologic lesion scoring, parasite load in tissues, behavioral changes, weight loss or titration of specific anti-*T. gondii* IgG antibodies have been used in previous *in vivo* studies in mice (Jungersen et al., 2002; Bezerra et al., 2019; Hamilton et al., 2019; Fernández-Escobar et al., 2021; Salman et al., 2021). However, most of these studies have been based in cell lines of human or murine origin or in murine experimental models, so there is a lack of information regarding the impact of these variables in ovine toxoplasmosis (Dubey, 2009). Furthermore, several differences in the host physiology, specifically on reproductive parameters or immune response caused that results from experimental models based on mice could not always be directly extrapolated to other species, such as sheep or pigs (Taniguchi et al., 2018; Sánchez-Sánchez et al., 2019a; Largo-de la Torre et al., 2022)

The main objective of the present PhD Thesis was to study the parasite-host interaction in ovine toxoplasmosis and to establish how genetic and phenotypic variability of *T. gondii* isolates could influence the development of the disease in sheep. The experimental part of this project was divided in two main experiments (Experiment 1 and 2) designed to develop and refine both *in vitro* and *in vivo* experimental models based in sheep and then used them to evaluate the interaction of *T. gondii* recently obtain isolates with the host. The isolates used in these experiments belong to archetypal types II and III and to the three most prevalent genotypes in Spanish livestock species (ToxoDB #3, #2 and #1) (Fernández-Escobar et al 2020a). These isolates were obtained by Saluvet group (Complutense University of Madrid) within the coordinated project where this PhD Thesis is framed. The results from the experiments that formed this PhD Thesis are shown and individually discussed in the different chapters of this manuscript. This discussion aims to summarize their main relevant findings and discuss them in an integrated context.

Previous studies on the same isolates used in the current PhD, have shown that there is actual genetic and phenotypic variability among them (Fernández-Escobar et al.,2020a; Fernández-Escobar et al.,2021; Largo-de la Torre et al., 2022). The results from the experiments carried out in this PhD Thesis also confirmed this variability but, interestingly enough, the results from genetic studies and characterization in murine models did not have a direct correlation with the results from the experimental models based in sheep. To be more precise, the isolate TgShSp1 (type II, ToxoDB#3) showed low mortality in mice (0%) and low parasite invasion rates in a cell line of ovine trophoblasts (AH-1) (Fernández-Escobar et al., 2021) and OvMØs (Chapter I). However, this same isolate caused the highest number of lesions in placentomes and fetuses, including the percentage of affected area at the CNS and also the highest parasite distribution and burden when it was tested in an ovine *in vivo* experimental model (Chapter III). On the other hand, TgShSp24 (type III, ToxoDB#2) and TgShSp16 (type II, ToxoDB #3) showed the opposite results. TgShSp24 had the most virulent profile in *in vitro* models with the highest invasion rates in ovine trophoblasts and OvMØs. Both TgShSp24 and TgShSp16 had the highest mortality rates in mice (around 20%) and parasite burdens when compared to the other recently obtained isolates (Fernández-Escobar et al., 2021). Besides, TgShSp24 in piglets infection had higher parasite burdens than TgShSp1 (Largo-de la Torre et al., 2022). However, in the experimental model of pregnant sheep (Chapter III), TgShSp16 was associated with a less number of lesions, and lower parasite detection levels and burden than TgShSp1, and the results of TgShSp24 infection were located, in terms of frecuency and parasite distribution and burden, between those of TgShSp1 and TgShSp16. These results reinforce the observation that it is not always possible to extrapolate the results from experimental models based in different species. Intra-genotype phenotypic variations suggest that deeper genetic characterization and studies, to correlate it with the phenotype of the isolate, are needed in order to identify genetic markers for virulence in the different species of interest.

