UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Genetic and phenotypic characterization of Toxoplasma gondii isolates obtained from sheep and Iberian pigs in Spain

Caracterización genética y fenotípica de aislados de Toxoplasma gondii obtenidos de ganado ovino y cerdo ibérico en España

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Mercedes Fernández Escobar

Directores

Luis Miguel Ortega Mora
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DOCTORAL THESIS

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DOCTORADO CON MENCIÓN INTERNACIONAL

La presente Tesis Doctoral cumple con los requisitos exigidos por la Universidad Complutense de Madrid para obtener la mención de Doctor Internacional:

1) Realización de una estancia mínima de tres meses en una institución de enseñanza superior o centro de investigación fuera de España:

- Centro receptor: Friedrich Loeffler Institut, Institut für Epidemiologie (IfE). Greifswald - Insel Riems (Germany).
- Investigador principal: Dr. Gereon Schares
- Duración de la estancia: 3 meses (15/09/2020-14/12/2020).

2) Los apartados correspondientes al resumen, la introducción, los resultados, la discusión y las conclusiones de la Tesis Doctoral han sido redactados en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta a cualquiera de las lenguas oficiales en España (inglés).

3) La Tesis Doctoral ha sido evaluada por dos expertos pertenecientes a alguna institución de educación superior o instituto de investigación no español.

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“Todo intelectual tiene una responsabilidad muy especial. Tiene el privilegio y la oportunidad de estudiar. A cambio, él le debe a la sociedad el compromiso de representar los productos de su estudio en el modo más simple, claro y modesto que pueda. Lo peor que pueden hacer los intelectuales -el pecado capital- es intentar erigirse en grandes profetas por encima de los demás seres humanos e intentar impresionarlos con filosofías enredadas. Quien no puede hablar con sencillez y claridad debería quedarse callado hasta que pueda hacerlo.”

Karl Popper, 1984. *Against Big Words.* Carta contenida en Karl Popper, 1992. *In Search of a Better World. Lectures and Essays from Thirty years.*

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PRESENTACIÓN DE LA TESIS DOCTORAL EN FORMATO DE PUBLICACIONES

La presente Tesis Doctoral es un compendio de tres estudios publicados en revistas científicas indexadas, en los que se recogen los resultados derivados de las investigaciones realizadas dentro del Programa de Doctorado en Veterinaria. La publicación de estos resultados en revistas cuyo índice de impacto se encuentra situado en el primer cuartil de la relación de revistas del *Journal Citation Reports* (JCR) para su área o áreas afines, supone un marcador de calidad, favoreciendo a su vez su internacionalización. Además, parte de la investigación bibliográfica llevada a cabo para la redacción de la sección introductoria será publicada como artículo de revisión.

La presentación de la Tesis Doctoral en formato de publicaciones se ha realizado cumpliendo con los criterios de idoneidad exigidos por la Comisión de Doctorado y el Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid. A continuación, se enumeran las referencias completas de las publicaciones incluidas en la Tesis Doctoral:

1) Autores: **Mercedes Fernández-Escobar**, Rafael Calero-Bernal, Julio Benavides, Javier Regidor-Cerrillo, María Cristina Guerrero-Molina, Daniel Gutiérrez-Expósito, Esther Collantes-Fernández, Luis Miguel Ortega-Mora.
   Título: Isolation and genetic characterization of *Toxoplasma gondii* in Spanish sheep flocks.
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   Área o categoría temática, cuartil e índice de impacto (JCR): Parasitología, Q1, 2.824.

2) Autores: **Mercedes Fernández-Escobar**, Rafael Calero-Bernal, Javier Regidor-Cerrillo, Raquel Vallejo, Julio Benavides, Esther Collantes-Fernández, Luis Miguel Ortega-Mora.
   Título: Isolation, Genotyping, and Mouse Virulence Characterization of *Toxoplasma gondii* From Free Ranging Iberian Pigs.
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   DOI: doi: 10.3389/fvets.2020.604782.
   Área o categoría temática, cuartil e índice de impacto (JCR): Veterinary Science, Q1, 2.245.

3) Autores: **Mercedes Fernández-Escobar**, Rafael Calero-Bernal, Javier Regidor-Cerrillo, Raquel Vallejo, Julio Benavides, Esther Collantes-Fernández, Luis Miguel Ortega-Mora.
   Título: *In vivo* and *in vitro* models show unexpected degrees of virulence among *Toxoplasma gondii* type II and III isolates from sheep.
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   Revista: *Veterinary Research*, 52(1):82.
   DOI: 10.1186/s13567-021-00953-7.
   Área o categoría temática, cuartil e índice de impacto (JCR): Veterinary Science, Q1, 3.357.

4) Autores: Mercedes Fernández-Escobar, Gereon Schares, Pavlo Maksimov, Maike Joeres, Luis Miguel Ortega-Mora, Rafael Calero-Bernal.
   Título: *Toxoplasma gondii* genotyping: a closer look into Europe.
   Revista: *Trends in Parasitology*, invited submission.

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LIST OF ABBREVIATIONS/ LISTADO DE ABREVIATURAS

AH	Aqueous humor
AIDS	Acquired immunodeficiency syndrome
Ala	Alanine
alt.	Alternative
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BAL	Broncho-alveolar lavage
BKI	Bumped kinase inhibitor
BMMφs	Bone-marrow-derived macrophages
bp	Base pair
BRC	Biological Resource Centre
BSA	Bovine Serum Albumin
CBM-SO	Centro de Biología Molecular - Severo Ochoa
CNS	Central nervous system
CO^2	Carbon dioxide
CSF	Cerebrospinal fluid
Ct	Cycle threshold
DALYs	Disability-adjusted life years
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DCs	Dendritic cells
DH	Definitive host
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post-inoculation
DT	Dye test
e.g.	*Exempli gratia*
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assays
ELISA PP	ELISA percentage of positivity
EU	European Union
F1 progeny	First filial generation
FBS	Foetal bovine serum
FeLV	Feline leukemia virus
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
g	Grams
g	Relative Centrifugal Force (RCF) or G-Force
G	Gauge number (gage)
GRAs	Dense granules secreted proteins
h	Hours
H&E	Haematoxylin and eosin staining
HIV	Human inmmunodeficiency virus
hpi	Hours post-inoculation

HRM	High-Resolution Melting
i.e.	*id est*
IC	Internal control
ID	Identification
IFAT	Indirect fluorescent antibody test
IFN	Interferon
IgG	Immunoglobulin G
IH	Intermediate host
IHA	Indirect haemagglutination assays
Ile	Isoleucine
IMC	Inner membrane complex
Int	Intermediately virulent
IP	Intraperitoneal; intraperitoneally
IR	Invasion rate
IRGs	Immunity-related GTPases
IRPC	Relative index per cent
ISAGA	Immunosorbent agglutination assay
ITS1	Internal transcribed spacer 1
IU	International units
JCR	Journal Citation Reports
LAT	Latex agglutination test
LD_{100}	Lethal dose 100%
LD_{50}	Lethal dose 50%
MAPA	Ministerio de Agricultura, Pesca y Alimentación
MAT	Modified agglutination test
Mb	Million of base pairs
Met	Metionine
MICs	Micronemes secreted proteins
min	Minutes
ml	Milliliter
MLS	Multilocus sequencing
mm	Milimeters
Mn-PCR-RFLP	Multilocus nested PCR-RFLP
MOI	Multiplicity of infection
MRA	Mixed, recombinant, or atypical RFLP patterns
MS	Microsatellite
NA	Data not available
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
ng	Nanograms
Non	Non-virulent
nPCR	Nested-PCR
ºC	Degree Celsius
p value	Probability value
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pInvR	Parasite invasion rate
pmol	Picomol

PO	*Per os* inoculation (oral inoculation)
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
qPCR	Real-time PCR
QTL	Quantitative trait locus
R.D.	Real Decreto / Royal Decree
R^2	Coefficient of determination
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
ROPs	Rhoptries secreted proteins
RT	Room temperature
s	Second (s)
SC	Subcutaneous; subcutaneously
Seq	Sequencing
SNP	Single nucleotide polymorphism
SRS	Super family of surface adhesins
SW	Swiss Webster
Tg	*Toxoplasma gondii* (prefix)
TLR	Toll-Like Receptors
ToxoDB	*Toxoplasma* database
TY	Tachyzoite yield
U	Units
UK	United Kingdom
ULE	University of León (Spain)
UPS	Upstream promoter insertion sequence
UTR	Untranslated region
Val	Valine
VH	Vitreous humor
Vir	Virulent
vs	*versus*
w/v	Weight/volume percentage
WB	Western-blot
$\bar{x}$	Average value
#	Number
µg	Micrograms
µl	Microliter
µm	Micrometer

CHAPTER I

SUMMARY

CAPÍTULO I

RESUMEN

Toxoplasma gondii is an apicomplexan parasite globally distributed with a heteroxenous life cycle that virtually comprises all homoeothermic animals, including humans, as intermediate hosts and felids as definitive hosts. The zoonotic, abortifacient, and foodborne nature of the parasite makes toxoplasmosis a relevant public and animal health concern worldwide.

A comprehensive research effort on *T. gondii* biology along with the rapid development of molecular techniques suitable for strains genotyping over the last decades, led to the initial description of a widely clonal European and North American *T. gondii* genetic population dominated by three main clonal genetic types (I, II, and III), in contrast to an extremely diverse South American population. However, the information available from Europe is limited, with frequent methodological deficiencies and important sampling disparities among regions. Briefly, the available European literature evidences a clear predominance of type II strains (comprising around 80% of samples) coexisting with much less abundant type III and recombinant strains or mixed infections, as well as minor proportions of type I and imported genotypes. In the specific case of Spain, the majority of the scarce investigations dealt with direct genotyping from clinical samples, with the subsequent limitations to classify the strains and the impossibility of extending its characterization.

The virulence of *T. gondii* strains has been conventionally determined by mortality in laboratory mice, with type I isolates traditionally classified as highly virulent (100% lethality regardless of the dose), and types II and III considered intermediate (99-30% lethality) and non-virulent (<30% lethality) in a dose-dependent manner, respectively. Nonetheless, just as *T. gondii* population genetic structure is now known to go beyond this simple clonality, with up to 16 haplogroups described worldwide and an enormous diversity existing in South America, the traditional virulence strains classification is up for debate. In addition to the scarce information on circulating genotypes in Spain, the knowledge about the phenotypic characteristics of such strains is virtually nil.

With this background, the general objective of the present Doctoral Thesis was to obtain a representative panel of isolates from Spanish livestock, namely sheep and Iberian pigs, that might enable us to study the genetic and phenotypic diversity of circulating strains in such farm animals by implementing molecular, *in vivo* and *in vitro* methodologies. In the first specific objective proposed, isolation procedures were implemented on ovine abortion-derived tissues occurred all over our country and submitted for *T. gondii* diagnosis, as well as on myocardial tissues from chronically infected adult sheep slaughtered for human consumption (Sub-objective 1.1). A total of 31 *T. gondii* DNA-positive foetal brains and 50 adult heart tissues associated with high antibody ELISA titres, were selected for bioassay in mice. Thereby, 10 isolates (TgShSp2-10 and TgShSp18) were obtained from five different abortion outbreaks, and 20 isolates (TgShSp11-17 and TgShSp19-31) were obtained from slaughtered adults raised in five different locations. Together with the TgShSp1 isolate available in our laboratory, the panel represented a significant cross-section of the *T. gondii* population infecting Spanish sheep flocks. Genetic characterization of the isolates (Sub-objective 1.2) was firstly carried out by the widely used PCR-RFLP method based on *SAG1, SAG2* (5'- 3' *SAG2*, and *alt. SAG2*), *SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1* and *Apico* markers. As a result, 90.3% (28/31) of the isolates were determined to present a genotype II PRU variant (ToxoDB #3), 6.5% (2/31) of them corresponded to a clonal genotype III (ToxoDB #2) and finally, only 3.2% (1/31) presented a clonal genotype II (ToxoDB #1). Thanks to the great effort on sample collection from ovine abortion outbreaks and the implementation of the PCR-RFLP method on all DNA-positive clinical samples (abortion-derived

tissues and myocardial sample digests, n = 151), a more complex genetic diversity was observed, revealing co-infection events and demonstrating the selection of certain strains during bioassay experiments. Furthermore, PCR-DNA sequencing of fragments of the *SAG3*, *GRA6* and *GRA7* polymorphic genes, led to the confirmation of RFLP results and to the detection of a widespread single-nucleotide polymorphism at the *SAG3* locus across the Spanish population, which had already been described in sheep French isolates, suggesting a common origin in livestock from both neighbouring countries.

Subsequently, and regarding the phenotypic characterization of some of the *T. gondii* isolates recently obtained from sheep (Sub-objective 1.3), a comprehensive virulence assessment in mice procedure including lethal (cumulative mortality) and non-lethal parameters (*i.e.*, cumulative morbidity, as well as tropism, parasite burden and histopathological lesions in different tissues), was designed and further implemented on 10 selected isolates. Moreover, as a valuable complement for the *in vivo* virulence evaluation, an ovine trophoblast cell line (AH-1) was chosen for the characterization of *in vitro* invasion rates and proliferation kinetics of six selected isolates. Thus, most type II isolates possessed non-virulent characteristics, although important intra-genotype differences were observed. The TgShSp16 isolate (#3) stood out with 21% cumulative mortality rate and a significant enhanced ability to disseminate *in vivo* to the brain, despite low-intermediate *in vitro* invasion and proliferation rates. On the other hand, the type III (#2) TgShSp24 isolate presented similar lethality and parasite burdens in mice, as well as the highest invasion rate along with a tachyzoite production at 72 hpi nine to three times higher than that of the rest of the isolates in AH-1 cells. Finally, the *CS3*/*ROP18*/*ROP5* allelic combination of isolates included in phenotypic characterization assays was investigated, resulting in a profile II/2/2 in those isolates with a RFLP-clonal genotype II or PRU variant, and in a profile III/3/3 in those isolates with a RFLP-clonal genotype III.

In the second specific objective, heart tissues from adult Iberian pigs collected at slaughterhouse were subjected to isolation procedures (Sub-objective 2.1). Thus, 15 myocardial tissues from animals with high antibody ELISA titres and reared in different locations of southwestern Spain were bioassayed in mice. As a result, five isolates (TgPigSp1-5) were obtained. Genetic characterization of the isolates (Sub-objective 2.2) by PCR-RFLP resulted in 60% (3/5) of the isolates presenting a genotype II PRU variant (ToxoDB #3) and 40% (2/5) corresponding to a clonal genotype III (ToxoDB #2). Aiming phenotypic characterization of *T. gondii* isolates obtained from Iberian pigs belonging to the two prevalent genotypes in the population (Sub-objective 2.3), the above-mentioned virulence in mice assay was implemented on TgPigSp1 (type III, #2) and TgPigSp4 (type II PRU variant, #3) isolates. Accordingly, the TgPigSp1 isolate was moderately/highly virulent in mice with notable cumulative mortality (87.5%) and morbidity (100%) rates, whereas TgPigSp4 was non-virulent (0% lethality) and triggered only mild clinical signs in 42.1% of seropositive mice. Parasite burdens and histopathological lesions found in mouse tissues supported the significant differences between both strains.

In short, in the different investigations that comprise this Doctoral Thesis, a representative panel of circulating isolates in sheep and Iberian pigs in Spain has been obtained, covering a wide part of the country and especially leading regions in sheep and Iberian pigs breeding. The genetic characterization findings agreed with the available European genotyping data on *T. gondii* strains infecting domestic animals and highlight the specific predominance of the genotype II PRU variant within the haplogroup 2 and the noteworthy prevalence of type III strains. Concerning the virulence assessment, type III isolates presented the most virulent profile among the strains

evaluated, although it should be noted the large intragenotype phenotypic variability within type II and type III genotypes in the *T. gondii* Spanish population. These findings directly contradict former classifications that considered type III strains as the least virulent among the three *T. gondii* clonal types and demonstrate that current widely used genetic characterization methods are not informative enough to sort out *Toxoplasma* population virulence. The *CS3*/*ROP18*/*ROP5* allelic combination of selected isolates showed no strong link with the degree of virulence shown, providing additional evidence that other genetic factors must be involved in the virulence of *T. gondii* in mice. The limitations of current genotyping methods have become apparent in present studies, emphasizing the need to implement next-generation tools (*e.g.*, whole-genome sequencing) that may allow us to obtain much more detailed, accurate, and resolutive genetic information of Spanish *T. gondii* isolates, which in turn may help to explain the wide phenotypic variability observed.

Toxoplasma gondii es un parásito apicomplejo de distribución mundial con un ciclo biológico heteroxeno que prácticamente comprende a todos los animales homeotermos, incluidos los seres humanos, como hospedadores intermediarios, y a los felinos como únicos hospedadores definitivos. El carácter de agente abortigénico, y la naturaleza zoonósica y de transmisión alimentaria de la infección por *T. gondii* hacen que la toxoplasmosis sea una preocupación importante para la salud pública y animal en todo el mundo.

Durante las últimas décadas, los importantes esfuerzos de investigación enfocados a estudiar la biología de *T. gondii* junto con el rápido desarrollo de técnicas moleculares adecuadas para el genotipado de los aislados, llevaron, inicialmente, a la descripción de una población de *T. gondii* predominantemente clonal en Europa y Norteamérica dominada por tres tipos genéticos clonales principales (I, II y III), en contraste con una población extremadamente diversa en Sudamérica. Sin embargo, la información disponible en Europa es bastante limitada, con frecuentes deficiencias metodológicas en los estudios e importantes disparidades de muestreo entre regiones. En resumen, la literatura europea disponible evidencia un claro predominio de cepas tipo II (que comprenden alrededor del 80% de las muestras) coexistiendo con cepas tipo III y recombinantes o infecciones mixtas mucho menos abundantes, así como muy escasos genotipos tipo I e importados. En el caso concreto de España, la mayoría de las escasas investigaciones realizaron el genotipado directo de muestras clínicas, con las consiguientes limitaciones para clasificar las cepas y la imposibilidad de ampliar su caracterización.

La virulencia de los aislados de *T. gondii* se ha determinado convencionalmente por medio de la determinación de la mortalidad en ratones de laboratorio tras la inoculación. Los aislados de tipo I son clasificados, tradicionalmente, como altamente virulentos (100% de letalidad independientemente de la dosis), y los tipos II y III son considerados de virulencia intermedia (99-30%) y baja (<30%) de una manera dependiente de la dosis, respectivamente. No obstante, así como actualmente se sabe que la estructura genética de la población de *T. gondii* va más allá de esta simple clonalidad, con hasta 16 haplogrupos descritos en todo el mundo y una enorme diversidad existente en América del Sur, la clasificación tradicional de los aislados por su virulencia en ratón está sujeta a debate. Además de la escasa información disponible sobre los genotipos circulantes en España, el conocimiento sobre sus características fenotípicas es prácticamente nulo.