There is scant information regarding how genetic variability of *T. gondii* isolates could influence the response of sheep to infection, and especially at cellular level. The only study carried out in ovine cells, specifically in ab ovine cell line of trophoblasts (AH-1) presented significant differences between TgShSp1 and TgShSp24, that had the

lowest and the highest invasion rates and tachyzoites yield, respectively (Fernández-Escobar et al., 2021). Besides trophoblasts, macrophages are one the main target cells of *T. gondii,* involved in both the control and dissemination of the parasite (Sasai et al., 2018). Moreover, the interaction between the parasite and the macrophage results in the phenotypic polarization of the latter. This polarization could be influenced by the genetic variability of the parasite (Jensen et al., 2011). This close relation between the macrophages and *T. gondii* advocated for the development of an *in vitro* experimental model based on macrophages in order to study the parasite-host interaction in toxoplasmosis. Furthermore, the development of a model based on ovine macrophages could greatly help in the study and understanding, of ovine toxoplasmosis. In Experiment 1 a panel of six isolates were studied in OvMØs at 6 hpi, both in terms of parasite internalization (cIR and McR) and transcription of cytokines and iNOS RNA. It is important to mention that all of the isolates investigated were recently obtained, with a low number of culture passage in order to avoid adaptation to cell culture and, thus, modification of their virulence (Sánchez-Sánchez et al., 2019a). TgShSp24 (type III isolate, ToxoDB#2) demonstrated the highest cIR and McR when compared to all the other type II isolates (TgShSp1, TgShSp2, TgShSp3, TgShSp11, TgShSp16). Similar to what has been described for other type II isolates infecting human monocytes, i.e. THP-1 cell line (Meneceur et al., 2008). However, this high rate of internalization into OvMØs did not correlate to an increased virulence in the ovine pregnant model, at least in terms of reproductive loses, as infection by TgShSp24 did not cause more significant reproductive losses than infection with TgShSp1 or TgShSp16. This *in vitro* experimental model of OvMØs showed differences in internalization and cytokine transcription not only between archetypal types (II and III) but also within isolates of the same archetype (type II). TgShSp1 and TgShSp3 (PRU II-A) isolates obtained from abortion samples, showed a lower internalization rate than TgShSp11 and TgShSp16 (PRU II-C) obtained from chronically infected sheep. Similar results were obtained when the same isolates were studied in the *in vitro* model based in ovine trophoblasts (Fernández-Escobar et al., 2021). It is not clear which could be the cause of these differences and, although the samples origin was different (i.e. abortions and chronic infected sheep), further characterization of these isolates is needed in order to draw conclusions.

Regarding the influence of the isolates on the polarization of macrophages, the *in* vitro model of OvMØs showed a correlation between a higher parasite internalization and the polarization towards a proinflammatory M1 profile. OvMØs infected by TgShSp24 showed a M1 profile, followed by TgShSp2 (clonal type II, ToxoDB#1). Both isolates caused the highest transcription of IL-12 and TNF-α by macrophages. These cytokines are crucial for regulating *T. gondii* proliferation in mice (Suzuki et al., 1988; Scharton-Kersten et al., 1995; Sturge & Yarovinsky, 2014). Besides, studies in mice with type III isolates resulted in an M1 profile too (Tuladhar et al., 2019). On the other hand, TgShSp1 and TgShSp3 (Type II PRU, ToxoDB#3) isolates induced a M2 polarization in OvMØs denoted by the transcription of IL-10, TGF-β and IL-4 mRNA (anti-inflammatory cytokines) and that was related to a lower parasite internalization. These results should be taken with care as the *in vitro* experimental model is based on just one population of cells and its results could not be extrapolated to the complexity of the immune response of the host and the plethora of cell interaction that it encompasses. However, bearing in mind that the only variable among the infections was the isolate inoculated into the culture of OvMØs, the most evident result from this study is that the different isolates used, all of the obtained from ovine samples, did show intra- and inter-genotypic variations in the phenotypic traits evaluated. Whether this variation could have any influence in the pathogenesis of ovine toxoplasmosis was addressed in Chapter III. The influence of isolate variability on the macrophage polarization to M1 or M2 has been associated with the presence of dense granule or rhoptry proteins that interact with host molecular pathways. It has been shown in *in vitro* studies with mice macrophages that are infected with ROP16-deficient *T. gondii* isolates produced an M1 phenotype, whereas ROP16-positive isolates induced an M2 phenotype (Jensen et al., 2013; Chen et al., 2020). Although these specific proteins have not been assessed in the isolates used in this PhD Thesis, they showed that genetic variation among isolates could have a direct impact in the response of infected macrophages. On the other hand, it has been also described that murine macrophages infected with type I and III isolates are mainly polarized towards a M2 profile (Jensen et al., 2011). This discrepancy in the results of macrophage infection supports the necessity of further characterization of the isolates used in experimental models, as well as their homogenization, so the results of the different

studies are comparable. Additionally, the increase of IL-17 transcription caused by the infection of TgShSp11 and TgShSp16 isolates (PRU II-C) is of particular interest as IL-17 has been associated with the control of murine toxoplasmosis (Moroda et al., 2017). It remains to be investigated which relevance could have this cytokine on the pathogenesis of ovine toxoplasmosis. On the other hand, this same cytokine has been suggested to participate in the control of ovine neosporosis, caused by *N. caninum*, a closely-related protozoan parasite (Gutiérrez-Expósito et al., 2020b). Specifically, the production of IL-17 has been associated with a decrease in vertical transmission. Taking together these results, it is tempting to hypothesize that the production of IL-17 induced by TgShSp16 isolate in the *in vitro* model could be related to the low number of infected fetuses found in the ovine pregnant model (Chapter III). Further research is needed regarding local and peripheral immune responses of the host when infected with each isolate.

In order to study the complex parasite and host interaction that takes place after infection in ovine toxoplasmosis, it is necessary to refine as much as possible an experimental model of pregnant sheep. Placenta and fetuses undergo significant morphological changes during gestation; this is why it is necessary to characterize the normal macroscopic and histological findings in the placenta and fetuses of healthy animals in this experimental model. A better understanding of the ovine placenta and fetal morphology, including their histological structure, would help to identify the changes caused after the infection of an abortifacient agent, especially when those lesions are mild. Regarding the macroscopic changes found in the ovine placenta in Chapter II, allantoic calculus found in almost all studied placentas was probable caused by desquamation from fetal membranes that would provide a nucleus for its formation, as it has been suggested to occur in horses, where similar structures have been named as hippomanes (Dickerson et al., 1967). The presence of amniotic plaques was another common finding that could lead to confusion, as they could resemble papillomatous lesions that could be caused by infectious agents (Russo et al., 2020). These plaques were located mainly around the umbilical cord, and they were actually useful for identifying the amniotic membrane of the placenta, as the plaques arise from this membrane, facing the fetus. This is a common finding in sheep and cows (Pourlis et al., 2008; Simões & Stilwell, 2021). The presence of meconium in the

amniotic fluid, also a common finding, is associated with fetal distress in ruminants and humans (Schlafer et al., 2000; Steer et al., 2022) and could help to assess the occurrence of fetal stress after infection. Histological changes that could be misinterpreted as lesions were only present in placentomes, and not in the intercotyledonary placenta, probably as it is the most active part of the ruminant placenta (Sammin et al., 2009). The occurrence of intracytoplasmic nonferrous pigment within trophoblasts, named in the literature as phagocytic trophoblast cells (Schlafer et al., 2000) was a strikingly common finding. This pigment is most probably a product of hemoglobin metabolism after erythrocyte phagocytosis by the trophoblasts (Myagkaya et al., 1984). Another common finding was the hyalinization of the caruncular septa. This change had been already described in sheep (Sammin et al., 2009), but it is not clear what is the cause. Caspase-3 immunohistochemestry ruled out that it has its origin in the apoptosis mediated by this molecule, so this degenerative process must be related to other non-apoptotic cell death pathways such as necroptosis or necrosis (Santagostino et al., 2021). Both changes (i.e. intratrophoblastic pigments and hyalinization of caruncular septa) were located in the arcade hematomata. This is why this area is especially complicated for the histological evaluation of the placentome in the ovine pregnant model. The morphological evaluation of the vessels in the placentomes has special importance in this experimental model because of the vascular lesions described in Chapter III. The identification of thickening of the blood vessels wall and the extravasation of proteins around vessels in caruncle was demonstrated to occur in healthy placentomes. These findings should be regarded with care when evaluating the placentomes, as well as the occurrence of mineralization, which was also a common finding in ovine placentomes and it is not necessary caused by infectious agents or abortive conditions (Sammin et al., 2009). Furthermore, infiltration with a moderate number of T cells and macrophages, most of them resident macrophages, was normal in the interdigitating zone of ovine placentomes, while B cells were scarce. Among all the immune cell populations evaluated in the ovine placentome, it is worth highlighting the absence of NK cells. Previous studies have also observed that this population of cells, specifically the NKp46+ cells, might not be playing a relevant role in ovine gestation, either in physiological conditions or after infection by *Chlamydia abortus* or *T. gondii* (Castaño

et al., 2019; Wattegedera et al., 2019). This is a relevant finding since in murine and human toxoplasmosis, NK cells have been shown to play an important role, as one of the main effectors of the innate immunity when controlling parasite dissemination (Gigley, 2016), and have even been used for the evaluation of the virulence *in vitro* assays in mice (Ivanova et al., 2016). Further research on the innate immune response against *T. gondii* in ovine toxoplasmosis is needed in order to elucidate the role of NK cells.