Con estos antecedentes, el objetivo general de la presente Tesis Doctoral fue obtener un panel representativo de aislados de *T. gondii* procedentes de especies ganaderas españolas, concretamente ganado ovino y cerdo ibérico, que permitiera estudiar la diversidad genética y fenotípica de la población de *T. gondii* circulante en dichas especies mediante la implementación de metodologías moleculares y modelos *in vivo* e *in vitro*. En el primer objetivo específico propuesto, se llevó a cabo el aislamiento del parásito a partir de tejidos derivados de abortos ovinos, así como de corazones de ovejas adultas crónicamente infectadas y sacrificadas para el consumo humano, procedentes de algunas de las principales zonas productoras de ovino españolas (Subobjetivo 1.1). Para ello, se seleccionaron 31 encéfalos fetales con presencia confirmada de ADN de *T. gondii* y 50 tejidos cardíacos adultos procedentes de animales con altos títulos de anticuerpos por ELISA para su bioensayo en ratón. De este modo, se obtuvieron 10 aislados (TgShSp2-10 y TgShSp18) de cinco brotes de abortos diferentes y 20 aislados (TgShSp11-17 y TgShSp19-31) procedentes de ovejas adultas criadas en cinco localizaciones

distintas. Junto con el aislado TgShSp1 disponible en nuestro laboratorio, estos aislados constituyen un panel representativo de la población de *T. gondii* que circula en los rebaños ovinos españoles. La caracterización genética de los aislados (subobjetivo 1.2) se realizó en primer lugar mediante el método ampliamente utilizado de PCR-RFLP basado en los marcadores moleculares *SAG1*, *SAG2* (5'- 3' *SAG2* y alt. *SAG2*), *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1* y *Apico* (Su et al., 2010). Como resultado, se determinó que el 90,3% (28/31) de los aislados presentaban la variante PRU del genotipo II (ToxoDB #3), el 6,5% (2/31) de ellos correspondía a un genotipo III clonal (ToxoDB # 2) y, finalmente, sólo el 3,2% (1/31) presentó un genotipo II clonal (ToxoDB #1). Gracias al esfuerzo en la recolección de muestras de brotes de abortos y la implementación del método PCR-RFLP en todas las muestras clínicas positivas a la detección de ADN del parásito (tejidos derivados de abortos y digestiones de muestras de miocardio, n = 151), se observó una diversidad genética más compleja en este tipo de muestras cuando se comparó con la información obtenida de los aislados, revelando eventos de coinfección y sugiriendo la selección de ciertas cepas durante los experimentos de bioensayo. Además, la secuenciación de fragmentos de los genes polimórficos *SAG3*, *GRA*6 y *GRA7* permitió confirmar los resultados obtenidos por PCR-RFLP y detectar un polimorfismo de un solo nucleótido en el locus *SAG3* muy extendido en la población española y que ya había sido descrito en cepas ovinas francesas, lo que sugiere un origen común en el ganado de ambos países vecinos.

Para la caracterización fenotípica de aislados de *T. gondii* seleccionados (10 aislados) procedentes de oveja (subobjetivo 1.3), se diseñó un protocolo de evaluación de su virulencia en ratón, incluyendo parámetros letales (mortalidad acumulada) y no letales (*i.e.*, morbilidad acumulada, así como el tropismo, la carga parasitaria y las lesiones histopatológicas en diferentes tejidos). Además, este estudio se completó con la evaluación *in vitro* en una línea celular de trofoblasto ovino (AH-1) de las tasas de invasión y la cinética de proliferación de seis aislados seleccionados. De este modo, la mayoría de los aislados de tipo II poseían características no virulentas, aunque se observaron importantes diferencias intra-genotipo. El aislado TgShSp16 (#3) destacó con una tasa de mortalidad acumulada del 21% y una capacidad significativamente mayor para diseminarse *in vivo* al cerebro, a pesar de las tasas de invasión y proliferación *in vitro* intermedias-bajas. Por otro lado, el aislado de tipo III (#2) TgShSp24 presentó una letalidad y cargas parasitarias similares en ratones, así como las mayores tasas de invasión y producción de taquizoítos a las 72 horas post-inoculación *in vitro*, esta última entre tres y nueve veces mayor que la alcanzada por el resto de los aislados en las células AH-1. Finalmente, se investigó la combinación alélica de los loci *CS3*/*ROP18*/*ROP5* en los aislados incluidos en los ensayos de caracterización fenotípica, determinándose un perfil II/2/2 en aquellos aislados con un genotipo II clonal o variante PRU, y un perfil III/3/3 en aquellos aislados con un genotipo III clonal.

En el segundo objetivo específico, el miocardio de cerdos ibéricos adultos fue recogido en matadero y procesado para el aislamiento del parásito (subobjetivo 2.1). Se inocularon en ratones 15 tejidos de animales con altos títulos de anticuerpos por ELISA y criados en diferentes localizaciones del suroeste de España. Como resultado del bioensayo, se obtuvieron cinco aislados (TgPigSp1-5). La caracterización genética de los aislados (subobjetivo 2.2) por PCR-RFLP resultó en un 60% (3/5) de ellos presentando la variante PRU del genotipo II (ToxoDB #3) y un 40% (2/5) correspondiente a un genotipo III clonal (ToxoDB #2). Con el objetivo de caracterizar fenotípicamente estos aislados (subobjetivo 2.3), se llevó a cabo el ensayo de virulencia en ratones antes mencionado con las cepas TgPigSp1 (tipo III, #2) y TgPigSp4 (variante PRU del tipo II, #3). En consecuencia, el aislado TgPigSp1 mostró una virulencia entre moderada y alta en ratón, con unas notables tasas de mortalidad (87,5%) y morbilidad (100%)

acumuladas, mientras que el aislado TgPigSp4 resultó avirulento (0% de letalidad) y causó únicamente signos clínicos leves en el 42,1% de los ratones seropositivos. Las cargas parasitarias y las lesiones histopatológicas encontradas en los tejidos de los ratones respaldaron las diferencias significativas entre ambas cepas.

En definitiva, en las diferentes investigaciones que integran esta Tesis Doctoral se ha obtenido un panel representativo de aislados de *T. gondii* circulantes en ovejas y cerdos ibéricos en España, que abarca una amplia superficie del país, y especialmente, las regiones líderes en la cría de ganado ovino y porcino ibérico. Los resultados de la caracterización genética son similares a los datos europeos disponibles sobre genotipado de cepas de *T. gondii* que infectan animales domésticos, y destacan el predominio específico de la variante PRU del genotipo II dentro del haplogrupo 2 y la notable prevalencia de las cepas de tipo III. En cuanto a la evaluación de la virulencia en ratón, los aislados de tipo III presentaron el perfil más virulento entre las cepas evaluadas, aunque cabe destacar la gran variabilidad fenotípica intra-genotipo dentro de los tipos II y los tipos III en la población española de *T. gondii*. Estos hallazgos están en contradicción con las clasificaciones convencionales que consideraban a las cepas de tipo III como las menos virulentas entre los tres tipos clonales de *T. gondii*, demostrando que los métodos de caracterización genética ampliamente utilizados en la actualidad no son suficientemente resolutivos para clasificar la virulencia de la población de *Toxoplasma*. La combinación alélica *CS3*/*ROP18*/*ROP5* de los aislados seleccionados no estuvo asociada con el grado de virulencia observado, proporcionando nuevas evidencias de que otros factores genéticos deben estar involucrados en la virulencia de *T. gondii* en ratón. En este estudio, se han puesto en evidencia las limitaciones de los métodos de genotipado actuales, poniendo de relieve la necesidad de desarrollar nuevas herramientas basadas en las tecnologías de secuenciación de nueva generación (*e.g.*, la secuenciación del genoma completo) que nos permitan obtener información genética mucho más detallada, precisa y resolutiva. Esto podría, a su vez, ayudar a explicar la amplia variabilidad fenotípica observada.

CHAPTER II

INTRODUCTION

CAPÍTULO II

INTRODUCCIÓN

1. *Toxoplasma gondii* and toxoplasmosis

1.1 *Toxoplasma gondii*

Toxoplasma gondii, the etiologic agent of toxoplasmosis, is an apicomplexan obligate intracellular parasite of major medical and veterinary importance. Since its discovery in 1908 by Nicolle and Manceaux, infecting tissues of the North African rodent *Ctenodactylus gundi*, an intense scientific work led to the description of its complex life cycle and the definition of the species as one of the most widespread and successful opportunistic pathogens worldwide (Dubey, 2010). *Toxoplasma gondii* can infect virtually all warm-blooded animal species, including humans, which defines it as an excellent example of the One Health concept (Djurković-Djaković et al., 2019). Its wide host range makes the parasite to be an important problem not only for public health, but also for livestock industry and wildlife conservation programmes. Although toxoplasmosis is normally associated with asymptomatic chronic establishment of the infection, clinical disease could imply serious damage to the foetus in pregnant host, and severe neurologic and pulmonary implications in immunocompromised individuals. Currently, there is only one available commercial live-attenuated vaccine indicated for veterinary use in sheep and no effective treatments are available for toxoplasmosis at present (Sánchez-Sánchez et al., 2018; Konstantinovic et al., 2019; Wang et al., 2019).

1.1.1 Taxonomic classification

Toxoplasma gondii is a protist parasite of the subphylum Apicomplexa, taxonomic group of endoparasites of animals characterized by the presence of an apical complex in its cellular structure, with more than 6000 members. The infections involving apicomplexan parasites represents a huge burden on public and animal health worldwide (*e.g., Plasmodium, Cryptosporidium*, *Sarcocystis*, and *Toxoplasma* genera) as well as a notable economic impact on agriculture (*e.g.*, *Eimeria, Besnoitia, Neospora, Babesia* and *Theileria* genera among others) (Swapna and Parkinson et al., 2017). Within Apicomplexa subphylum, *T. gondii* belongs to the Coccidia subclass, as other parasites whose life cycle includes merogony, gametogony and sporogony phases. *Toxoplasma gondii* was thought to only parasitize extraintestinal tissues of a wide range of warm-blooded hosts, until 1970 when Frenkel and his collaborators found the parasite as an intestinal coccidium of cats with an isosporan-like oocyst stage (Frenkel et al., 1970). Within Coccidia subclass, *T. gondii* is included into the family Sarcocystidae, which comprises other genera of cyst-forming parasites with heteroxenous life cycles including stages of sexual and asexual replication and the oocysts sporulation in the environment, such as *Neospora*, *Besnoitia*, and *Hammondia* (Tenter et al., 2002). It is the only species of the genus. The taxonomic classification of *T. gondii* is detailed in the **Figure 1**.

1.1.2 Morphology

There are three known invasive stages in the complex heteroxenous life cycle of *T. gondii*: tachyzoites, bradyzoites contained within the tissue cysts, and sporozoites inside the oocysts.

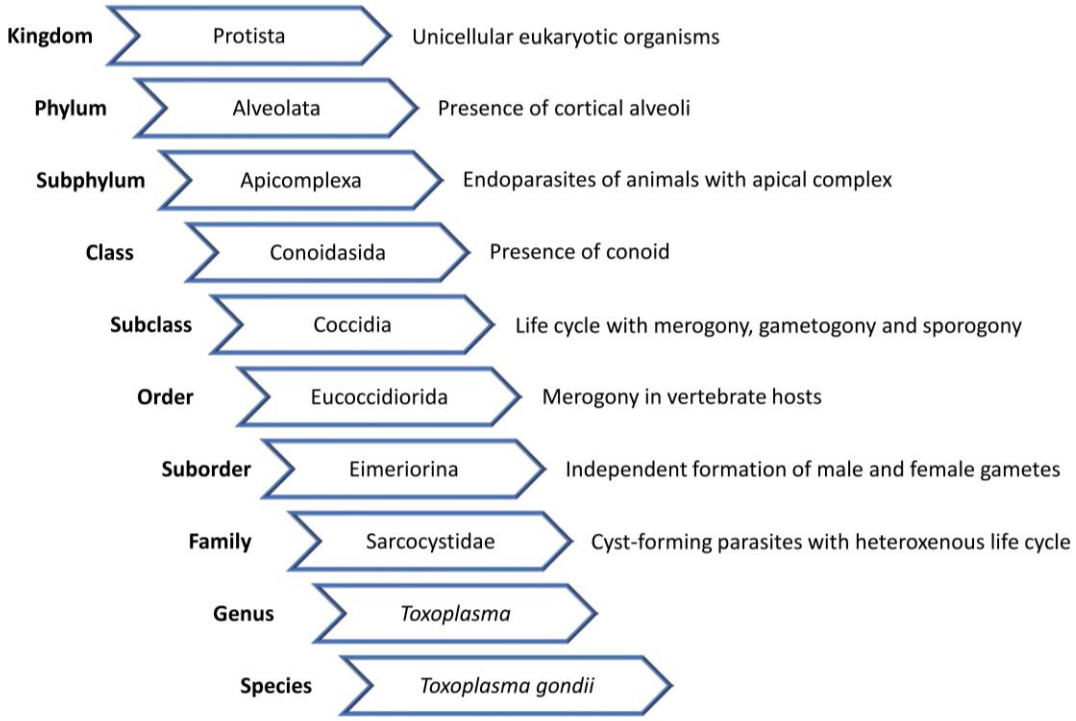

Figure 1. Taxonomic classification of *Toxoplasma gondii*.

Tachyzoites are the rapidly multiplying stage of *T. gondii*, responsible of the acute phase of the infection, intraorganic dissemination, and tissue damage in the host. Tachyzoites are crescent-shaped cells of approximately 2 x 6 µm, capable of invading almost all types of nucleated cells, developing inside a structure called parasitophorous vacuole. Here, the parasite asexually replicates repeatedly by endodyogeny until host cell lysis, when egression and invasion of adjacent cells occur (**Figure 2A, B**). Thus, continuous lytic cycles together with the related immunopathologic consequences lead to the acute clinical disease (Dubey et al., 1998; Dubey, 2010).

Bradyzoites are the slow-replicating stage of the parasite. They are responsible for the chronification of the infection, remaining confined inside tissue cysts specially in immunoprivileged host tissues like the central nervous system (CNS), the eye, and the skeletal muscle (**Figure 2C, D**). Tissue cysts grow and remain intracellular, as the bradyzoites divide inside by endodyogeny up to several hundreds, varying in diameter and form depending on the time since formation and the tissue invaded; in the brain, cysts are often spheroidal and rarely reach a diameter of 70 µm, whereas intramuscular cysts are elongated and may be 100 µm long. The tissue cyst wall is elastic, argyrophilic, and < 0.5 µm thick (Dubey et al., 1998; Dubey, 2010). Bradyzoites represent the quiescent life stage of the parasite and can persist inside tissue cysts without causing clinical signs for the entire life of the host, although relapse cases have been well documented, in relation with immunodepression conditions such as cancer, transplant treatments (*e.g.*, corticosteroids) or immunosuppressive infections (*e.g.*, HIV) (Delhaes et al., 2010b; Stajner et al., 2013; Pena et al., 2017).

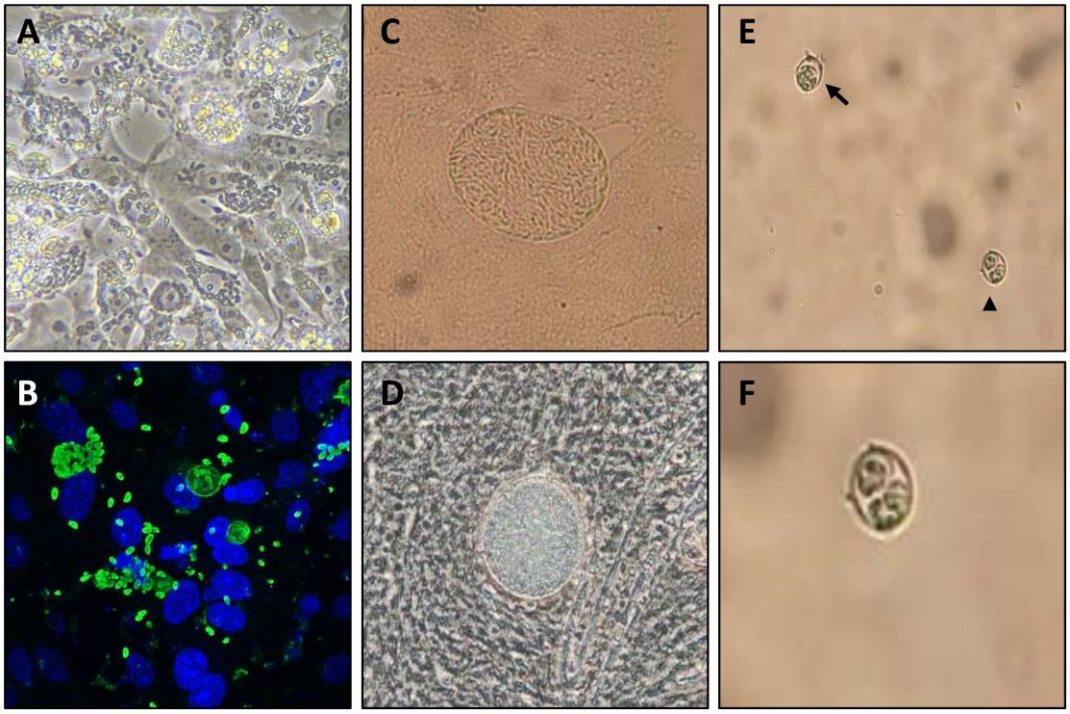

Figure 2. Invasive stages in the life cycle of *Toxoplasma gondii*. (**A**) Tachyzoites (unstained, 40x); (**B**) tachyzoites (immunolabelled, 40x). (**C**, **D**) Tissue cysts in mouse brain (unstained, 40x). (**E**) Unsporulated (arrow) and sporulated (arrowhead) oocysts (unstained, 40x). (**F**) Sporulated oocyst (unstained, 100x). Source: SALUVET and Fernández-Escobar et al. 2021, *Veterinary Research* (p. 87).

Finally, the **sporozoites** are contained within the sporulated oocysts, the environmentally resistant stage of *T. gondii*. The oocysts are excreted unsporulated to the environment in faeces by the definitive host (only members of the Felidae family) (Martorelli et al., 2019). Sporulation occurs within 1 to 5 days after shedding (depending on aeration and temperature), requiring an event of sporogony after which the oocysts become infective and environmentally resistant. As mentioned above, *T. gondii* possesses an isosporan-like oocyst stage, with spherical shape and approximately 11 x 13 µm in diameter, characterized by containing two ellipsoidal sporocysts and four sporozoites and a residual sporocystic body inside each of them (**Figure 2E, F**) (Dubey et al., 1998; Dubey, 2010).

To refer to any stage of *T. gondii* life cycle the general term zoite is used. *Toxoplasma gondii* zoites present a complex ultrastructure that includes several key organelles, differing slightly from each other (Dubey et al., 1998). Apart from typical organelles of any eukaryotic cell, *T. gondii* zoites present structures unique to apicomplexan parasites (**Figure 3 A-E**):

- The pellicle: a three-layered structure composed by the outer plasma membrane (plasmalemma) and the underlying inner membrane complex (IMC). It interacts with the glideosome (an actin-myosin motor complex) and with subpellicular microtubules powering motility and invasion (Dubey, 2010; Frénal et al., 2017).