The main finding in ovine fetuses, besides the occurrence of hematopoiesis in the liver and lymph nodes, was the vacuolation of epithelial cells. This feature had been previously described only in the upper part of the small intestine in fetuses of cows, rats and pigs (Moon, 1972; Mathan et al., 1976; Asari et al., 1987). However, at least in sheep, this vacuolation seems to be more common, occurring also in fetal kidneys, eye and esophagus. This finding, together with the presence of inflammatory cells, consistent with mature lymphocytes, in various organs such us liver or muscles, should not be misdiagnosed as degenerative or inflammatory changes caused by *T. gondii* or any other infectious agent.

Regarding Chapter III, an ovine pregnant model was used for an oral infection with a low dose (10 oocysts) of three isolates: TgShSp1 (Type II, ToxoDB#3), TgShSp16 (Type II, ToxoDB#3) and TgShSp24 (Type III, ToxoDB#2). All the infected groups suffered similar reproductive losses (i.e. early or late abortions and stillbirths). These results show that the characterization of the isolate virulence cannot be extrapolated between species, as type III isolates were regarded as less virulent than type II in mice (Dubey, 2009) and also whether the differences observed in other phenotypic markers in the pregnant sheep when characterizing the virulence of the isolate could be an indication of the risk for reproductive failure after infection. After infection of sheep with TgShSp16 and TgShSp24 isolates, these animals showed fever and seroconversion significantly earlier than those infected with TgShSp1. Similar findings were found in an *in vivo* experimental model in piglets (Largo-de la Torre et al., 2022). A rise in body temperature and specific serological antibodies against *T. gondii* are main components of the host responses to an active *T. gondii* infection (Innes et al., 2009). Thus, the difference in the time when analyzing these parameters between groups, could suggest that TgShSp16 and TgShSp24 isolates were recognized by the host immune

response earlier than TgShSp1. However, further investigation on the onset of parasitemia and on the peripheral immune response against each isolate would help to elucidate these differences between groups. At 14 dpi, there were no histological lesions in the placentomes of any of the infected groups, while they were found, although with different frequency, at day 28 dpi. This would confirm that the oral infection with sporulated oocysts would take between 15 and 28 days to cause lesions in this organ (Benavides et al., 2017) at least with the isolates studied so far. Another relevant finding at 28 dpi was that PCR detection showed higher sensitivity in detecting parasite DNA than histological analysis in detecting lesions. This seems to suggest that the invasion of the placenta by the parasite precedes the development of lesions (Gutierrez et al., 2010) and most probably the latter are a consequence of the former, as it has been previously suggested (Castaño et al., 2016). In any case, it is relevant to highlight that, despite similar results regarding the occurrence of reproductive failure, the infected groups showed differences in several parameters, such as the frequency of lesions or the parasite distribution and burden. To summarize, it could be said that those animals infected TgShSp1 showed evidences that suggest the occurrence of a more severe disease than those infected with TgShSp24 and, especially, TgShSp16. It could be hypothesized that TgShSp16 and TgShSp24 may show a delayed placental and fetal invasion, that could be caused by a slower dissemination of the parasite through the host and/or a better control of the parasite by the host immune response. Actually, TgShSp1 has been shown to induce a stronger proinflammatory immune response that TgShSp24 when infecting piglets (Largo-de la Torre et al., 2022). The immune responses mounted in sheep after *T. gondii* is far to be completely understood and its characterization deserves further investigation.

The previously unacknowledged occurrence of vascular changes in ovine toxoplasmosis was remarkable, as it was found at 28 dpi in all infected groups, mainly affecting the maternal vessels of the interdigitating zone of the placentomes. Reports of vascular lesions caused by *T. gondii* infection are uncommon and mainly liked to immunosuppressive conditions in humans or to ocular toxoplasmosis (Vidal, 2019). The parasite could damage the endothelial cells and trigger the occurrence of subsequent vasculitis and thrombosis (Konradt et al., 2016; Smith et al., 2021). Bearing this in mind, it could then be suggested that the vascular lesions found in the placenta of the

current study might be related to the local action of the parasite, taking advantage of the modulation of the immune response that it is thought to occur at the placenta during the second half of gestation. However, it is not clear whether such modulation occurs, or which could be its influence, on sheep (Wattegedera et al., 2019). Although the pathogenesis of these lesions is not clear, it is worth highlighting that the occurrence of early abortions in ovine toxoplasmosis, where the parasite is not detected by PCR, is closely linked to vascular lesions in the placenta, i.e. infarcts (Castaño et al., 2014). Further characterization of these lesions, especially the investigation of the role of endothelial cells and whether it could be caused by the direct effect of the parasite or by indirect mechanisms, such as the deposit of immunocomplexes or oxidative damage to the endothelial cell membrane, would help to elucidate the early mechanisms for toxoplasma abortion.

The Chapter IV of this PhD Thesis was focused on the characterization of the CNS lesions associated to *T. gondii* infection in fetuses, and also in the study on how this infection could influence the numbers of cell populations found in the brain. Similarly, as occurred in the placenta, the infection by TgShSp1 caused the most severe lesions in the brain in terms of percentage of CNS affected area, as a consequence of the higher number and size of pathological foci in the studied samples. Besides, the number of microglial cells, astrocytes and the occurrence of apoptosis were higher in those fetuses infected by TgShSp1, followed by those infected with TgShSp24. It is worth highlighting that fetuses infected with TgShSp16 showed no histological lesions in the CNS and there were no differences in the brain cell populations, or occurrence of apoptosis, with the CNS from the control animals. Differences in the severity of *T. gondii* histological lesions had previously been related to isolates variability (Dubey et al., 2016; Fernández-escobar et al., 2021). Further research is needed in order to clarify whether the variations observed between the groups are linked to differences in the dissemination of the isolates through the hosts or because their virulence once reached de CNS is different.

Regardless the isolate infecting the fetus, the glial foci found in the brain were formed by microglial cells and not recently recruited macrophages, as there was no labelling for calprotectin in these cells (Chilosi et al., 1990). Also, it is remarkable that meningitis and perivascular inflammatory lesions were mainly formed by cells with a

macrophage phenotype, which highlights the relevance of this cell population in the response against *T. gondii* infection in the brain. It remains to be analyzed whether these cells are activated towards a proinflamatory response (M1) or favor parasite replication with an antinflamatory polarization (M2) (Dellacasa-Lindberg et al., 2011) which would promote the dissemination of the parasite (Bhandage et al., 2019). Furthermore, the occurrence of more severe lesions, i.e. more numerous and larger glial foci, was directly related to a decrease in the number of neurons and an increase in the number of astrocytes, microglial cells and caspase-3 apoptotic units. It has been shown that astrocytosis could be triggered by activated microglia or the presence of damaged tissue (Sofroniew & Vinters, 2010; Still et al., 2020) without the necessity of the actual invasion of the brain by the parasite. Actually, the reaction of astrocytes has also been found in the brain of early aborted fetuses, a clinical presentation of ovine toxoplasmosis where the parasite has scarcely reached the placenta (Gutiérrez-Expósito et al., 2020a). On the other hand, the astrocytes are also one of the main target cell of *T. gondii* and a reactive estate of these cells has also been found after direct invasion of the brain by the parasite (Hulinska et al., 1990). The neuronal decrease observed in this study was related to an increase in caspase-3 expression, which could in turn have been triggered after the direct invasion of the neurons by the parasite, a mechanism already described (Cabral et al., 2016). Another possibility is that the activated astrocytes or microglial cells could have produced mediators such as pro-inflammatory cytokines and chemokines, which could also be triggering apoptosis (Zhang et al., 2014; Wang et al., 2019a).

This PhD Thesis brings new insights into the influence of *T. gondii* isolates on the pathogenesis of ovine toxoplasmosis, pointing a broken linkage between virulence and genotype, as well as between *in vitro* and *in vivo* experimental models. The inter-species differences observed contribute to elucidate the lack of correlation between animal species. Physiological morphological changes in ovine placentas and fetuses have been described, greatly assisting *T. gondii* experimental infections in the ovine pregnant model. Furthermore, newly described vascular lesions and brain cell populations changes open the door to new research in order to fill the knowledge gaps regarding abortion pathogenesis and ovine *T. gondii* neuropathogenesis respectively.