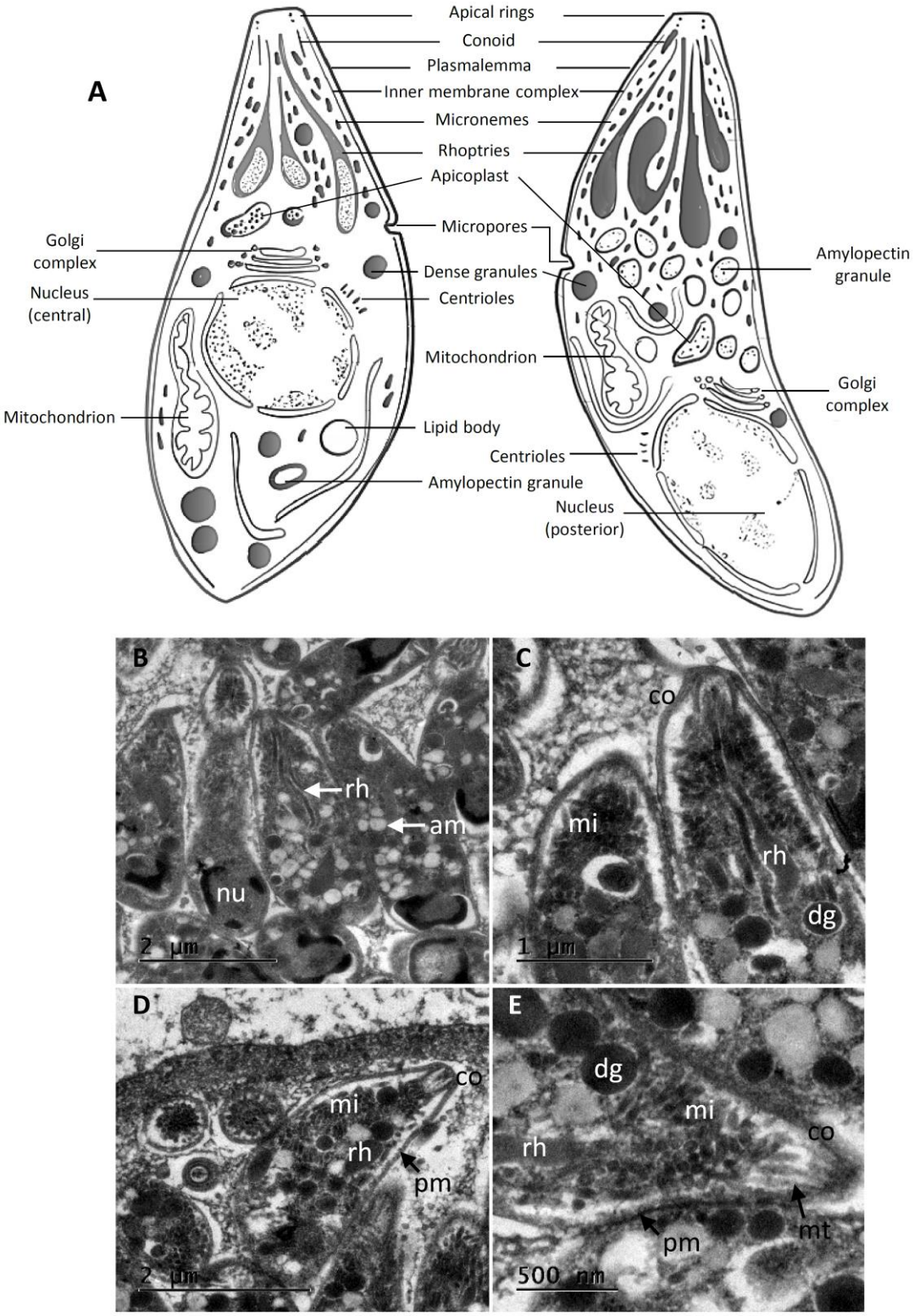

Figure 3. (**A**) Schematic drawings of the ultrastructure of *Toxoplasma gondii* tachyzoites (left) and bradyzoites (right). Adapted from Marta García-Sánchez, Doctoral Thesis, 2019. (**B**-**E**) Transmission electron microscopy micrographs of *Toxoplasma gondii* bradyzoites inside a tissue cyst showing ultrastructural details. Note, nucleus (nu), rhoptries (rh), amylopectin granules (am), conoid (co), micronemes (mi), dense granules (dg), plasmalemma (pm), microtubules of the conoid (mt). Source: SALUVET.

- The apical complex: characteristic set of cytoskeletal structures essential for parasite motility, attachment, and invasion. The subpellicular microtubules, the conoid, two apical rings and two polar rings compound the cytoskeleton of the cell, driving the support and movement of the parasite (Hu et al., 2006).
- Cytoplasmic organelles:
    - Micronemes and rhoptries: secretory organelles associated to the apical end of the cell. Micronemes are small vesicles that secrete proteins (MICs) involved in the recognition and adhesion to the host cell, whereas proteins secreted by the rhoptries (ROPs) have a key role in the formation and maturation of the parasitophorous vacuole (PV) as well as in parasite survival by modulating host immune and metabolic responses (Venugopal and Marion, 2018).
    - Dense granules: secretory organelles distributed throughout the cell, but mainly at the posterior end. Dense granules secreted proteins (GRAs), similarly to ROPs, are essential to maintain the structure and integrity of the PV, and to ensure parasite survival by modulating several host cells functions (Venugopal and Marion, 2018).
    - Amylopectin granules.
    - The apicoplast: a relict non-photosynthetic plastid with importance as a therapeutic target, since it contains genetic material codifying for essential pathways of parasite metabolism, such as fatty acids biosynthesis (Sheiner et al., 2013).

1.1.3 Life cycle

Toxoplasma gondii is a ubiquitous parasite capable of infecting an unusual wide range of hosts. The complex life cycle of *T. gondii* is defined as facultative heteroxenous, with virtually all warm-blooded animals as intermediate hosts (IH), including most livestock species and humans, and members of the Felidae family (wild and domestic cats) as definitive hosts (DH). The life cycle is divided into three well-defined stages: an enteroepithelial sexual stage, an exogenous stage in the environment, and an extraintestinal asexual stage (Figure 4).

- Enteroepithelial sexual stage

Toxoplasma gondii carries out the sexual portion of its life cycle exclusively in the small intestine of the definitive host (Martorelli et al., 2019). After the ingestion of tissue cysts by cats, the tissue cyst wall is digested by proteolytic enzymes in the stomach and small intestine. The released bradyzoites (haploid) penetrate the epithelial cells of the small intestine and initiate the asexual development of numerous generations of *T. gondii*. Five morphologically distinct asexual types of *T. gondii* (types A to E) develop in intestinal epithelial cells in a continuous asexual replication cycle of merozoite-schizont by schizogony before gametogony begins. After fertilization of haploid macrogametes by haploid microgametes, resulting in a diploid zygote formation, an oocyst wall is developed around the parasite and epithelial cells lysis permits the release of the unsporulated oocysts to the lumen (Tenter et al., 2000; Dubey, 2010).

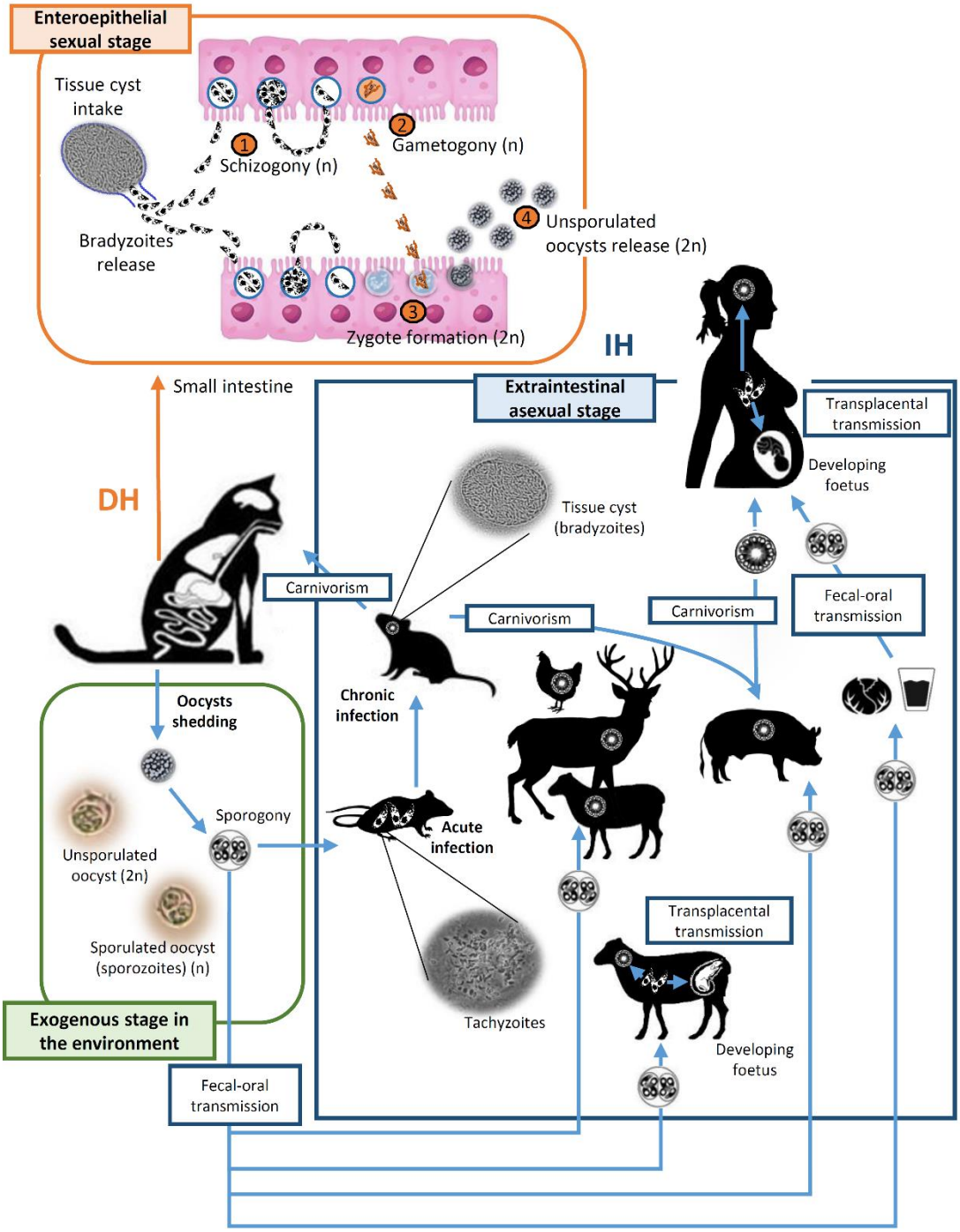

Figure 4. *Toxoplasma gondii* life cycle and transmission routes.

- Exogenous stage in the environment

Felids shed unsporulated non-infectious oocysts in their faeces, but sporogony occurs in the environment within 1 to 5 days given suitable conditions of aeration, humidity, and temperature. Sporogony involves meiosis (postzygotic) and sporulation, ultimately producing two sets of four haploid sporozoites, contained within a second set of walled structures, called sporocysts. Sporulated oocysts are infectious for both the IH and the DH (Dubey, 2010; Ramakrishnan et al., 2019).

- Extraintestinal asexual stage

After ingestion of sporulated oocysts by an IH, sporozoites excyst, penetrate enterocytes and goblet cells of the intestinal epithelium, and move to the lamina propria where they multiply asexually by endodyogeny in a variety of nucleated cells until dividing into tachyzoite forms. Tachyzoites disseminate generally and transform into bradyzoites to persist inside tissue cysts in a wide variety of organs as a chronic infection, commonly for the lifetime of the host. Bradyzoites continue multiplying slowly by endodyogeny inside the tissue cyst. The bradyzoite-induced cycle in the intermediate host is similar to that of the oocyst-induced cycle but bradyzoites are apparently less infective than sporozoites (Dubey, 2006; Dubey, 2010).

As we mentioned above, DH could also get infected by sporulated oocysts. It has been hypothesized that sporozoites first convert to tachyzoites in the intestinal epithelium of felids, then tachyzoites convert to bradyzoites, and when tissue cysts rupture, a few bradyzoites return to the intestinal epithelium to initiate the enteroepithelial sexual cycle. *Toxoplasma gondii* has adapted to an oocyst-oral route in intermediate hosts and a tissue cyst-oral route in carnivores, especially in the cat. *Toxoplasma gondii* oocysts are less infective and less pathogenic for the cat than for other hosts, conversely to bradyzoites (Dubey, 1996; Dubey 2006).

Thus, *T. gondii* may be transmitted from definitive to intermediate hosts, from intermediate to definitive hosts, as well as between definitive and between intermediate hosts (Figure 4). The continuity of the cycle is not limited to the presence of a certain host species, remaining indefinitely by transmission of tissue cysts between intermediate hosts (even in the absence of definitive hosts) and also by transmission of oocysts between definitive hosts (even in the absence of intermediate hosts) (Tenter et al., 2000).

1.2 Toxoplasmosis

1.2.1 Epidemiology

As described previously, the three biological stages, tachyzoites, bradyzoites, and sporozoites are infectious with a variable degree of efficiency (Dubey, 2005; Dubey, 2006) for both intermediate and definitive hosts, which may acquire the infection via one of the following routes (Dubey et al., 1998) (**Figure 4**):

1) Fecal-oral route: horizontal transmission by oral ingestion of sporulated oocysts from the environment (*i.e.*, contaminated water, soil or food) (acquired toxoplasmosis).

2) Carnivorism: horizontal transmission by oral ingestion of tissue cysts contained in raw or undercooked meat or viscera of intermediate hosts (acquired toxoplasmosis).
3) Transplacental route: vertical transmission by tachyzoites transference from a pregnant host to the foetus (congenital toxoplasmosis).
4) Iatrogenic transmission: transmission by tachyzoites via transfusion of packed leukocytes (ordinary blood transfusion is virtually free from risk) or laboratory accidents, as well as transmission by tachyzoites/bradyzoites via transplantation (*e.g.*, solid organs).

Toxoplasma gondii infection in humans and animals is widespread all over the world. *Toxoplasma* infection has been described for more than 350 host species, mammals and birds, with the vast majority of them living in a wild environment (Robert-Gangneux and Dardé, 2012). The seroprevalence in felids is a crucial issue because they are the only hosts that can excrete the environmentally resistant oocysts and can directly transmit the parasite to humans and livestock. Seroprevalence of *T. gondii* infection varies widely according to the age, lifestyle (stray, wild, or domestic), breed and country ranging between 0 to 100% of seropositivity. Conclusions are difficult to make but low values appear to be more frequent in Asian countries like Thailand or Korea, and high values appear to be typical of African or South American surveys from countries like Egypt, Ethiopia, Mexico or Brazil (reviewed in Dubey et al., 2020a). In the case of Spain, seroprevalence ranged from 10 to 84.7 % in domestic cats (Millán et al., 2009a; Miró et al., 2011), and from 62.8 to 80.7 % in wild felids studied (Millán et al., 2009b; García-Bocanegra et al., 2010a).

Prevalence of *T. gondii* infection as well as the main transmission route in each IH is influenced by several factors, mainly the presence of felids in their environment, the climate conditions (favouring sporulation and survival of oocysts in the environment), susceptibility to *Toxoplasma* infection (some species may be more resistant) or the diet and feeding behaviour of the host species (Robert-Gangneux and Dardé, 2012). It is considered that approximately one-third of human population is infected with *T. gondii* worldwide although figures vary notably between countries (from 10 to 80%). The lowest seroprevalences are found in countries of North America, South East Asia, and northern Europe (10 – 30%), intermediate values normally come from central and southern Europe (30 – 50%), whereas the highest rates are detected in Latin America and in tropical African countries (Pappas et al., 2009). In Spain, seroprevalence studies carried out all over the territory between 1992 and 2010 showed figures ranged from 11.2 to 42.4%, in concordance with above mentioned classification (reviewed in Calero-Bernal et al., in preparation).

Special attention must be paid to meat-producing animals due to the zoonotic character of the parasite, knowing that human infections are mainly acquired after ingestion of raw or undercooked meat containing viable *T. gondii* tissue cysts (Cook et al., 2000; Opsteegh et al., 2011; Belluco et al., 2018). *Toxoplasma gondii* is considered a specific risk for food safety in the European Union (EU) (EFSA, 2007; EFSA, 2011; De Berardinis et al., 2017) and the second causal agent of foodborne illness in the USA (Scallan et al., 2011). As farm animals represent simultaneously a major source of infection for humans and reservoirs of *T. gondii* for wildlife predators, there is an emerging concern about it, with efforts made in improving our knowledge on real prevalence, main risk factors, economic impact, characterization of strains circulating in livestock, developing vaccines and even in obtaining "*Toxoplasma*-free" meat (Hiszczyńska-Sawicka et al., 2014; Djokić et al., 2016; Stelzer et al., 2019; Dubey et al., 2020b,d; Gutiérrez-Expósito et al., submitted).

1.2.2 Pathogenesis, clinical signs and lesions

Toxoplasma gondii infection outcome varies depending on the genetic background and immune status of the host, as well as on the parasite genotype. The resistance or susceptibility to infection seems to differ depending on the host species and even the subspecies, as has been demonstrated in the case of rodents (Hassan et al., 2019; Mukhopadhyay et al., 2020). There seems to be a pattern in which those hosts that have historically evolved together with the parasite develop greater resistance to the disease. Proof of this is the fatality of the infection in Australian marsupials, whose evolutionary history has practically developed in the absence of felids (Innes, 1997). In the same way, the genotype of the strains to which the host is exposed is also decisive. For example, type I strains are more prevalent in North and Southeast Asia, where *Mus musculus castaneus* and *M. m. musculus* are also more abundant. Type I strains do not kill these mice but are extremely virulent to *M. m. domesticus*, which is the dominant subspecies in Europe and North America. By contrast, type II and III strains, which predominate in Europe and North America, generally do not kill *M. m. domesticus* (Shwab et al., 2014; Mukhopadhyay et al., 2020). Other animals are more resistant to *Toxoplasma* infection, with the infection usually being inapparent or producing only transient mild symptoms during the acute phase, although the host remains chronically infected for its lifetime. This group includes cattle, pigs, and humans, among others. This could be explained by the long-time co-evolution of these species with the domestic cat derived from their domestication by man. In essence, an evolutionary arms race of reciprocal selection pressure between virulence factors of the parasite and immune defences of the host might drive the species-dependent susceptibility to toxoplasmosis (Gazzinelli et al., 2014; Mukhopadhyay et al., 2020). Obviously, the immune status of the host also influences the development of the disease. *Toxoplasma gondii* is considered the most frequent opportunistic pathogen in AIDS patients and there are numerous reports of infections after immunosuppressive treatments or transplants (Collazos, 2003; Ajzenberg et al., 2002b, 2009). In addition, the imbalance of the immune response during pregnancy is one of the main causes of the different manifestations that *Toxoplasma* infection has during pregnancy.

- Acquired toxoplasmosis

Toxoplasma gondii infection is usually subclinical but could lead to severe clinical manifestations in immunodepressed or pregnant hosts. Natural infections are mainly acquired by ingestion of meat containing tissue cysts or oocyst-contaminated food or water. Thus, during the first days after infection (acute stage) bradyzoites or sporozoites penetrate the intestinal epithelial cells, multiply, and spread locally to mesenteric lymph nodes and to distant organs by invasion of lymphatics and blood (parasitemia). This acute phase occurs between 4- and 12-days post-infection and may cause unspecific clinical signs such as mild fever, dyspnea, arthralgia, fatigue, or lymphadenopathy. The enteroepithelial invasion develops into enteritis and necrosis lesions of the intestine and mesenteric lymph nodes. Necrosis is caused by the intracellular growth of tachyzoites, and could also occur in diverse organs (*e.g.*, lungs, liver, eyes, heart, or adrenals, among many others, mainly visceral tissues) after general dissemination of the parasite.

After the acute period, the host normally controls the infection due to acquisition of humoral and cellular immunity against the parasite, resulting in inflammation and the start of the chronic phase. After this inflammation stage, infection clearance starts and tachyzoites migrate from mostly visceral tissues to immunoprivileged organs (*e.g.*, brain, eyes, muscle tissues), where transform into encysted bradyzoites remaining safe from host immune response (Dubey, 2010). Intact tissue

cysts probably do not cause any harm and persist for the lifetime of the host. However, there are evidence of cysts ruptures, with clusters of tissue cysts observed in brain tissues or even reactivation of the disease (Ajzenberg et al., 2009; Dubey, 2010; Calero-Bernal and Gennari, 2019). The mechanism of relapse has been linked to immunosuppression conditions but remains largely unknown (Dubey, 2010). Cases of repeat shedding of oocysts in cats are documented in literature (Dubey, 2010). Ocular toxoplasmosis is one of the most common clinical manifestations of *T. gondii* infection, mainly characterized by necrotizing retinitis with secondary choroiditis (retinochoroiditis), occurring adjacent to a pigmented retinochoroidal scar. It is frequent in both congenital infections and in immunocompromised hosts, but it may also occur in immunocompetent patients (Butler et al., 2013).

In case of immunosuppressed host, the acute phase of enteroepithelial invasion and dissemination could result in exacerbated outcomes involving vital organs (*e.g.*, toxoplasmic pneumonitis, encephalitis or myocarditis, among others), or even in a fatal acute toxoplasmosis, with multi-organ failure and death. Three immunosuppression clinical settings are considered here: certain immunosuppressive viral infections (*e.g.*, human immunodeficiency virus [HIV]) in humans; feline immunodeficiency virus [FIV] and feline leukemia virus [FeLV] in cats), transplantation, and therapy of malignant diseases (*e.g.*, immunosuppressive chemotherapy) (Davidson et al., 1993; Dubey, 2010; Calero-Bernal and Gennari, 2019). In addition to primary infection, in immunocompromised hosts is more likely the disease reactivation by tissue cysts rupture (from a previous subclinical infection) (Ajzenberg et al., 2009; Calero-Bernal and Gennari, 2019). Cerebral toxoplasmosis is the most common cause of expansive brain lesions in AIDS patients, and also constitutes a potentially lethal risk for other immunocompromised patients usually associated to the reactivation of a latent past cerebral toxoplasmosis (Schlüter and Barragan, 2019).

- Congenital infection

In pregnant hosts that acquire the infection, vertical transmission by tachyzoites transference to the foetus (congenital transmission) occurs. Parasitemia during pregnancy may result in placentitis, tachyzoites bypassing the placental blood barrier and invasion of the foetal organs compromising the developmental process. As during a chronic reactivated infection, the congenital infection manifests mainly in the CNS (of the foetus in this case) (Schlüter and Barragan, 2019). Congenital infection can lead to a wide variety of manifestations depending on the stage of gestation when infection occurs, ranging from early embryonic death with reabsorption (early gestation) to stillbirth or neonatal death (mid-gestation), or even the birth of transplacentally infected progeny (late gestation) (Dubey, 2010; Khan and Khan, 2018). *Toxoplasma gondii* induces typical histologic lesions in the placenta, consisting of multifocal necrosis and mineralization of cotyledonary villi, as well as in the foetus, generally involving infiltrates of diverse immune cells often accompanied by necrosis in multiple organs (Dubey, 2010).

Toxoplasma gondii vertical transmission has been especially studied in humans and small ruminants due to its higher prevalence and impact in these species. In humans, common signs reported in congenitally infected infants are hydrocephalus or microcephalus, cerebral calcifications, retinochoroiditis and long-term disabling sequelae; retinochoroiditis or neurological involvements could also appear later in life (Dubey and Jones, 2008). Likewise, the expulsion of small mummified foetuses or the birth of weak lambs is common in sheep when

infection takes place at middle or late gestation, respectively (Benavides et al., 2017; Dubey et al., 2020d). Cases of congenital transmission in relation to toxoplasmosis reactivation during pregnancy have been documented in women (Ladas et al., 1999; Silveira et al., 2003; Garweg et al., 2005), and while recrudescence is frequent in successive goat pregnancies, its importance is under debate in pregnant sheep (Dubey, 1982; Benavides et al., 2017). Cattle and horses are considered highly resistant to clinical toxoplasmosis with scarce reports on reproductive failure, whereas sheep and goats are highly susceptible, and pigs remain at an intermediate degree of susceptibility (Dubey, 2010; Sah et al., 2019; Nayeri et al., 2021).

1.2.3 Diagnosis and control

The diagnosis of *T. gondii* infection is crucial for the surveillance, prevention, and control of toxoplasmosis. Regarding clinical diagnosis, typical clinical signs of the infection (if present) (*i.e.*, fever, dyspnea, arthralgia, fatigue, or lymphadenopathy) are non-specific and non-pathognomonic; even in abortion or complications during pregnancy cases, a wide variety of viral, bacterial and parasitic pathogens could be involved, especially in livestock species. Thus, laboratory analyses have a key role in *T. gondii* diagnosis, and could be divided as follows (Liu et al., 2015):

- Direct diagnostic techniques

  - Microscopic diagnosis

    These techniques imply the detection of the parasite based on light microscopy. Despite having been traditionally used, they have a low sensitivity and require skilled personnel to obtain reliable detection results. Oocysts could be identified on faecal (felids), water, soil, or food (*i.e.*, susceptible to *T. gondii* presence like vegetables or fruit) samples, and even in aerosols, after filtration and centrifugation processes (Lass et al., 2009; Sroka et al., 2010; Mancianti et al., 2015; Caradonna et al., 2017; Lass et al., 2017). On the other hand, direct observation of tachyzoites is possible in different tissues and body fluids from infected host like broncho-alveolar lavage (BAL), cerebrospinal fluid (CSF), aqueous humor (AH), vitreous humor (VH), amniotic and peritoneal/ascitic fluids or skin aspirates, among others (De Salvador-Guillouët et al., 2006; Stajner et al., 2013; Pena et al., 2014). In addition, brain smears are traditionally used to detect tissue cysts directly through optic microscopy, normally in experimentally infected mice.

  - Bioassay

    The implementation of bioassays using laboratory animals (*i.e.*, mice and cats) is traditionally considered as the gold standard for toxoplasmosis diagnosis (Ghosn et al., 2003; Costache et al., 2013). Cats are the most sensitive bioassay model for the detection of *T. gondii* in meat because an animal can be fed with much larger volumes of tissues (500 g or more) and can excrete millions of oocysts after ingesting only one bradyzoite (Dubey, 2010). However, the complex, expensive and time-consuming character of the technique has favoured the first selection of serological or molecular methods for *Toxoplasma* diagnosis, but it remains an invaluable way to obtain *T. gondii* isolates by bioassay in mice (Su and Dubey, 2020; Dubey et al., 2020c).

- Molecular methods based on specific-DNA detection

PCR-based diagnosis methods cover the inherent limitations of traditional diagnostic methods, being much more sensitive and specific. Nested-PCR targeting multicopy genes such as *B1* gene, the 529-bp repetitive element and the internal transcribed spacer 1 (*ITS1*), are usually used for the detection of *T. gondii* in biological samples, where in some cases the parasite burden is extremely low (*e.g.*, blood samples) (Grigg and Boothroyd, 2001; Castaño et al., 2014). Real-time PCR is useful to quantify parasite burden in particular clinical samples as well as to evaluate toxoplasmosis progression or treatment efficacy; real-time PCR protocols have been designed to amplify multicopy genes like *B1* and the 529-bp repetitive element (Burg et al., 1989; Teixeira et al., 2013; Castaño et al., 2016).

- Histopathology and immunohistochemistry assessments

Histopathology and immunohistochemistry evaluations have been implemented as supporting techniques in different clinical toxoplasmosis diagnosis scenarios, highlighting the case of abortions in sheep and goats. Placenta and foetal brain are the preferred materials for histologic examination (Uggla et al., 1987; Pereira-Bueno et al., 2004; Kim et al., 2009; Partoandazanpoor et al., 2020; Dubey et al., 2020e).

- Serological assays based on circulating antigens detection

Due to the sometimes difficult interpretation of serological results based on specific antibodies detection (indirect diagnosis) to determine the time point of infection (recent/chronic), a valuable alternative is the detection of circulating antigens in serum (direct diagnosis). The sandwich enzyme-linked immunosorbent assay (sandwich ELISA) has been designed for that purpose, involving a specific antibody coated-well exposed to the serum sample and an enzyme-conjugated antibody that recognize the formed antibody-antigen complex (Liu et al., 2015). Although its use is not common, several interesting instances and applications are reported in the literature (Attallah et al., 2006; Dautu et al., 2008).

- Indirect diagnostic techniques

- Serological assays

Serological tests are crucial not only for particular diagnosis but also for epidemiology studies. Serology methods have been applied over adult or foetal serum samples, or even over other fluids susceptible to contain antibodies, such as foetal fluids (*e.g.*, thoracic fluids) or meat juices (Gazzonis et al., 2020). A variety of serological assays, such as dye test (DT), modified agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA), immunosorbent agglutination assay (ISAGA), indirect fluorescent antibody test (IFAT), indirect haemagglutination assays (IHA), latex agglutination test (LAT), and Western-Blot (WB) have been developed to detect different antibody classes (Liu et al., 2015). However, the most widely used techniques in clinical and scientific reports focused on any host are ELISA, IFAT, and MAT (Calero-Bernal and Gennari, 2019; Dubey et al., 2020b,d; Nayeri et al., 2021).

The binding (avidity) of specific antibodies to antigens changes during the course of the infection, from low avidity values during the initial stage to increased values with the progress of the infection. IgG avidity ELISA tests discriminate between high and low avidity IgG proteins in serum making possible to differentiate between a recent or a chronic infection (Villard et al., 2013; Caballero-Ortega et al., 2008).

When it comes to toxoplasmosis control, actions to develop effective drugs and vaccines as well as to control transmission must be considered. All drug therapies against toxoplasmosis currently used in clinical practice (*e.g.*, combined regimens based on drugs such as pyrimethamine, sulfadiazine, clindamycin or azithromycin, among others) are active against the rapidly dividing tachyzoite stage of the parasite, but do not exert any significant activity against the tissue cysts formation, and of course cannot clear persistent infections; in addition, failure rates and related side-effects remain significant (Wang et al., 2019). Immunoprophylaxis is considered a better alternative for efficient long-term disease control but to date there is only one commercially available vaccine, Toxovax™ (MSD), licensed for use specifically in sheep and with demonstrated potential to reduce congenital toxoplasmosis and tissue cyst development at some extent (Katzer et al., 2014). The development of an effective, safe, and durable vaccine against *T. gondii* infection remains a necessity to facilitate the control of toxoplasmosis.

Due to the limited development of drug therapies or vaccines, the main strategies for controlling of toxoplasmosis involve transmission control. Transmission control strategies depends on the host species on which we were focused but they are always based on reducing the exposure to *T. gondii* oocysts in the environment and avoid the ingestion of meat potentially contaminated with tissue cysts. In humans, this is applicable to our consumption habits, preventing to eat raw or undercooked meat in which possible tissue cysts remain viable or washing vegetables and fruit before consumption, as well as to the way we handle domestic cats. In the case of livestock species, control measurements consist basically of improving management conditions to reduce the exposure of herbivore animals to oocyst in the environment and also to possible sources of infected meat (*e.g.*, small mammals or birds, animal carcasses) in the case of omnivore animals.

1.3 Toxoplasmosis in sheep

1.3.1 Epidemiology

The global seroprevalence of *T. gondii* in sheep flocks varies amply (0–100%) between regions and values are strongly dependent on factors such as the age of the ewes, the geographic and climatic conditions, or the management system (Dubey et al., 2020). Many publications support the idea that the *T. gondii* prevalence in sheep flocks increases with age, indicating a strong post-natal transmission (Dubey and Kirkbride, 1989; Katzer et al., 2011; Hutchinson et al., 2011). Climate conditions favouring the sporulation and survival of oocysts, as well as extensive management systems with outdoor access for animals are also demonstrated relevant risk factors that increase the seroprevalence in sheep (Dubey, 2010; Stelzer et al., 2019; Dubey et al., 2020d). In Spain, individual seroprevalence updated figures ranged between 41.2–62.3% in sheep flocks (Almería et al., 2018; Jiménez-Martín et al., 2020), in agreement with rates found in other Mediterranean countries (Stelzer et al., 2019).

Sheep are strict herbivores; therefore, the main source of infection is the ingestion of water, particles of soil or food contaminated with cat faeces (felids are common in farm surroundings). However, transplacental route is also of special relevance, since sheep are especially susceptible to abortions due to toxoplasmosis.

1.3.2 Economic and health impacts

The economic impact associated to *Toxoplasma* infection in small ruminants comes mainly from the economic losses derived from abortion outbreaks. Natural infections in livestock species are usually asymptomatic, unlike in the case of pregnant animals, outstanding sheep. Toxoplasmosis is known to be one of the main causes for ovine reproductive failure, responsible of 10 to 23% of ovine abortions in Europe or USA (Dubey, 2009b; Dubey et al., 2020d). Concretely, in Europe, *T. gondii*-specific DNA have been detected in sheep abortion tissues submitted for diagnosis in distinct countries, *i.e.* in 10% of the ovine abortion-derived tissues from Ireland (Gutierrez et al., 2012), 6–11% from UK (Carson et al., 2018), 11.1–18.1% from the Sardinia region, Italy (Masala et al., 2003, 2007), 10.6% from Germany (Steuber et al., 1995), and in 5.4–18.9% from Spain, as observed in previous reports (Hurtado et al., 2001; Pereira-Bueno et al., 2004; Moreno et al., 2012). Abortion storms can result in notable losses to sheep producers, but the real cost is difficult to estimate; only a few studies report data such as Katzer et al. (2011), pointing out that *T. gondii* could be responsible for between 680.000 and 1.360.000 abortions annually in the EU, or Innes et al. (2009), reporting similar values (1.5 million of lambs/year). In addition, the importance in nature of recrudescence of the infection with repeated congenital infections is also under debate (Dubey, 1982; Innes et al., 2009; Dubey, 2010).

On the other hand, the high seroprevalence figures reported claim attention on the potential of ovine meat (lamb/mutton) as a source of infection for humans, with a number of studies that evidence the presence of *T. gondii* in multiple sheep tissues (*e.g.*, heart, diaphragm or shoulder muscle, among others) in the case of slaughtered animals destined for human consumption (Halos et al., 2010; Rani et al., 2020; Gazzonis et al., 2020) or directly in meat products collected at the supermarket (Aspinall et al., 2002; Lafrance-Girard et al., 2018; Dawson et al., 2020). *Toxoplasma gondii* DNA has also been detected in semen, milk, and once from blood of sheep, being relevant the presence of the parasite in milk as another possible source of human infection (Vismarra et al., 2017a; Dubey et al., 2020d).

1.3.3 Pathogenesis, clinical signs and lesions

Thanks to the development of standardized experimental sheep infection models, the knowledge about the pathogenesis of ovine toxoplasmosis has increased considerably over the last years (Benavides et al., 2011, 2017). Infection in sheep is usually subclinical, although mild fever, respiratory distress, diarrhoea, nasal discharge, and short episodes of anorexia have been described as occasional clinical signs; animals usually recover at 14 days post-infection, coinciding with seroconversion (Dubey, 2010; Benavides et al., 2011). Benavides et al. (2011) proposed an experimental model of lambs oral infection for investigating acquired toxoplasmosis pathogenesis. Histopathological lesions were detected mainly in the brain, characterized by glial foci and perivascular cuffs (mostly located in the cerebral cortex of the forebrain and in the midbrain), and in the heart, denoted by foci of interstitial myositis. Furthermore, tissue cysts and tachyzoite-like structures were observed in the brain, heart, lungs and lymph nodes.

Since acquired infection in sheep is normally asymptomatic, investigations are focused on pathogenesis of congenital infection, in which the stage of pregnancy when *T. gondii* infection takes place in the dam is crucial in determining the consequent clinical outcome (Castaño et al., 2016). Congenital infection can lead to a wide variety of manifestations, ranging from early embryonic death with reabsorption (early gestation) to stillbirth or neonatal death with simultaneously expulsion of small mummified foetuses (mid-gestation), or even the birth of transplacentally infected weak or even clinically normal lambs (late gestation) (Benavides et al., 2017; Dubey et al., 2020d). Both maternal and foetal immune response changes during pregnancy are believed to be the main responsible of the different abortion clinical manifestations, with foetus immunity not developed until mid-gestation and a strong immunomodulation taking place over the placenta from this stage of pregnancy. At early gestation, active peripheral maternal immune response delays dissemination of the parasite, but once it invades the immunologically immature foetus, abortion is unavoidable. At mid- and late gestation, the parasite disseminates more easily to the placenta probably due to the modulation of the maternal immune response, invading the foetus earlier and causing more severe lesions. However, the maturation of the foetal immune system at mid-gestation contributes to the control of parasite multiplication, evidenced by increased number of inflammatory cells as the main component of the lesions and by a higher foetal survival rate. The specific mechanisms involved in these maternal-foetal immune response fluctuations during pregnancy remain unknown (Castaño et al., 2016; Benavides et al., 2017). Typical microscopic lesions comprise of non-purulent inflammation and necrosis, regardless of the tissue (placental o foetal) or the period of gestation when infection occurred, although a later period and the time post-infection are related with more severe lesions. In the foetal tissues, the brain is the most frequent location of lesions, which appear as scattered foci of necrosis in early infections, or foci of gliosis with central areas of necrosis and occasional mineralization predominately in later infections. Skeletal muscle, heart, lungs, and liver are other locations where lesions may be found (Benavides et al., 2017).

In addition to the clinical and economic relevance of "classical" ovine toxoplasmosis, new presentations of the disease also present challenges to veterinarians. An "early" version of ovine toxoplasmosis during pregnancy involving occurrence of abortion 7-12 days after infection (instead of 28 days as expected), and associated with up to 100% of foetal mortality has been described in experimental models (Buxton, 1998; Castaño et al., 2016). While necrotic lesions with a variable degree of inflammatory infiltration are the hallmark histological finding in the placenta and foetal brain in "classical" *T. gondii* ovine abortions, in the case of early abortions placental infarcts associated with thrombosis in the placental vessels and periventricular leukomalacia in foetal brains are described. In addition, these lesions are not associated with evidence of parasite replication (Benavides et al., 2017; Gutiérrez-Expósito et al., 2020).