CONCLUSIONS

1. The different isolates of *Toxoplasma gondii* causes variations in the cell/parasite interaction and cytokine transcription after *in vitro* experimental infection of ovine macrophages derived from peripheral blood monocytes, suggesting a different polarization of macrophages in response to infection according to the isolate.

2. The oral infection of pregnant sheep with a low dose of sporulated *Toxoplasma gondii* oocysts at day 90 gestation is a useful experimental model that has proven its efficacy on the evaluation of the influence of isolates variability in ovine toxoplasmosis. The study of the placenta and fetuses at days 14 and 28 post infection, especially the latter, is an adequate approach to investigate the early events in both the dissemination of the parasite and development of lesions.

3. The oral infection of pregnant sheep with *Toxoplasma gondii* isolates showing inter- and intra-genotypic differences previously studied *in vivo* and *in vitro* assays, triggers variations on the pathogenesis of the disease. There are differences on the onset of fever, serological response, lesion development or parasite detection and burden, but all the isolates cause similar reproductive failure in the sheep.

4. The placenta and fetuses from non-infected control sheep show macroscopic and histological changes that might lead to misinterpretation when analyzing samples for pathological examination in an experimental model of pregnant sheep.

5. The scarcity of natural killer cells and B lymphocytes within ovine placentomes suggest that, in this species, these cell populations might not play a relevant role during the second half of gestation.

6. Experimental oral infection of pregnant sheep with *Toxoplasma gondii*, regardless the isolate inoculated, causes previously unacknowledged vascular lesions in the placenta at day 28 post infection.

7. Besides the necrotic and inflammatory lesions already described, astrogliosis, microgliosis and a decrease in the numbers of neurons, together with an increase of caspase-3 apoptotic events are changes associated with *Toxoplasma gondii* infection in the fetal brains.

CONCLUSIONES

1. Los diferentes aislados de *Toxoplasma gondii*, tras la infección experimental *in vitro* de macrófagos ovinos derivados de monocitos de sangre periférica, provocan variaciones en la interacción de la célula con el parásito y en la transcripción de citoquinas, apuntando a una polarización diferente de los macrófagos dependiendo del aislado.

2. La infección de ovejas con una dosis baja de ooquistes esporulados de *Toxoplasma gondii* a los 90 días de gestación es un modelo experimental útil y apropiado para el estudio de la influencia de la variabilidad de aislados en la toxoplasmosis ovina. El estudio de la placenta y el feto a los 14 y 28 días de infección, especialmente de este último, es una estrategia adecuada para investigar tanto la diseminación del parásito como el desarrollo de las lesiones en el periodo temprano de la enfermedad.

3. La infección oral de ovejas gestantes con aislados de *Toxoplasma gondii* que previamente presentaban diferencias intra- e inter-genotípicas en estudios *in vivo* e *in vitro*, modifica la patogenia de la toxoplasmosis. Ocasiona diferencias en el momento en el que aparece la fiebre y la respuesta serológica, en el desarrollo de lesiones y en la detección del parasito y su carga. Sin embargo, el fallo reproductivo es similar con todos los aislados.

4. En la placenta y fetos procedentes de ovejas sanas en la segunda mitad de gestación se observan hallazgos macroscópicos e histológicos que se deben diferenciar de lesiones en estos tejidos en un modelo experimental de oveja gestante.

5. El número reducido de células “natural killer” y linfocitos B presentes en los placentomas procedentes de ovejas sanas sugiere que, al menos durante la segunda mitad de la gestación, estas poblaciones celulares no desempeñan un papel importante en la placenta de esta especie.

6. La infección oral de ovejas gestantes mediante ooquistes de *Toxoplasma gondii* causa, a los 28 días post infección, lesiones vasculares en la placenta no descritas previamente e independientemente del aislado.

7. Además de las lesiones necróticas e inflamatorias en los encéfalos ovinos se ha observado que la infección por *Toxoplasma gondii* puede ocasionar también astrogliosis, microgliosis, una disminución del número de neuronas y un aumento de la apoptosis.