1.3.4 Diagnosis

Clinical signs during ovine toxoplasmosis are non-specific and useless for diagnosis; even in ovine abortion cases, a wide variety of viral (*e.g.*, Border disease virus, Schmallenberg virus), bacterial (*e.g.*, *Chlamydia abortus*, *Brucella melitensis*, *B. ovis*, *Coxiella burnetii*) and parasitic (*e.g.*, *Neospora caninum*) pathogens can be involved (García-Bocanegra and Zafra, 2019).

Regarding laboratory techniques, due to the short period during which *T. gondii* multiplies freely in the host and the low cyst burden present in tissues, serological tests are the basis of diagnosis. Among the wide range of serology methods applied, ELISA, MAT, and IFAT assays are the most

widely used in the literature focused on sheep (Dubey, 2010; Dubey et al., 2020d). Opsteegh et al. (2010) found excellent correlation between MAT and different commercial ELISA test using naturally infected sheep sera, similarly to what Glor et al. (2013) showed between IFAT and IHA tests using sera from Swiss naturally and experimentally infected sheep. Serology assays are broadly used, and notable efforts have been made for their optimization, but are limited in some cases, such as in acute phases of the disease (7-11 days after infection) with non-detectable specific antibodies against *T. gondii* in host sera (Castaño et al., 2016); furthermore, a positive serologic result is not necessarily related with an active infection in an adult (García-Bocanegra and Zafra, 2019). PCR-based post-mortem diagnosis is more specific but much less sensitive than serology in acquired toxoplasmosis, as demonstrated in comparative studies (Dubey et al., 2020d).

Concerning ovine abortion cases, in addition to the detection of anti-*T. gondii* antibodies in foetal fluids or serum, the detection of *T. gondii* DNA by PCR in placental and foetal tissues (mainly the brain) is also widely implemented. In abortion, stillborn or congenitally infected born alive lamb cases, the detection of anti-*T. gondii* antibodies is directly confirmatory of an active infection due to the particular ovine placental structure preventing the maternal antibodies to be transferred to the foetus during pregnancy (acquired immunity through colostrum) (García-Bocanegra and Zafra, 2019). Although foetal serology and DNA detection are useful aids, in abortion cases histopathology and immunohistochemistry evaluations are essential to establish a cause–effect association, because *T. gondii* can be passively transmitted transplacentally but abortion can occur due to other concomitant pathogens or stress conditions (Pereira-Bueno et al., 2004; Dubey, 2010; Benavides et al., 2017).

1.3.5 Control

The control of toxoplasmosis can be addressed in different ways, including transmission control, drug therapy and immunoprophylaxis, but the combination of different approaches is known to be the optimal strategy. The implementation of farm biosecurity protocols, hygienic measures and management practices should be adopted in all farms, mainly for reducing the level of environmental contamination with *T. gondii* oocysts via cat faeces or limiting the access of felids to abortion-derived tissues. Basic measurements are: avoiding cat access to farm areas, especially to those housing pregnant ruminants, troughs, food warehouses and water supplies; promptly removing any abortion-derived tissue as well as appropriately disposing of animal carcasses; and establishing rodent control (Sánchez-Sánchez et al., 2018).

Regarding immunoprophylactic control of the infection, to date consist in the establishment of a vaccination programme with the only available vaccine, Toxovax™ (MSD), indicated for veterinary use in sheep in order to reduce congenital toxoplasmosis and tissue cyst development (Katzer et al., 2014). The vaccine is based on a live attenuated S48 strain, which was originally isolated from a case of ovine abortion in New Zealand, and after around 3000 passages in laboratory mice was shown to have lost the ability to develop tissue cysts in the infected host or even oocysts in the cat (Buxton and Innes, 1995). It has been described that following subcutaneous inoculation of naïve sheep, the parasite multiplies in the local draining lymph nodes, causing a mild febrile response with peak titres of antibody reached by 6 weeks. No viable parasite was recovered from tissues of vaccinated sheep examined at 10 days or 6 months post-infection. The immunity induced by this vaccine is likely to involve both CD4+ and CD8+ T cells and IFN-γ (Buxton and Innes, 1995). Vaccination induces immunity that protects against abortion for at least 18 months after the initial vaccination (Buxton et al., 1993).

Finally, a vast panel of drug compounds (including spiramycin, monensin, sulphamezathine, pyrimethamine or decoquinate, among others), have been evaluated in pregnant ewes to reduce the outcome of exogenous transplacental transmission after sheep experimental infection, as this is the main concern in ovine toxoplasmosis (reviewed by Sánchez-Sánchez et al., 2018). Promising safety and efficacy results were achieved by Sánchez-Sánchez et al. (2019a) testing the treatment with a bumped kinase inhibitor (BKI-1294) in a pregnant sheep experimental infection model. Complete safety, 71% decrease in foetal/lamb mortality, and significant vertical transmission reduction were demonstrated. All these studies were focused on the acute phase of the infection (*i.e.*, tachyzoite stage) but not in the chronic phase, against the formation of tissue cysts. Montazeri et al. (2018) published a systematic review on drugs and compounds against tissue cysts in the last three decades (1987 to 2017), but only one of the studies considered was an attempt in sheep, demonstrating that 44.4% of lambs receiving toltrazuril treatment presented muscle tissues free of cysts after infection with Me49 strain oocysts (Kul et al., 2013). Nevertheless, despite all these efforts, no safe and effective drug is available for animal toxoplasmosis at present.

1.4 Toxoplasmosis in pigs

1.4.1 Epidemiology

Serological surveys have indicated that up to 30% of domestic pigs have been exposed to *T. gondii* worldwide (Dubey et al., 2020b). Prevalence figures vary amply depending on demonstrated important risk factors like the age, pig category, geographical origin, and management system (Stelzer et al., 2019). Prevalence values increase significantly with the age due to pigs normally acquiring the infection postnatally (Dubey et al., 2020b). Another relevant risk factor is the management system, with quite low prevalence figures (<1%) in pigs reared indoor with controlled management conditions, preventing access of rodents or cats, whereas free-ranging pigs, reared in farms with less controlled conditions allowing outdoor access and in backyard holdings present seroprevalence values above 60% (De Berardinis et al., 2017; Dubey et al., 2020b). In Iberian pigs raised in Spain, anti-*T. gondii* antibodies have been detected with a frequency from 9.5 to 58.2% (Hernández et al., 2014; Pablos-Tanarro et al., 2018; Castillo-Cuenca et al., 2020).

In swine, the main routes of transmission are the ingestion of water, particles of soil or food contaminated with oocysts, and carnivorism (scavenging). A controversial issue is the emerging interest on producing organic and semifree-range pigs for getting meat products with high-quality nutritional components and valuable organoleptic aspects, but conversely, compromising food safety with the exposure to not only *T. gondii* but also other zoonotic parasites such as *Trichinella* spp.

1.4.2 Economic and health impacts

Pig industry is of major importance for the economy of many countries, particularly, USA, China, and European countries like Germany, Spain, and France. Concretely, Spain is the second largest pork producer in the EU and the fourth worldwide (Castillo-Cuenca et al., 2020). *Toxoplasma* infections in domestic pigs, as in most of livestock species, are usually asymptomatic; however, several cases of clinical disease after natural infection as well as reports of reproductive failure

due to toxoplasmosis have been recorded worldwide (Dubey, 1986, 2009a; Dubey and Beattie, 1988; Dubey et al., 2020b). Most of clinical toxoplasmosis cases have been documented from Asian countries (*i.e.*, Japan, Taiwan, China, Korea, Thailand), but outbreaks have been also described in countries such as Germany (Klein et al., 2010), Italy (Gelmetti et al., 1999) and Brazil (Olinda et al., 2016). Especially noticeable is the case of China, where numerous cases have been documented (Li et al., 2010; Dong et al., 2018; Dubey et al., 2020b), some of them with important mortality and morbidity rates, becoming a serious concern. The ToxoDB #9 (*Chinese 1*) and ToxoDB #10 (clonal type I) genotypes have been detected frequently in association with clinical toxoplasmosis manifestations as well as in tissues from slaughtered pigs in China, suggesting a causal relation (Dubey et al., 2020b).

Contrary to the vast knowledge about the importance of vertical transmission of *T. gondii* in small ruminants and humans, the role of the parasite as cause of reproductive disorders in sows and the epidemiological significance of transplacental transmission are less understood (Basso et al., 2015, 2017). In China, abortions presumably caused by *T. gondii* in sows are considered common and assumed to cause important economic losses (Pan et al., 2017); nevertheless, European reports of reproductive problems due to *T. gondii* infection in pigs are scarce. A large epidemiological study in 94 pig breeding farms in Germany suggested an association of *T. gondii* with reproductive failure in sows, with significantly higher seroprevalence in farms experiencing reproductive disorders (*e.g.*, repeat-breeders, abortion, neonatal mortality) (Damriyasa et al., 2004). Furthermore, *T. gondii* was detected in the placenta or in foetuses of 34 out of 113 sows that had aborted or delivered a high number of stillborn or weak piglets in Switzerland (Basso et al., 2015).

On the other side, the European Food Safety Authority (EFSA) recognized *T. gondii* as one of the public health hazards in swine to be assessed during meat inspection (EFSA, 2011). After chicken, pork is the most frequently consumed meat in the world and is ranked first in some European countries such as Norway and Switzerland (OECD, 2021). This fact, together with not inconsiderable seroprevalence figures, draws attention to the role of pork meat as a source of infection for humans, with wide evidence of parasite detection in slaughtered animals (Djokić et al., 2016; Dubey et al., 2020b) or pork meat collected at retail stores (Aspinall et al., 2002; Berger-Schoch et al., 2011). Nonetheless, serological or parasitological surveys based on abattoir samples do not provide a true assessment of risk to humans, because storage and other post-harvest treatments such as dry curing (Genchi et al., 2017), and obviously, freezing or cooking thoroughly the meat, can reduce viability or kill *T. gondii* (Olsen et al., 2020). A recent extensive study in the United States aimed to isolate by bioassay in mice viable parasites from pork and lamb samples collected at grocery stores, with a success rate of 0.13% (1/750) (Dubey et al., 2020b), in agreement with a previous similar survey attending beef, chicken, and pork meat with a 0.33% rate (Dubey et al., 2005c).

1.4.3 Pathogenesis, clinical signs and lesions

Clinical disease is believed to occur only during the acute phase of infection as a result of necrotic and inflammatory processes during tachyzoites multiplication in several tissues. Clinical manifestations seem to occur more frequently in neonatal and weaned pigs, but also cases of clinical toxoplasmosis affecting sows have been described. Common signs observed in clinically infected pigs include anorexia, appetite loss, apathy, fever, ocular and nasal discharge, lymphadenopathy, dyspnoea, cyanosis, diarrhoea, limb weakness, neurological signs and

sometimes death (Dubey, 1986, 2009a; Dubey and Beattie, 1988), although none of these signs is pathognomonic for toxoplasmosis. Chronically infected animals do not have clinical signs, but they represent an important source of infection for humans, in particular if undercooked pork or insufficiently treated meat products containing tissue cysts are consumed (Dubey, 2009a). Is well known the association of certain immunosuppressive viral infections and toxoplasmosis, with the parasite as an opportunistic pathogen. In some cases, viral infections such as Porcine Circovirus type 2 (Klein et al., 2010) and Porcine Parvovirus (Basso et al., 2015) have been associated with clinical manifestations of toxoplasmosis in pigs. Regarding pathological lesions observed in acute acquired infections, Jungersen et al. (1999) in an experimental 7-week-old pigs infection model pointed out focal fibrinonecrotising pneumonia, focal necrotising hepatitis, splenitis and lymphadenitis of bronchial and mesenterial lymph nodes.

As mentioned previously, *T. gondii* infections in sows may be associated with reproductive failure characterized by abortion, foetal mummification, stillbirth and neonatal mortality (Dubey, 1986, 2009a; Dubey and Beattie, 1988; Basso et al., 2015). However, experimental attempts to reproduce congenital toxoplasmosis in pigs have not been consistently successful (Basso et al., 2017). Dubey et al. (1990) experimentally induced congenital toxoplasmosis in two sows fed with 1000 *T. gondii* oocysts and associated histopathological lesions were studied. The predominant lesions were necrotizing placentitis, nonsuppurative encephalomyelitis, and myocardial degeneration, necrosis and mineralization. Furthermore, numerous tachyzoites were seen in trophoblast cells lining areolae in placenta. Diverse pathological results have been obtained in other pregnant sows experimental infection models using different routes of infection, doses and *Toxoplasma* strains (Moller et al., 1970; Jungersen et al., 2001).

1.4.4 Diagnosis

Regarding diagnosis of acquired and congenital toxoplasmosis in pigs, the same considerations as in the case of sheep toxoplasmosis should be given. *Toxoplasma gondii* infection evidence is based mainly on serologic and PCR-based methods, as well as on histopathology in abortion cases. Likewise, between the wide number of serological techniques, ELISA and MAT are the most commonly used in pig diagnostic and seroprevalence studies (Dubey, 2010; Dubey et al., 2020b). Comparative efficacy studies carried out in naturally and experimentally infected pigs, as well as in retail pork samples, claimed that serological tests (ELISA and MAT) are the most sensitive followed by bioassay and molecular methods (real-time PCR and nested-PCR), with a significant lack of sensitivity in the case of histopathology (Yai et al., 2003; Hill et al., 2006; García et al., 2006; Tsutsui et al., 2007; Bezerra et al., 2012).

1.4.5 Control

In the EU, there is no official requirement for surveillance or control for *T. gondii* in pigs or pork, although the EFSA recommends auditing of biosecurity and implementing serological surveys for the identification of high-risk farms (EFSA, 2011). Again, biosecurity protocols, hygienic and management good practices for reducing the level of environmental contamination with *T. gondii* oocysts should be implemented in pig farms. Indeed, management conditions take special relevance, since seroprevalence data show how systems with outdoors access and without controlled conditions are strongly related to higher rates (Stelzer et al., 2019). Carnivorism and rooting habits in pigs state the access to outdoors and the presence of rodents or even birds in the facilities as relevant risk factors for acquiring the infection. Furthermore, the emerging interest

on producing organic and free-range pigs for getting presumably more valuable meat products complicates the scenario (Olsen et al., 2020).

As described above, leaving apart the Toxovax™ (MSD) vaccine indicated for veterinary use in sheep, to date no drugs or vaccines are prescribed for toxoplasmosis in animals. As abortion storms due to toxoplasmosis are not considered relevant in pig (unlike in sheep), and in non-pregnant animals the infection is normally asymptomatic, no special efforts have been made to develop compounds against acute or chronic phases of the infection in this species (Montazeri et al., 2018). However, some attempts to evolve a specific vaccine for pigs have been made, being the most widely used experimental model for the development of vaccines against *Toxoplasma* even ahead of the ovine model (Hiszczyńska-Sawicka et al., 2014). Burrels et al. (2015) demonstrated the effectiveness of vaccination with S48 strain tachyzoites (strain in which Toxovax™ vaccine is based on) in the reduction of viable *T. gondii* tissue cysts within tissues from pigs after challenging the animals with oocysts of the M4 strain.

2. Genetic diversity of *Toxoplasma gondii*

2.1 Genetic diversity and population structure

The study of the genetic diversity and population structure of pathogens is crucial for understanding their epidemiology and pathogenicity, as well as for implementing suitable disease control strategies (Beck et al., 2009). *Toxoplasma gondii* life cycle, as for the rest of apicomplexan parasites, is largely haploid, with the exception of a brief diploid stage during the sexual phase in the intestine of its DH. Sporozoites are the result of postzygotic meiosis and seem to follow classical mendelian laws (Dubey, 2010). Thus, most stages of the life cycle are characterised by allelic homozygosity, facilitating genetic recombination and supporting direct measurements of population-level heterozygosity. It is important to note that, for many apicomplexan parasites, (*e.g.*, *Cryptosporidium*, *Eimeria*), the sexual phase is thought to be mandatory, whereas in the case of *Toxoplasma* and *Neospora* genera, zoites can propagate by asexual replication indefinitely (Beck et al., 2009). The total haploid genome of *T. gondii* (Me49 strain used as reference) contains 13 chromosomes and more than 8300 protein coding genes identified, with a total genome size above 65 million of base pairs (Mb) (Khan et al, 2005; Reid et al. 2012; Lorenzi et al., 2016; Xia et al., 2021) (**Table 1**). Comparative genomic studies carried out with *T. gondii* and several members of the Apicomplexa subphylum have demonstrated how *T. gondii* is demarcated from its closest relatives by the tandem amplification and diversification of certain groups of genes involved in host-parasite interactions, determining also key differences among the 16 major clades defined for the species. These determinant groups include genes encoding MIC (involved in host cell attachment), GRA and ROP (modulation of host immunity) secretory proteins, as well as members of the SRS super family of surface adhesins (adherence and immune evasion) (Lorenzi et al., 2016).

Prior to the development of methods based on the characterization of specific genetic markers, *T. gondii* isolates were grouped by their virulence to outbred mice. Furthermore, evidenced differential expression of polymorphic antigenic peptides and zymodemes between *T. gondii* strains suggested differences in particular genes, and were used to draw some pioneer population structures (Dardé et al., 1988, 1992; Bohne et al., 1993; Meisel et al., 1996). Simultaneously during the 1980s and 1990s, methods were developed to recognize genetic differences among *T.*

gondii strains and to associate them to the virulence observed in mice (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). Restriction fragment length polymorphism (RFLP) method based only on *SAG2* gene, allowed pioneer researchers to initially describe a clonal population structure with three genetic types (I, II, and III) linked to mouse virulence observed in isolates obtained from human patients (Howe and Sibley, 1995). Cited authors proposed that type I isolates were 100% lethal to mice, irrespectively of the dose, and that types II and III were generally non-virulent (<30% lethality in a dose-dependent manner) (Howe et al., 1996). Since then, global population structure and genetic variability of *T. gondii* have been extensively investigated. At first, the studies were strongly biased by the fact that isolates included were mainly from human patients and domestic animals originating from France and the USA (Dardé et al., 1992; Howe et al., 1997). In addition, monolocus typing strategies promoted the misidentification of atypical and recombinant strains circulating (Dubey et al., 2005a,b). Nevertheless, in subsequent studies, valuable efforts made on collecting numerous and geographically distant isolates from different hosts, and specially the implementation of multilocus genotyping methods, revealed a much more complex population structure comprising a higher genetic diversity than previously thought (Ajzenberg et al., 2004; Dubey et al., 2008; Frazão-Teixeira et al., 2011). The rapid development of multilocus-sequencing methods, and the description of a wide panel of new PCR-RFLP and microsatellite (MS) markers led to the consolidation of the general predominance of the three initial clonal types, but new concepts appeared on the scene (Ajzenberg et al., 2002a, 2005; Khan et al., 2005, 2007; Su et al., 2006).

Table 1. Comparison of features of the *Toxoplasma gondii* genome with close related apicomplexan species. Data extracted from Lorenzi et al. (2016), Xia et al. (2021), Reid et al. (2012), and Blazejewski et al. (2015).