SUMMARY

Toxoplasmosis is a protozoan infectious disease caused by the parasite *Toxoplasma gondii*. It is an important disease in both in Europe and globally due to its zoonotic potential and the reproductive failure it triggers in livestock, mainly in sheep, which results in heavy economic losses. Despite the fact that there are multiple methods for controlling this disease, such as management, vaccination or treatment; toxoplasmosis is still a very relevant animal health and public problem. There are still many unknown mechanisms of the pathogenesis of ovine toxoplasmosis, mainly regarding the occurrence of abortions. On the other hand, there are evidences that the variability of *T. gondii* isolates could influence the pathogenesis of the disease in murine models and humans' observational studies. However, little is known regarding the influence of this variation in sheep. Bearing in mind that extrapolation of results between species is not always possible, it is necessary to study how the genotypic and phenotypic variability of *T. gondii* isolates could influence the development of the disease in pregnant sheep. For this, it is essential to use an experimental model of pregnant sheep, as the placenta and fetus are the main targets of the parasite. In order to clearly identified lesions caused by the disease, a detailed analysis of the morphological and physiological findings in both placenta and fetuses is also necessary.

Thus, the aim of this PhD Thesis is to analyze the influence of *T. gondii* isolates variability on parasite/host interaction in ovine toxoplasmosis and the consequences it may have on pregnant sheep. For this purpose, four studies have been conducted, focused in four specific objectives and based on two different experiments (Experiment 1 and 2). An *in vitro* assay with ovine macrophages derived from peripheral blood monocytes (OvMØs) was developed for the evaluation of the influence of isolates variability on these cells (Experiment 1). Then an *in vivo* experimental model with pregnant sheep was carried out, where the animals were orally infected with low doses of three genetic and phenotypically different *T. gondii* isolates (Experiment 2).

Chapter I is based on Experiment 1 with the specific aim of developing an *in vitro* model of OvMØs and study the influence that genetic variability of different *T. gondii* isolates may have on their response (objective 1). In this study, OvMØs were infected with six *T. gondii* isolates, obtained from Spanish sheep. Afterwards, the

subsequent cell infections rates and transcript expression of cytokines and iNOS were evaluated. TgShSp24 isolate (Type III, ToxoDB#2) had a higher internalization/infection rate, followed by TgShSp2 (Type II, ToxoDB#1); compared with the rest of type II isolates: TgShSp1, TgShSp3, TgShSp11 and TgShSp16 (Type II, ToxoDB#3). Moreover, these two isolates (i.e. TgShSp24 and TgShSp2) also exhibited increments in cytokines that favored inflammation by a M1 macrophages polarization. TgShSp1 and TgShSp3 (Type II, ToxoDB#3 isolated from abortions) exhibited highest levels of anti-inflammatory cytokines. Isolates coming from abortions (fetal brains) triggered a higher iNOS expression. These results show intra- and inter-genotypic differences in the parasite/macrophage relationship, proving that more genotypic and phenotypic traits must be evaluated for the study of the virulence of *T. gondii* isolates. Besides, macrophages polarization could be affected depending on the infecting isolate.

Chapter II, III and IV are based in the results from Experiment 2, where seventy-three pregnant sheep were divided in four groups an orally inoculated at day 90 of gestation according to the following distribution: 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. Once inoculated clinical sings were evaluated and early and late abortions were noted. Five animals per group were culled at 14 and 28 dpi. The remaining sheep were left until delivery occurred (i.e. lambs or stillbirths).

Specifically, Chapter II is based on the description of the main macroscopic and microscopic findings in healthy non-infected ovine placentas and fetal viscera from the control group, together with the evaluation of the main cell populations found in the placentomes (objective 2). In order to achieve this, control placentas and fetuses were examined macroscopically and microscopically by H-E. Besides, immunohistochemical evaluation of macrophages (Iba1, calprotectin), B (CD20) and T cells (CD3), Natural killer (NK) cells and caspase-3 was carried out on the placentomes. Macroscopically, the placentas showed placentomes with variations in terms of shape and size. The presence of amniotic plaques and allantoic calculi was fairly common too. Regarding histological evaluation, all placentomes showed hyalinization of caruncular septa and intracytoplasmic pigments in the trophoblasts at the hematomata arcade. Moreover, almost half of the placentomes evaluated showed hemorrhages, foci of cellular debris,

protein extravasation and mineralization. Moderate levels of T cells and Iba1 positive macrophages, and scarce B cells were present in the interdigitating zone of the placentome. The most striking finding was the absence of NK cells in the placentome. Cellular debris and areas of hyalinization were not immunolabelled for caspase-3. In fetal organs the most common findings were hematopoiesis, lymphocyte infiltrates and vacuolation of the epithelial cells of the eye, esophagus, intestine and kidney. It is relevant to establish that all these macroscopic and histological findings are found under normal conditions, as they might lead to misinterpretations when analyzing samples from an experimental model of pregnant sheep.

Chapter III is aimed to study the influence of the genetic and phenotypic variability of previously mentioned three *T. gondii* isolates on the development of toxoplasmosis in pregnant sheep at mid gestation (objective 3), and it is based on the examination of all the animals from Experiment 2. The isolates used in this experiment had shown intra- and inter-genotypic variations in previous *in vitro* and *in vivo* experimental studies. In this study, the clinical outcome of the infection up to the end of gestation was investigated, together with the occurrence of histological lesions, the parasite distribution and burden at 14 and 28 dpi. Reproductive failure including early or late abortions and stillbirths was similar for all the groups. However, the onset of fever and seroconversion occurred later for sheep infected with TgShSp1 Isolate compared with those infected with TgShSp16 isolate and TgShSp24 isolate. Regarding serial culling, *T. gondii*-DNA was only detected in few placentomes from TgShSp1 infected sheep at 14 dpi, while at 28 dpi, the parasite was detected in all the groups in at least one animal. However, at 28 dpi, parasite detection and burdens were higher for the TgShSp1 infected group, followed by the infected with TgShSp24. Histological lesions in placentomes and fetal lesions from euthanized animals were only detected at 28 dpi and were more frequent at the TgShSp1 infected group. Infection with TgShSp16 isolate was associated with the lower values of all of these phenotypic traits. Regardless the isolate inoculated into the sheep, vascular lesions (i.e. vasculitis and thrombi) previously unacknowledged in placentomes at 28 dpi were found. These findings suggest that the variability in the infecting isolates could influence the pathogenesis of the disease. However, the outcome of the disease (i.e. reproductive failure) is similar in all the groups, despite the isolate used for infection.

Chapter IV is focused on the characterization of the histological lesions and the changes affecting the resident cell populations in the CNS of fetuses in ovine toxoplasmosis (objective 4). For this study, fetal brains from selected animals of Experiment 2 were used. Those fetal brains showing histological lesions consistent with *T. gondii* infection were characterized by digital analysis in H-E stained sections. Furthermore, the same region of the brain of five fetuses from each group was immunohistochemically labeled for the detection of neurons (NeuN), astrocytes (GFAP), macrophages and microglial cells (Iba1) and recently recruited macrophages (calprotectin). Together with the detection of apoptosis (caspase-3) and axonal damage (APP). Lesions caused by the TgShSp1 isolate in the brain were larger and more numerous and were associated to a larger percentage of damage area in the CNS. These lesions were mainly characterized by glial foci, with or without necrosis that together with the perivascular cuffs, were mainly formed by microglial cells. Moreover, histological lesions were also associated with a higher number of astrocytes, microglial cells and caspase-3 mediated apoptosis in the midbrain. These findings were accompanied by a reduction in the number of neurons. These results demonstrate that the variability of *T. gondii* isolates could influence the severity of the histological lesions in fetal brains. Besides, it seems that there is relation between the severity of histological lesions and the occurrence of microgliosis, astrocytosis, caspase-3 apoptosis and reduction in the number of neurons.

Taken all together, the results of this PhD Thesis demonstrate that the inter- and intra-genotype variability inherent to recently obtained *T. gondii* isolates could influence the host/parasite relationship in ovine toxoplasmosis, and also that findings from experimental models in given species might not be directly extrapolated to others. An experimental model based on OvMØs has demonstrated to be adequate for the investigation of isolate virulence. Besides, macrophages polarization is influenced by the isolate of *T. gondii*. Furthermore, this PhD Thesis provides an advance in the understanding of ovine toxoplasmosis specially regarding abortions, as previously unknown vascular changes were observed early after infection (i.e. 28dpi) and, regarding fetal nervous lesions, as the previous information on brain lesions and resident cell population changes was scarce.

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