Genome features	*Toxoplasma gondii* (Me49 strain)	*Neospora caninum* (Liverpool strain)	*Sarcocystis neurona* (SO SN1 strain)
Genome size (Mb)	65	62	127
Chromosomes	13	13	NA
G+C content (%)	52.2	54.8	51.5
Protein coding genes	8322	6936	7093
Percent of genome encoding proteins (%)	60.5	59	50.9

NA, data not available.

Multilocus typing methods brought out strains that possess types I, II or III alleles, identical to those found in the three major lineages, but these have segregated differently among the loci analysed. These strains were considered to be the result of recombination events between strains of the major lineages and were called “recombinant strains”. In addition, strains with unique polymorphisms at some loci (not detected in the three predominant lineages) were also described and designated as “atypical” (Ajzenberg et al., 2004). The database ToxoDB (http://toxodb.org/toxo/) allows the identification, collection, and assignation of a recognizable code for all deposited isolates and genetic variants according to the combination of alleles of 11 PCR-RFLP markers, known as ToxoDB genotype number (#); likewise, specific designation related with origin could be attributed to some genetic profiles defined by 15 MS markers (*e.g.*, *Africa 1*, *Caribbean 3*). Except for main lineages and some unique strains, equivalence between RFLP- and MS-defined genotypes is still unclear.

The genome-wide polymorphism rate between the three main lineages has been estimated to be approximately 1%, characterised by an extensive bi-allelism falling into type I, II and III SNPs (Grigg et al., 2001; Khan et al., 2005; Boyle et al., 2006; Sibley and Ajioka, 2008). The origin of this clonality, low genetic diversity within each lineage and low divergence between them has been suggested to be due to a recent emergence/expansion from a common ancestor only 10000 years ago (Su et al., 2003), in addition to an extensive bypass of sexual cycle with a continuous asexual propagation (Sibley and Ajioka, 2008). Generally, it has been assumed that *T. gondii* genetic population is the consequence of scarce but crucial meiotic/genetic crosses between highly similar parental strains, with extensive expansion through asexual reproduction by direct oral infection between different IH. The capacity of a unique zoite to undergo complete sexual development and self-fertilization in the cat, combined with relatively few cats simultaneously infected with multiple strains, limit the chances for genetic exchange (Boyle et al., 2006; Sibley and Ajioka, 2008; Wendte et al., 2010). Nevertheless, this theory is not applicable to the South American model, where a notably higher prevalence of the infection along with an increased diversity of wild felids might have promoted more frequent recombination events resulting in the contrasting extreme diverse non-clonal population there (Bertranpetit et al., 2017).

After the great advances in molecular typing techniques, there have been several comprehensive attempts to unravel the population structure of the parasite. Combining strains from Europe, North and South America, in a phylogenetic analysis of intron sequences, Khan et al. (2007) differentiated 11 haplogroups, including the three major clonal lineages (renamed as haplogroups 1, 2 and 3) extended in North America and Europe, and other haplogroups that emerged as successful recombinant/atypical strains that spread clonally over South America. Similarly, Khan et al. (2011) re-evaluate the population structure of *T. gondii* in North America using sequenced-based phylogenetic and population analyses and defined a new clonal lineage (lineage 12) that included American strains previously classified as atypical by PCR-RFLP typing. On the other hand, Pena et al. (2008) focused on Brazilian situation (at some extent representative of the tropical status) and analysed a total of 125 Brazilian isolates by PCR-RFLP typing, identifying 48 genotypes sorted in only four successfully expanded clonal lineages (types BrI, BrII, BrIII and BrIV) and a large group of divergent highly diverse strains. Then, in an even more extensive and in-deep study, Su et al. (2012) typed 950 isolates from all over the world using PCR-RFLP and MS markers as well as sequencing of introns from housekeeping genes, comprising 138 genotypes. Phylogenetic analysis of the data identified 16 well-defined haplogroups (belonging to 6 major clades A-F), mostly in agreement with previous results reported by Khan et al. (2007, 2011). Africa and Asia are largely unexplored in comparison with the other continents and were underrepresented in mentioned phylogenetic analyses. An extensive recent review exposed that type II and III isolates are also common in African territories (especially type III), coexisting with other less abundant genotypes identified by MS markers as *Africa 1* (haplogroup 6) and *Africa 3* (haplogroup 14), or by PCR-RFLP markers such as the ToxoDB #20 (Galal et al., 2018). Finally, Chaichan et al. (2017) carried out a comprehensive review about the Asian *T. gondii* population structure, considering 390 strains (36 different PCR-RFLP genotypes), and concluding that 82.6% (322/390) belonged to type I, II, III or *Chinese 1* (ToxoDB #9, haplogroup 13) clonal lineages. Overall, in North America and Europe the population structure of *T. gondii* appears to be dominated by three clonal lineages (I, II and III), which coexist with much more scant, genetically diverse isolates. A fourth clonal lineage (the above-mentioned lineage 12) is largely confined to North America, where it is more common in wild animals. In contrast, much greater genetic diversity is observed in South America, where the population fits an epidemic structure, with a few major clonal complexes and abundant less related isolates, without signs of the recent genetic

bottleneck and clonal structure seen in other continents. It could be said that African and Asian situations are a mixture between both scenarios, with abundance of isolates belonging to type I, II, and III clonal lineages, coexisting with other clonal groups that emerged from the strong expansion of recombinant or atypical isolates, but exhibiting a less divergent character than in South America (Lorenzi et al., 2016; Su et al., 2012). Phylogenetic and geostatistical approaches led Bertranpetit et al. (2017) to hypothesize with a South American origin of *T. gondii* and its initial spread through North America, Asia, Europe and finally Africa, through different migration routes, linked to the co-evolution of Felidae family members and humans, what could at some extent explain the different population structure found between South America and the rest of the continents.

It is important to clarify that, the classification of an isolate into clonal, recombinant, or atypical is a sensitive and non-definitely settled issue, because it depends on the number and the discriminating power of markers used for genotyping. In literature, especially that from Europe, a large percentage of genotyping studies based on PCR-RFLP or PCR-sequencing apply less than four molecular markers, what contributes to underestimate genetic diversity and limit our knowledge about the genetic structure of the population.

2.2 Genotyping methodologies

In order to genetically characterize *T. gondii* strains circulating in a wide range of hosts all over the world, different molecular technologies have been developed, including the widely used PCR-RFLP, microsatellites typing and PCR-sequencing (MLS) typing methods, and less frequently applied RAPD-PCR, pyrosequencing, and High-Resolution Melting (HRM) analysis methods (Liu et al., 2015). Furthermore, as previously mentioned, scientists promptly realized that monolocus typing strategies were underestimating genetic diversity and simplifying population structure of the genus. Thus, a rapid description of new genotyping targets was carried out, not only regarding PCR-RFLP markers (Khan et al., 2005), but also MS regions (Ajzenberg et al., 2002a, 2005), intron sequences from housekeeping genes (Khan et al., 2007), or sequences from protein-coding genes of interest (Dubey et al., 2011). MS, PCR-RFLP and MLS typing methods cumulate most of global *T. gondii* strains genetic characterization data, and therefore, are the most useful for comparison and for drawing conclusions.

- PCR-RFLP methodologies

The PCR-restriction fragment length polymorphism (RFLP) analysis is based on the ability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs) present in PCR products and subsequently display distinct DNA banding patterns on agarose gels electrophoresis (Su et al., 2010). The technique was initially designed to identify SNPs that are biallelic among archetypal strains. PCR-RFLP analysis has been applied to the genetic typing of a large number of isolates and clinical samples of animal and human origin, being the most widely used method in *T. gondii* strains genotyping (Howe and Sibley, 1995; Pena et al., 2008; Shwab et al., 2014).

The conventional multilocus PCR-RFLP analysis method relies on single-copy polymorphic DNA sequences, requiring a relatively large amount of starting parasite DNA; thus, it has been difficult to apply in clinical samples, due to the extremely low parasite burden usually present in infected tissues. The development of the multiplex (multilocus) nested PCR-RFLP (Mn-PCR-

RFLP) method made possible to double amplify these single-copy regions, solving the problem. In such reliable method, 11 markers are pre-amplified at the same time by multiplex PCR using external primers in a single reaction, and the pre-amplified PCR product is used as the template in subsequent individual nested PCRs (nPCR) with specific internal primers (Su et al., 2010).

Actually, PCR-RFLP method only distinguishes between the major clonal lineages (and some variants) but does not include the so called "fingerprinting level" of discrimination. More than 250 SNP-RFLP markers, mapped across the 13 chromosomes of the *T. gondii* genome, have been designed (Khan et al., 2005). The most suitable strategy should be to amplify the well-known and widely used *SAG1*, *SAG2* (both 5'- and 3'- ends), alt. *SAG2*, *SAG3*, *BTUB*, *GRA6*, *C22-8*, *C29-2*, *L358*, *PK1*, and *Apico* markers, but under the view of the available literature, especially in former studies, normally only one or two of these regions are examined, highlighting the predominance of both ends of *SAG2* region (Howe et al., 1997; Fuentes et al., 2001; Su et al., 2006, 2010). The limited discriminatory power of this assay is balanced by the manageability of the technique, since no automated sequencer is needed and small amounts of DNA present in clinical samples are less problematic thanks to multiplex nested-PCR amplification.

- MS regions analysis

The MS sequences are tandem repeats of short (1 to 6 bp) DNA motifs that are ubiquitous in eukaryotic genomes and undergo length changes due to insertion or deletion of one or multiple repeat units. The most commonly proposed mutation mechanism for MS sequences is strand slippage, occurring predominantly during DNA replication. Therefore, for the same locus there may be different alleles, with a different length depending on the number of repetitions of the motif. Thus, the analysis of the different MS for each sample is carried out by determining the exact size of the amplicon for each marker. First, MS loci are amplified by PCR using fluorescently labelled forward and unlabelled reverse primers. Then, the dye-labelled products are separated by size using automated (capillary) electrophoresis and identified by fluorescence detection (Ajzenberg et al., 2002a, 2010).

This method has two different levels of discrimination. The first step of discrimination is the performed at the typing level, to distinguish between the major clonal lineages (type I, II, and III genotypes) from atypical strains. The second level of discrimination is the "fingerprinting level", representing a high degree of discriminatory power for differentiating closely related strains belonging to the same haplogroup or lineage. This high-resolution analysis is required for establishing a common source of infection in outbreaks, possibly discriminating geographical origin of the organism, or even identifying laboratory contaminations issues during diagnosis (Ajzenberg et al., 2010).

A group of 15 MS markers are routinely used to genotype *T. gondii* strains. It includes eight "genotyping markers" (*TUB2*, *W35*, *TgM-A*, *B18*, *B17*, *M33*, *IV.1*, and *XI.1*) which are able to distinguish between major clonal lineages, and seven "fingerprinting markers" (*M48*, *M102*, *N60*, *N82*, *AA*, *N61*, and *N83*), with enhanced discrimination power. Characterization studies generally cover five of them or all 15 different MS regions. Ajzenberg et al. (2010) developed an easy-to-use method for the amplification of the 15 MS regions in a single multiplex-PCR assay in which the 15 pairs of primers needed are included simultaneously. The main limitation of this assay is the requirement for an automated sequencer.

- Multilocus sequence (MLS) typing analysis

The MLS typing analysis is based on DNA sequence polymorphisms detected in genomic regions of interest, including SNPs, and insertion or deletion events (Liu et al., 2015). Multilocus PCR-RFLP and microsatellites typing are preferred tools for characterization of *T. gondii* in epidemiological studies, but when the amount of DNA for *T. gondii* isolates is not limited, the MLS typing is the method of choice because it has the highest resolution power among all typing methods. While PCR-RFLP and MS assays are focused on only limited known mutated positions, under-representing the true genetic diversity, MLS typing analysis covers the whole variability of a sequenced region. Noteworthy studies using this approach revealed important hallmarks in *T. gondii* population structure (Khan et al., 2007, 2011), emphasizing the importance of a sequencing approach for studying the population genetics and phylogeny of the parasite.

MLS typing analysis could be based on intron (*e.g.*, *UPRT1*, *UPRT2*, *UPRT7*, *MIC2*, *BTUB*, *HP2* and *EF1*, among others) and coding regions (*e.g.*, *SAG1*, *SAG3*, *ROP18*, *BSR4*, *GRA6* and *GRA7*, among others) (Khan et al., 2007; Prestrud et al., 2008; Gao et al., 2017; Bertrandpetit et al., 2017). In addition, Castro et al. (2020) developed a protocol to generate PCR-RFLP profiles from DNA sequence data, via *in silico* digestion of known PCR-RFLP marker sequences by identification of restriction enzyme motifs. Thus, the integration of data generated by these two different typing methods is now possible.

2.3 Genetic diversity of *Toxoplasma gondii* in Europe

Europe, along with North and South America, is the region most widely screened for circulating *T. gondii* strains genetic characterization. However, despite the large number of studies carried out in the continent, involving numerous samples from a wide variety of hosts and domains (*i.e.*, human, domestic animals, wildlife, and environment), the data are worryingly limited due to several factors (Lebov et al., 2017). To note, MS and PCR-RFLP typing are the most widely used methods without a clear tendency, but except for predominant lineages and some unique strains, equivalence between assigned genotypes by each technique remains at some extent confusing; thus, remarks should be given separately.

A comprehensive literature review allowed us to extract the data summarized below. This task will be completed with some of the results obtained from the present Doctoral Thesis and will lead to a future review article on the available *T. gondii* genotyping data in continental Europe aiming to provide an overall picture of the circulating strains distribution within the European context (**Appendix 1**).

- *Toxoplasma gondii* genetic diversity based on PCR-RFLP or PCR-sequencing methodologies

The most problematic issue is the use of an insufficient number of molecular markers for typing, especially in the case of PCR-RFLP and PCR-sequencing methodologies. Unfortunately, an important percentage of the studies implemented monolocus typing methods (mainly based on the *SAG2* marker) despite being completely outdated and possessing major limitations for reliable strain classification. Concretely, almost half of the studies carried out on European samples analysed by PCR-RFLP or PCR-sequencing

methods (40/99) are based on one marker, and among the rest only 49 studies were based on four or more genomic regions (Herrmann et al., 2014) (**Table 2**). Some valuable reviews regarding *T. gondii* diversity in other continents, established a "cut-off" in at least five molecular markers analysed in an investigation to be considered in the review analysis (Lorenzi et al., 2016; Chaichan et al., 2017; Galal et al., 2018). However, within those studies implementing an efficient number of markers, sometimes there is no coincidence, making impossible to compare data. On the one hand, the most frequently used marker is *SAG2* (5'- and 3'- ends of the gene) probably because it was among the first described, supposing a milestone on *Toxoplasma* genetic research (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). On the other hand, PCR-RFLP assays are not very informative when based on genes infrequently used such as *ROP1* (Haque et al., 1999; Turčeková et al., 2013), or on markers mostly applied in certain type of original sample like in the case of *B1* gene in environmental samples (*i.e.*, water, soil, air, vegetables, or fruit) (Burg et al., 1989; Sroka et al., 2008, 2009, 2010).

Aiming to describe the reliable information available about genotypes circulating in Europe, a minimum number of regions analysed should be established, as above mentioned. If the analysis of four genetic markers is accepted as cut-off, the reliability of data increases considerably. Isolates and specimens have been both considered. From that premise, the available *T. gondii* isolates and specimens typed are unlikely to be representative of the parasite genetic diversity in Europe, with vast regions and important hosts not taken into consideration. From a One Health concept approach, four "domains" or "compartments" (*i.e.*, humans, domestic and wild animals, and environment) could be taken into account. Concerning strain types detected in humans, only three countries are represented (Germany, Poland and Serbia) in five studies with a total of 33 samples typed (Djurković-Djaković et al., 2006; Nowakowska et al., 2006; Stajner et al., 2013; Marković et al., 2014; Herrmann et al., 2014). Among them, almost 90% (29/33) corresponded with type II strains, only one type III was detected, and recombinant or mixed infections (sometimes indistinguishable only by PCR-RFLP methods) were described in three cases. The presumed predominance of type II in Europe is evident but not conclusive since data could be representative only of central Europe.

Most European (geno)typed samples have been collected from infected domestic (pets and livestock) and free-living animals. Regarding domestic animals, the range of countries represented is wider but not enough, with molecular studies from Austria, Czech Republic, Denmark, France, Germany, Italy, Ireland, Poland, Portugal, Serbia, Switzerland, and The Netherlands (21 studies with a total of 335 samples) (**Table 2**). Likewise, studies could be sorted according to the host, including data from sheep, goat, cattle, pig, horse, chicken, dog, and cat, standing out chicken and pig species in terms of sampling effort, with 102 and 71, samples typed, respectively. Type II strains were detected in 81.5% (273/335) of samples, together with an 8% (27/335) of type III, 3% of type I (10/335) and 7.5% (25/335) of mixed, atypical/non-canonical or recombinant infections. Concerning wild animals, European studies include data from Croatia, Czech Republic, Denmark, Germany, Italy, Norway, Poland, Scotland, Serbia, Spain, and the UK, with a total of 261 samples collected in 25 different studies. It involves data from a wide variety of hosts such as rodents, marine mammals, wild cats, wild swine, mesocarnivores, wild ungulates, and wild avian species. It could be highlighted the group of mesocarnivores with the highest number of studies (eight) and samples analysed (144).

Strains circulating in wildlife animals/sylvatic cycles corresponded approximately to 66% of type II (172/261), 20.7% of mixed, atypical or recombinant infections (54/261), 12.6% of type III (33/261), and 0.8% of type I (2/261).

Regarding genotypes present in environmental samples, the situation is even more restricted, with only two studies meeting the requirements and accounting for a total of nine samples. Slany et al. (2019) detected type II strains in seven samples of vegetables in the Czech Republic whereas Wojcik-Fatla et al. (2015) found type I alleles in DNA extracted from two ticks (*Dermacentor reticulatus*) collected in field areas of Poland. Once again, data is scarce and circumscribed to central Europe.

Table 2. Prevalence of the *Toxoplasma gondii* genetic types in Europe according to the four compartments within the One Health concept and based on PCR-RFLP or PCR-sequencing data. Percentages are given in brackets

	Human samples	**Domestic animal samples**	**Wildlife animal samples**	**Environmental samples**	**TOTAL**
Type I	0 (0)	10 (3)	2 (0.8)	2 (22.2)	14 (2.2)
Type II	29 (87.9)	273 (81.5)	172 (65.9)	7 (77.8)	481 (75.4)
Type III	1 (3)	27 (8)	33 (12.6)	0 (0)	61 (9.6)
Recombinant, atypical strains or mixed infections	3 (9.1)	25 (7.5)	54 (20.7)	0 (0)	82 (12.8)
TOTAL	33	335	261	9	638

As a whole, it could be claimed the complete predominance of type II strains circulating in Europe, that supposes 75.4% (481/638) of the total samples collected in 49 different studies included (**Table 2**). Type I strains are truly scarce, representing 2.2% (14/638) of samples, whereas type III strains imply almost 10% of total samples (61/638). Finally, recombinant, atypical or mixed infections suppose an unexpected 12.8% (82/638) of the records. Despite limitation of data, it could be pointed out the enhanced burden of type III strains, as well as recombinant/mixed infection in the case of wildlife animal species in comparison with the rest of European matrices considered. Germany, Italy and Serbia are the countries with the highest number of genotyping investigations in their territories. Sampling disparities exist between regions of the continent, and vast areas remain unexplored; truly little genetic data are available from human infection cases. It should be noted the complete absence of data from Spain except from wildlife animal studies (wild boar, rodents and foxes) (Calero-Bernal et al., 2013, 2015; Fernández-Escobar et al., 2020).

- *Toxoplasma gondii* genetic diversity based on MS methodologies

Regarding MS typing procedures, the number of markers is not such a problematic issue since the use of five “genotyping” markers or the complete panel of 15 “genotyping” plus “fingerprinting” markers is quite widely used. Under the view of the available literature, publications that reported genotyping data based on less than five MS markers are due to

amplification failures and not to an initial deficient methodological approach (Fekkar et al., 2011; Marcer et al., 2019); fortunately, the number of samples is non-significant compared to the 831 samples typed in 42 different studies by using more than five MS markers (**Table 3**). Here, as done in the previous section, a criterion for the minimum number of markers used for genotyping (five) was implemented; clinical and environmental samples along with isolates were included, and the prevalence of genetic types was again addressed from a One Health point of view. Apart from type I, II, III or recombinant/mixed infections, by MS typing is also possible to identify specific genotypes such us *Africa 1*, *Caribbean 2, Caribbean 3* even characterizing only five loci (*B18*, *TUB*, *Tg-MA*, *W35* and *B17*).

Unlike the previously exposed methods, the MS-based methodology has been widely used in the genetic characterization of human samples, involving a total of 428 specimens in 20 different studies. However, it is important to point out that despite the participation of a greater number of European countries, France clustered almost 80% of the human samples analysed (Ajzenberg et al., 2009, 2015), followed by far by Portugal (12%) (Ajzenberg et al., 2009; Vilares et al., 2017), Denmark (4.5%) (Jokelainen et al., 2018), and Belgium (4.5%) (Gisbert-Algaba et al., 2020); most of the other countries contributed with a single isolate or similar (Austria, England, Germany, Romania, Serbia, and The Netherlands). Concerning strain types detected in human population, 86.4% corresponded with type II strains, the types I and III were found in similar low proportions (2.6 and 3% respectively), and those cases involving recombinant, atypical or mixed infections corresponded to 6.3% of cases (**Table 3**). In addition, six cases of human infection with *Africa 1* strains and one case with *Caribbean 2* were detected in Belgium, Denmark, and France (Ajzenberg et al., 2010; Fekkar et al., 2011; Su et al., 2012; Jokelainen et al., 2018). The predominance of type II in Europe is again clear but once more it should be borne in mind that extensive areas of the continent are still not represented.

The second most studied category was that of domestic animals, involving a total of 238 samples in 15 different investigations. Once again, France and Portugal stood out in the number of genotyped samples, together with Austria, accounting for approximately 25% of the samples each. Data from Finland, Germany, Italy, Romania, Serbia, and The Netherlands are also available. In respect of the different hosts studied, most of the samples were collected from chicken and sheep (Verma et al., 2015; Bertranpetit et al., 2017; Shwab et al., 2018). In pets and livestock type II strains were detected in nearly 90% (217/238) of samples, together with an 7% (16/238) of type III and 2% of type I (4/238). Apart from that, only one sample presented a recombinant or mixed profile (0.4%, 1/238). Regarding wildlife, European studies included data from Belgium, Czech Republic, England, Finland, France, Italy, Norway, Portugal, Serbia, and Spain, with a total of 160 samples collected in 15 different publications; a wide variety of hosts were included in such surveys, highlighting the red foxes (*Vulpes vulpes*) (n=54) (Aubert et al., 2010; De Craeye et al., 2011) and wild boars (*Sus scrofa ferus*) (n=44) (Richomme et al., 2009; Gisbert-Algaba et al., 2020). Among strains circulating in wild animals, 88.8% corresponded to type II (142/160), 6.2% (10/160) to mixed or recombinant infections and 3.8% (6/160) to type III. Only one case of type I and another of *Caribbean 3* were detected (0.6% each, 1/160) in a pigeon from Portugal and a wild boar from Italy, respectively (Vilares et al., 2014; Sgroi et al., 2020).

As occurred in PCR-RFLP or PCR-sequencing data, environmental samples are quite scarce. Only Santoro et al. (2020) reported genotyping results from Mediterranean mussels (*Mytilus galloprovincialis*) collected in southern Italy, with four samples surprisingly belonging to type I and one sample showing a recombinant or mixed profile. As this is the only study, including such a small sample size, general conclusions should be drawn after further complementary surveys.

Table 3. Prevalence of the *Toxoplasma gondii* genetic types in Europe according to the four compartments within the One Health concept and based on MS data. Percentages are given in brackets

	Human samples	**Domestic animal samples**	**Wildlife animal samples**	**Environmental samples**	**TOTAL**
Type I	11 (2.6)	4 (2)	1 (0.6)	4 (80)	20 (2.4)
Type II	370 (86.4)	217 (91.2)	142 (88.8)	0 (0)	729 (87.7)
Type III	13 (3)	16 (7.8)	6 (3.8)	0 (0)	35 (4.2)
Africa 1	6 (1.4)	0 (0)	0 (0)	0 (0)	6 (0.8)
Caribbean 2	1 (0.2)	0 (0)	0 (0)	0 (0)	1 (0.1)
Caribbean 3	0 (0)	0 (0)	1 (0.6)	0 (0)	1 (0.1)
Recombinant, atypical strains or mixed infections	28 (6.3)	1 (0.5)	10 (6.2)	1 (20)	39 (4.7)
TOTAL	428	238	160	5	831

On balance, the prevalence figures obtained from reviewing the available data on *T. gondii* strains genotyped by MS in Europe are quite similar to those obtained by PCR-RFLP and PCR-sequencing methods. The predominance of type II strains in Europe is also evident by MS genotyping methods, involving 87.7% (729/831) of the total samples analysed in 42 studies that meet the criteria of at least five markers characterized **(Table 3**). As seen in previous section, type I strains remain infrequent, representing 2.4% (20/831) of samples. On the other hand, the prevalence of type III and recombinant or mixed infections slightly decrease until 4.2% (35/831) and 4.7% (39/831) of total records, respectively. Finally, MS-typing was able to resolve other non-canonical haplogroups, *i.e.*, *Caribbean 1*, *Caribbean 3*, or *Africa 1*, allowing to identify *T. gondii* strains possibly imported to Europe (1%, 8/831), either by human migration or trade. There are important sampling biases in the currently available data; not all countries are covered to the same extent and not all compartments are covered per country. Overall, France, Portugal, Austria, and Belgium are the countries with the highest number of surveys carrying MS genotyping in their territories; on the contrary, there are large areas of the continent from which there is no information, especially northern European countries. Again, it should be emphasized the lack of data from Spain except from an isolate (TgA21067) obtained from a yellow-legged gull (*Larus michahellis*), and fully characterized by 15 MS markers (Gamble et al., 2019).

2.4 Genetic diversity and virulence within *Toxoplasma* genus

In addition to study the global genetic diversity and population structure of *T. gondii*, genetic characterization studies aim to find a link between genetics and virulence features of the parasite. It would be useful for detecting genotypes or genetic features associated with more severe outcomes or specific clinical manifestations (Grigg et al., 2001; Carme et al., 2002, 2009; Demar et al., 2007; Gilbert et al., 2008). It appears to be a relation between type I isolates (and its variants) as well as atypical genotypes, and an incremented virulence in human and mice. In a study performed in the USA, an unusual bias toward type I alleles was found in the genotyped isolates from ocular toxoplasmosis patients (Grigg et al., 2001). Some reports on severe clinical toxoplasmosis patients in Europe were also related with "atypical" strains (Delhaes et al., 2010a, b), although the predominance of clonal type II in this continent is undeniable and there is no clear association (Fekkar et al., 2011; Jokelainen et al., 2018). Likewise, in Brazil there is a disproportionate association of congenital infections with the development of ocular toxoplasmosis, presumably related with the epidemic population structure dominant in this country (Gilbert et al., 2008). Finally, special attention deserves the French Guiana territory, where most divergent isolates have been detected in association with severe clinical cases of toxoplasmosis ("Amazonian toxoplasmosis") (Carme et al., 2002, 2009; Demar et al., 2007; Blaizot et al., 2019).

As in clinical human toxoplasmosis, different haplogroups show strong phenotypic differences also in the laboratory mouse, which provides a suitable model for acute and chronic infection. As will be explained in detail (see section 3 in this chapter), virulence degree of *T. gondii* strains has been conventionally determined according to cumulative mortality rate in outbred laboratory mice; in this regard, *T. gondii* clonal lineages I, II and III have been traditionally classified into highly virulent (100%, $LD_{100} = 1$; type I), intermediate virulent (99-30%, $LD_{50} \geq 1000$; type II) and non-virulent (< 30%, $LD_{50} > 10^5$; type III) (Sibley and Boothroyd et al., 1992; Su et al., 2002). Likewise, Pena et al. (2008) observed different degrees of virulence in mice in the different clonal groups described in Brazil: type BrI was considered as highly virulent, type BrIII as non-virulent, whilst type BrII and BrIV lineages were described as intermediately virulent.

In order to determine the major genetic effectors in mice virulence, the ability of *T. gondii* to undergo meiosis in the DH has been exploited to develop experimental genetic approaches based on co-infection of a cat with two separate well characterized parasite strains (Khan et al., 2005). Quantitative trait locus (QTL) mapping analyses of the virulence in mice of F1 progeny derived from sexual recombination experiments of representative strains of the three *T. gondii* archetypal genotypes (I×II, I×III and II×III crosses) resulted in the identification of some members of a family of serine/threonine protein kinases found in rhoptries (ROPs) as key determinants of acute virulence in mice (Saeij et al., 2006; Taylor et al., 2006; Reese et al., 2011; Behnke et al., 2011, 2015). *ROP18*, *ROP5,* and *ROP16* genes encode for three polymorphic rhoptry protein kinases that in a different but synergic manner contribute to the evasion of host immune response controlling the accumulation of interferon-γ induced immunity-related GTPases (IRGs) on parasitophorous vacuole membranes (PVM) and subsequent parasite destruction (Niedelman et al., 2012; Behnke et al., 2012). The proven role played by these effectors motivated the interest to develop molecular typing markers based on their sequences to quickly infer the degree of virulence of *Toxoplasma* strains. Consequent studies concluded that the allelic combination of *ROP18/ROP5* is highly predictive of virulence in mice across globally distributed *T. gondii* isolates (Dubey et al., 2014; Shwab et al., 2016). In another genetic mapping and linkage analysis

study, *CS3* locus located in chromosome VIIa (as *ROP18* gene) was defined to be also strongly linked to acute virulence in mice (Khan et al., 2005); posterior Brazilian and Chinese studies demonstrated this correlation of type I or II alleles present in strains with high mouse-mortality rates, and type III alleles associated with low or null values (Pena et al., 2008; Wang et al., 2013a; Rocha et al., 2018).

Host resistance and parasite virulence processes are linked in a continuous dynamic equilibrium (sometimes unstable), imposing intense selective pressures on each other (Gazzinelli et al., 2014). *Toxoplasma gondii* possess a wide host range, and host-parasite relationships are consequently highly specialized. Therefore, although general machineries of host immunity have been extensively studied, each host species has its own peculiarities derived from a specific mechanism of co-adaptation. For instance, although the IRG gene family is widely distributed in vertebrates, the IRG gene diversity found in rodents is remarkably higher. In addition, genes encoding Toll-Like Receptors (TLR) 11 and TLR12, proteins that along with IRGs are essential elements for detection and immune control of *T. gondii* in mice, are absent in the genome of other mammals such as humans, sheep, goat, cattle, cats or dogs (Gazzinelli et al., 2014). Because of that, it should be highlighted that virulence factors abovementioned have been described in mouse models and therefore are not directly applicable to other hosts and conclusions should be drawn with caution.

3. Phenotypic diversity in *Toxoplasma gondii*

The phenotype is defined as the set of traits of an organism (*e.g.*, morphology, host-interaction mechanisms, physiology) that results from the expression of its genotype under the influence of the environment in which it develops (Johannsen, 2014). *Toxoplasma gondii* possess a significant genetic and phenotypic diversity that have been proposed as responsible for the variation in clinical presentations. In the same manner as with genetic markers, parasite strain specific differences could be approached through the called "phenotypic markers" (Weiss and Kim, 2020). Lethality in laboratory mice is the most remarkable phenotypic marker, that has been well defined for the three archetypal clonal types of *T. gondii*. As mentioned previously, *T. gondii* lineage I strains have been traditionally classified into highly virulent (100% cumulative mortality, $LD_{100} = 1$), type II strains are considered intermediate virulent (99-30%, $LD_{50} \geq 1000$), and lineage III strains are defined as non-virulent (< 30%, $LD_{50} > 10^5$) (Sibley and Boothroyd et al., 1992; Su et al., 2002). Likewise, most of South American divergent haplogroups (4 - 10) have been also characterized as highly virulent using virulence in mice phenotypic marker (Grigg and Suzuki, 2003; Khan et al., 2007). Nevertheless, the biological diversity of *Toxoplasma* extends far beyond lethality in mice and it has been studied from multiple points of view, almost always linked to the interaction with the host. Both *in vitro* (*e.g.*, invasion, growth, tachyzoite-bradyzoite conversion, and plaque formation assays) and *in vivo* (*e.g.*, host immune response, weight loss, lesions severity, parasite burden in tissues) approaches have been implemented.

When it comes to strains phenotypic characterization, it should be considered that apart from parasite genetic background, both environmental factors and host genetics can influence on the outcome of the infection (Mukhopadhyay et al., 2020). There is wide evidence of maintained laboratory conditions influencing parasite biological behaviour (Khan et al., 2009a; Sánchez-Sanchez et al., 2019b), as well as completely different infection outcomes in different hosts challenged with the same isolate or between different infection routes implemented (Oliveira et al., 2016; Yang et al., 2017a; Taniguchi et al., 2018; Hassan et al., 2019; Sánchez-Sánchez et al.,

2019b). Besides, under the light of literature, while genetic characterization methodologies have been harmonized, phenotypic characterization procedures have not been subject of the same criticism and standardization. Only the calculation of cumulative mortality rate as suggested by Saraf et al. (2017) has been accepted as a consensus for virulence in mice assessment. Apart from that, there is a lack of harmony in the biological parameters measured as well as in the experimental conditions such as doses or timing under study. As a consequence, it is difficult to derive general conclusions and it becomes evident that an in-depth review of methodologies is needed.

3.1 Phenotypic characterization in *in vitro* models

Currently, scientific investigations are framed within strict animal welfare policies like those promoted by the EU (Directive 2010/63/EU), that aim to minimize the use of laboratory animals (*i.e.*, the three Rs principle: reduction, replacement, refinement). In this context, the use of *in vitro* models represents an excellent alternative for the study of intracellular organisms such as *T. gondii*. Concretely, *in vitro* models allow to study the host cell infection process by the tachyzoite stage, namely the lytic cycle, mimicking the dissemination of the parasite during the acute phase of the disease (Black and Boothroyd, 2000). The lytic cycle of *T. gondii* is a tightly regulated process which includes adhesion to the host cell, invasion, PV formation, multiplication, and egress (Sibley, 2010).

Proliferative stages of the parasite have been cultured *in vitro* employing a variety of cell culture lines (*e.g.*, HeLa, Vero, HFF, BeWo), primary cell lines, and even complex explants and organoid-derived monolayers (ODMs) approaches (Scheidegger et al., 2005; Holthaus et al., 2021); target cells or tissues (*e.g.*, trophoblast and nervous cell lines or explanted tissues, dendritic cells [DCs], macrophages) should be highlighted (Guimarães et al., 2008; Dellacasa-Lindberg et al., 2011; Witola et al., 2014; Mammari et al., 2014; Barbosa et al., 2015; Da Silva et al., 2017; Pacheco et al., 2020). Most of publications on *T. gondii* implementing *in vitro* assays are focused on safety and efficacy assessment of potential antiparasitic drugs (Basto et al., 2017; Murata et al., 2017; Radke et al., 2018) or on demonstrating the role of different host and parasite effectors in *T. gondii* lytic cycle (Camejo et al., 2014; Bai et al., 2018; Guo et al., 2019; Wang et al., 2020). *In vitro* models are considered also suitable first approaches to phenotypically characterize apicomplexan parasite strains (Conrad et al., 1993; Regidor-Cerrillo et al., 2011; Müller and Hemphill, 2013; Dellarupe et al., 2014; Frey et al., 2016; Jiménez-Pelayo et al., 2017; García-Sánchez et al., 2019). However, only a small proportion of the publications addresses the virulence characterization of *T. gondii* isolates *in vitro* (Sánchez-Sánchez et al., 2019b; Bernstein et al., 2020; Uzelac et al., 2020; Salman et al., 2021).

Contreras-Ochoa et al. (2012) carried out a systematic review of literature to compare studies that had used mouse and human glial cell cultures to determine *T. gondii* invasion and replication rates in these cells. The wide experimental heterogenicity found hampers to draw conclusions, but type II strains (Me49 and Prugniaud [PRU]) seem to be less invasive than type I (RH and BK) in such central nervous system-derived cells. Several publications have characterized some *in vitro* virulence parameters of the par excellence *T. gondii* strain RH in different cell types, and it has been extensively used as an experimental control; however, as evidenced previously, it belongs to the less globally expanded clonal lineage (type I), not representative of *Toxoplasma* diversity, and its continuous laboratory manipulation has drastically modified its original biological

behaviour (Khan et al., 2009a). Comparative studies of other different laboratory strains have been also published (Appleford and Smith, 1997; Diana et al., 2004; Fux et al., 2007; Lambert et al., 2009; Cañedo-Solares et al., 2013; Mammari et al., 2014). Overall, it is generally claimed that type I strains present enhanced proliferation capacity and lower host immune system stimulation than type II isolates (Mammari et al., 2014). Regarding the *in vitro* virulence assessment of non-laboratory isolates, the number of studies is relatively low; some studies that deserve attention are summarized in **Table 4**. One of the key issues is that there is no consensus in experimental conditions (multiplicity of infection [MOI], number of passages, time points for infection, cell culture lines, methods of analysis, etc.), yielding non-comparable results (Contreras-Ochoa et al., 2012). *In vitro* phenotypic evaluation is mostly based on parameters such as parasite invasion rate, proliferation kinetics, tachyzoite yield (TY), or plaque formation, tachyzoite-bradyzoite conversion and spontaneous cyst-formation capabilities (Regidor-Cerrillo et al., 2011; Sánchez-Sánchez et al., 2019b; Uzelac et al., 2020; Salman et al., 2021).

3.2 Phenotypic characterization in *in vivo* models

Due to the wide host-range of *T. gondii*, a large number of animal models of toxoplasmosis have been described, based on many different species and with different purposes (Dubey, 2010). Mouse models are the most frequently used due to the ease of handling, low cost, extensive availability of molecular and immunological reagents and vast scientific understanding about its genetics (*e.g.*, complete sequence of the mouse genome, easy access to genetically modified animals, outbred and inbreed lineages) (Mouse Genome Sequencing Consortium, 2002). Different sub-species of mouse have been used for *Toxoplasma* infection *in vivo* modelling, such as Swiss Webster, CD-1, C57BL/6, BALB/c or Kunming strains (Wang et al., 2013a; Taniguchi et al., 2018; Fukumoto et al., 2020; Uzelac et al., 2020).

Toxoplasma gondii infection has been widely studied using cell-culture derived tachyzoites that are intraperitoneally (IP) or subcutaneously (SC) inoculated into naive laboratory mice (Howe et al., 1996). Despite it does not constitute a natural route, this model has the advantages of reproducibility, ease of administration, and accurate quantification (Sibley et al., 1999). However, variants of this procedure have been also implemented in the literature, including IP injection of cysts (Taniguchi et al., 2018; Gatkowska et al., 2019), or *per os* (PO) inoculation of tissue cysts (McLeod et al., 1984; Khan et al., 2007; Taniguchi et al., 2018; Arcon et al., 2021) and oocysts (Yang et al., 2017b; Chiebao et al., 2021). These variants are inherently less reproducible due to the variable size and bradyzoite contents of a given tissue cyst, or the great difficulty in guaranteeing the oral dosage, as well as less approachable due to the complexity of oocysts obtention; but none-the-less, these are much more valuable and natural routes of infection (Sibley et al., 1999).

Mouse infection models have been useful for many aims, such as for examining the kinetics of parasite distribution following inoculation (Derouin and Garin, 1991), the role of different host or parasite effectors in pathogenicity *in vivo* (Mimura et al., 2012; Händel et al., 2012; Rochet et al., 2019), the evaluation of the immunoprophylactic potential of designed vaccines (Gatkowska et al., 2019; Arcon et al., 2021) or the effectiveness of some drugs for acute treatment (Müller et al., 2017; Montazeri et al., 2019), but also for phenotypic (virulence) characterization of isolates (Howe et al., 1996; Taniguchi et al., 2018; Chiebao et al., 2021; Bernstein et al., 2020; Fukumoto et al., 2020).

Table 4. Some examples of studies that addressed *in vitro* virulence assessment of non-laboratory *Toxoplasma gondii* isolates.

Isolates ID (host)/reference strains included	**Country**	**Genotype (ToxoDB#) or any genetic information**	***In vitro* assays**		***In vivo* assays**	**Reference**
			Cells employed	Parameters evaluated		
BRC TgH38034A, BRC TgH20017A, BRC TgH20018A, BRC TgH20002A (human)/ RH and PRU strains	France	Type II. By MS (*TUB2*, *W35*, *TgM-A*, *B18*, *B17*, and *M33*) and PCR-RFLP typing (*SAG1*, *SAG2*, and *GRA7*)	HFF cells	Invasion rate, parasite replication and spontaneous cyst formation	No	Brenier-Pinchart et al. (2010)
CIBM1 (human)/RH strain	Colombia	No genetic data from CIBM1 isolate	THP1 and Vero cells	Growth and invasion rates	No	Cuellar et al. (2012)
TgCtwh3 and TgCtwh6 (cat)/ RH and PRU strains	China	#9	Primary BMMφs and mouse macrophages Raw 264.7 cells	Cytokine profile and activation induced in macrophages	No	Zhang et al. (2013)
TgCkBrRN2 (chicken), TgCkBrRN3 (chicken), TgPgBrRN1 (pig)/ RH, Me49 and VEG strains	Brazil	No type determined. RFLP-*CS3* alleles III, I and II, respectively	Raw 264.7 cells	Cytopathic effect	Yes	Oliveira et al. (2016)
TgShSp1 (sheep)/Me49	Spain	#3	Marc-145, Vero, and HFF cells	Growth rate, plaque formation and tachyzoite-bradyzoite conversion	Yes	Sánchez-Sánchez et al. (2019b)
TgCkBrAL01, 02 (chicken), TgPigBrAL01, 02, 03 (pig) / RH, Me49	Brazil	#277, #144	Marc-145 cells	Tachyzoite-bradyzoite conversion assay	No	Ribeiro-Andrade et al. (2019)
EQ39 (horse), EQ40 (horse), G13 (pigeon), K1 (chicken)	Serbia	#54, non ToxoDB classificated	Vero cells	Growth rate, plaque formation, ENO2 expression	Yes	Uzelac et al. (2020)
TgSb (New-World monkey) and TgMr (marsupial)/RH, Me49 and VEG strains	Argentina	#163 and #14, respectively.	Vero cells	Growth and invasion rates	Yes	Bernstein et al. (2020)
TgCatJpOk1, TgCatJpOk2, TgCatJpOk3 and TgCatJpOk4 (cat)/RH and Me49 strains	Japan	*UPRT1*, *UPRT7*, *HP intron 2*, *GRA6*, *GRA7*, *SAG1* characterization	HFF cells	Invasion rate and tachyzoite-bradyzoite conversion capacity	Yes	Fukumoto et al. (2020)
TgCatJpObi1 (cat)/Me49	Japan	#4	HFF cells and primary mouse peritoneal macrophages	Growth rate and spontaneous cyst formation	Yes	Salman et al. (2021)

Concerning *in vivo* phenotypic characterization of *T. gondii* isolates, it has been traditionally reduced to the calculation of the cumulative mortality rate in laboratory outbred mice, procedure that was reviewed by Saraf et al. (2017) who proposed a standard operating procedure. According to the authors, cumulative mortality rate calculation implies the use of outbred mice (*e.g.*, Swiss Webster [SW] or CD-1 mouse strains), at least three consecutive doses of IP inoculated tachyzoites and further recording of the casualties among those successfully infected animals at day 30 pi. However, important improvements can be introduced by evaluating additional non-lethal parameters such as cumulated morbidity rate and severity of clinical signs according to normalized scoring, parasite burden and pathological lesions detected in different tissues (*e.g.*, CNS), anti-*T. gondii* IgG antibodies and haptoglobin levels in serum, cystogenic capacity, or even animal behavioral changes (Jungersen et al., 2002; Djurković-Djaković et al., 2012; Dubey et al., 2016; Hamilton et al., 2019; Bezerra et al., 2019; Salman et al., 2021).

Since the reproductive failure is a relevant manifestation of toxoplasmosis, special attention should be paid to animal pregnant models; as would be expected, mouse pregnant model is the most frequently implemented, characterized for a short period of gestation and a high number of pups (Vargas-Villavicencio et al., 2016). Müller et al. (2017) established an excellent mouse model for congenital toxoplasmosis based on oral infection with oocysts and demonstrated its applicability for investigating chemotherapeutic options. Experimental models for toxoplasmosis based on superior animals have been also developed, but its complexity and expensiveness have reserved its use for specific and advanced purposes such as testing the effectiveness of a drug or vaccine (Cornelissen et al., 2014; Sánchez-Sánchez et al., 2019a; Le Roux et al., 2020), or investigating the particular pathogenesis and parasite-host interaction mechanisms triggered in a specific relevant host (Dubey et al., 1996; Powell and Lappin, 2001; Benavides et al., 2011, 2017; Castaño et al., 2016; Basso et al., 2017; Gutiérrez-Expósito et al., 2020).

3.3 Phenotypic diversity of *Toxoplasma gondii* global population

Most of the isolates from which phenotypic data are available, understood as virulence in mice, originate from South America, especially from Brazil (relevant data has been summarized in **Appendix 2**) (Dubey et al., 2014; Shwab et al., 2016). More than half of these isolates, including isolates from Argentina, Brazil, Colombia, Costa Rica, French Guiana, Nicaragua, Puerto Rico, or Uruguay, presented 100% lethality (*e.g.*, #6, #21, #19, #14, #38, among many other genotypes); however, also intermediate, and non-virulent (< 30% mortality) South American isolates have been described (Howe and Sibley, 1995; Dubey et al., 2002, 2003, 2004, 2006a,b, 2007a,b,c,e, 2009; Fux et al., 2003; Khan et al., 2007; De Oliveira et al., 2009; Yai et al., 2009; Shwab et al., 2016; Bernstein et al., 2020). These facts are in concordance with the highly diverse genetic population found in South America wherein many genotypes coexist with none being notably dominant and where the prevalence of atypical and recombinant strains is notable. Isolates from North America subjected to virulence in mice assessment correspond in a high proportion to strains obtained from wild animals; consequently, it seems to be logical that many of them presented a highly virulent character (Dubey et al., 2014; Dubey et al., 2015; Shwab et al., 2016; Verma et al., 2016). Asian reports of virulence in mice are the second most abundant but the scenario is completely different with an abundance of nonvirulent isolates (*e.g.*, #2, #4, #9/*Chinese 1*, #18, #20, among others) (Dubey et al., 2007d,f; Shwab et al., 2016; Yang et al., 2017b; Salman et al., 2021) and rare cases of intermediate/high virulence (*e.g.*, #9, Haplogroup 2) (Wang et al., 2013a,b; Yang et al., 2017a; Taniguchi et al., 2018; Fukumoto et al., 2020;), in

agreement with a prevalent clonality in the population. It could be said that probably only South American and Asian records are enough abundant to result representative of the population diversity in such regions. Virulence studies on African isolates show both non-virulent isolates (*e.g.*, type III or variants, #132, #137, #15) as well as highly virulent strains (≥ 90% lethality) corresponding with the prevalent *Africa 1* and *Africa 3* genotypes (Velmurugan et al., 2008; Dubey et al., 2008; Mercier et al., 2010; Shwab et al., 2016). European data are also limited (**Table 5**), and only a few studies included non-laboratory isolates from Denmark (n=16), Spain (n=1), and Serbia (n=4); the majority of the strains were nonvirulent in mice (mainly corresponding with type II isolates) except for some moderately virulent isolates mostly associated with atypical/recombinant genotypes detected in Serbia (**Table 5**).

Table 5. European *Toxoplasma gondii* isolates phenotypically characterized in mice models. Laboratory-adapted and non-laboratory adapted strains have been considered separately.

Isolate ID	Original host	Country	Type (ToxoDB#)	Doses, parasite stage, route	Mouse strain	Virulence (% mortality)	Other parameters evaluated	Reference for virulence assessment
Non-laboratory isolates								
SSI 119	Adult pig	Denmark	Serotype II	10^2 tachyzoites IP	BALB/c J Bom	Non-virulent (11.1%)	Body weight gain, haptoglobin and IgG levels	Jungersen et al. (2002)
SVS P14	Adult pig					Non-virulent (0%)		
SVS F17	Adult cat					Non-virulent (0%)		
SVS O14	Aborted lamb					Non-virulent (0%)		
SVS Fox2	Adult fox					Non-virulent (0%)		
SVS O12	Adult sheep					Non-virulent (0%)		
SVS O15	Adult sheep					Intermediate (67%)		
SVS O16	Adult sheep					Non-virulent (0%)		
SVS O17	Adult sheep					Non-virulent (0%)		
SVS O18	Adult sheep					Non-virulent (0%)		
SVS O10	Aborted lamb					Non-virulent (0%)		
SVS O11	Weak-born lamb					Non-virulent (0%)		
SVS O13	Aborted lamb					Non-virulent (0%)		
SVS O14	Aborted lamb					Non-virulet (0%)		
SVS O20	Stillborn lamb					Non-virulent (0%)		
SVS O21	Aborted lamb					Non-virulent (0%)		
TgShSp1	Sheep (abortion)	Spain	Type II variant (#3)	$1\text{-}10^5$ tachyzoites IP	CD-1	Non-virulent (0%)	Virulence evaluation in pregnant mice and sheep models after PO oocysts dosage	Sánchez-Sánchez et al. (2019b)

Table 5 (continued)

Isolate ID	Original host	Country	Type (ToxoDB#)	Doses, parasite stage, route	Mouse strain	Virulence (% mortality)	Other parameters evaluated	Reference for virulence assessment
Non-laboratory isolates								
G13	Feral pigeon	Serbia	Recombinant	10^2-10^4 tachyzoites IP	SW	Non-virulent (10.7%)	*ROP18*/*ROP 5* alleles combination	Uzelac et al. (2020)
EQ39	Horse		Atypical (#54)			Non-virulent (6.8%)		
EQ40	Horse		Recombinant			Intermediate (69.4%)		
K1	Chicken		Recombinant			Intermediate (38.8%)		
Laboratory isolates								
Moredun M4	Sheep (abortion)	UK	Type II variant (#3)	2 x 10^2 tachyzoites IP	CD-1	Non-virulent (20%)	Parasite burden and histopathological lesions	Hamilton et al. (2019)
BOF	Human	Belgium	Atypical (#6)	5-10 cysts PO	CD-1	Non-virulent (8.3%)	Cyst oral infectivity	Khan et al. (2009b); Khan et al. (2007)
DEG	Human	France	Type II variant (#3)	5-10 cysts PO	CD-1	Non-virulent (0%)	-	
MAS	Human	France	Atypical (#17)	10^1- 10^3 tachyzoites IP	CD-1	Highly virulent (100%)	Cyst oral infectivity	
FOU	Human	France	Atypical (#6)	10^1- 10^3 tachyzoites IP	CD-1	Highly virulent (100%)	Cyst oral infectivity	
ENT	Human	France	Haplogroup 1	10^1- 10^3 tachyzoites IP	CD-1	Highly virulent (100%)	-	
MOR	Human	France	Haplogroup 1	10^1- 10^3 tachyzoites IP	CD-1	Highly virulent (100%)	-	Khan et al. (2009b)
GPHT	Human	France	Atypical (#6)	5-10 cysts PO	CD-1	Highly virulent (100%)	Cyst oral infectivity	Khan et al. (2007)

CHAPTER III

HYPOTHESIS AND OBJECTIVES

CAPÍTULO III

HIPÓTESIS Y OBJETIVOS

Toxoplasma gondii (subphylum Apicomplexa) is one of the most widespread and successful opportunistic parasites worldwide, and it is of major medical and veterinary importance (Dubey, 2010). *Toxoplasma gondii* can infect virtually all warm-blooded animal species, what makes the parasite to be an important concern not only in public health, but also in livestock industry and wildlife management and conservation programmes. Although toxoplasmosis is normally latent and asymptomatic, it can involve serious clinical manifestations in immunocompromised and pregnant hosts. *Toxoplasma gondii* is among the leading causes of abortion in sheep, involving a loss of around 1 million of lambs/year in the European Union (Innes et al., 2009; Katzer et al., 2011). In addition to its relevance in sheep, toxoplasmosis is a zoonotic disease and its notable prevalence in important meat-producing animal species such as pigs, poultry or goats is a major public health concern (Stelzer et al., 2019). Toxoplasmosis imposes a substantial disease burden in humans all across the world, with an estimated third of the global population infected and seroprevalence figures above 60% in some parts of South America, Africa, and South-East Asia (Pappas et al., 2009). While asymptomatic in most patients, toxoplasmosis is an important cause of choroiditis in immunocompetent patients and a potentially life-threatening illness in immunocompromised patients and foetuses (Montoya and Liesenfeld, 2004). Furthermore, congenital toxoplasmosis cases have been estimated in about 200,000 per year globally, equivalent to a burden of 1.20 million DALYs (disability-adjusted life years lost) burden (Torgerson and Mastroiacovo, 2013). The impact of human toxoplasmosis is evident and notably higher in South America where the different clinical manifestations tend to be not only more frequent but also more severe, presumably due to the genetic heterogeneity that characterizes *T. gondii* strains of this subcontinent (Gilbert et al., 2008; Carme et al., 2009; Blaizot et al., 2019).

Genetic and phenotypic diversity in *T. gondii* population has been broadly demonstrated. Biological differences are expected due to a global distribution, a complex life cycle including diverse environments and a vast host range, along with the capacity for genetic recombination during the sexual reproduction inside the DH's small intestine. The genetic exchange between different strains may occur when the DH becomes simultaneously infected with more than one strain. The degree of sexual exchange determines the population genetic structure and can highly influence phenotypic variability in the species. The study of the genetic diversity and population structure of *T. gondii* can be crucial for understanding its epidemiology and pathogenicity, as well as for implementing suitable disease control strategies (Beck et al., 2009).

Implementing first *T. gondii* genotyping methods, during the 1990s pioneer researchers originally described a clonal population structure shaped by three genetic types (I, II, and III) linked to virulence in mice observed in isolates obtained from human patients (Howe and Sibley, 1995). Thus, type I isolates were 100% lethal to mice regardless the dose, and types II and III were usually considered non-virulent (<30% lethality in a dose-dependent manner) (Howe et al., 1996). Since then, global population structure and genetic variability of *T. gondii* have been extensively investigated. Important efforts on isolation, and both molecular and phenotypic characterization of the parasite, revealed a much more complex population structure with at least 16 haplogroups worldwide (Su et al., 2012; Lorenzi et al., 2016), and a virulence degree classification that is now under review. Regarding *T. gondii* European population structure, similarly to that in North America, it appears to be dominated by three clonal lineages (I, II and III), which coexist with much more scant, genetically diverse isolates. This contrasts completely with the enormous genetic diversity observed in South America, where many genotypes coexist with none being dominant, and where atypical and recombinant strains are highly prevalent (Su et al., 2012; Lorenzi et al., 2016). Nevertheless, if a comprehensive review of the literature is carried out, the

limitation in genotyping data in Europe is evident. The clear predominance of type II strains in Europe, that supposes more than three quarters of records, is undeniable but important sampling disparities exist between regions of the continent, and vast areas remain unexplored. Furthermore, there is a lack of consensus over the methodologies and markers applied, resulting in incomplete, not very reliable and noncomparable results in many cases. Finally, minimal information exists about the *T. gondii* genotypes circulating in Spain. If studies applying less than four genotyping markers are discarded, only a few investigations focused on wildlife (wild boar, rodents and foxes) are available (Calero-Bernal et al., 2013, 2015; Fernández-Escobar et al., 2020).

Marked biological differences also occur among isolates of *T. gondii* in virulence traits, whose characterization has been traditionally approached through the calculation of the cumulative mortality rate in laboratory outbred mice (Saraf et al., 2017). Leaving apart lethality in mice, additional *in vitro* (*e.g.*, invasion, growth, and plaque formation assays) and *in vivo* (*e.g.*, host immune response, loss of weight, parasite burden in tissues) approaches have been carried out, but there is an important lack of consensus in experimental conditions (*e.g.*, MOI *in vitro*, timing, cell culture lines or mouse strains, dosages, inoculation routes, or methods of analysis), making comparisons almost impossible. Studies on biological characterization of European non-laboratory strains are limited; only one Spanish isolate (TgShSp1, genotype II PRU variant, ToxoDB #3) has been phenotypically characterized at some extent (Sánchez-Sánchez et al., 2019b), revealing important differences in the *in vitro* and *in vivo* virulence in comparison with the reference strain Me49 (clonal genotype II, #1) despite their almost identical genetic RFLP-profiles.

In this scenario, the general aim of the present Doctoral Thesis was to obtain a representative panel of *T. gondii* isolates from sheep and Iberian pig livestock in Spain and to achieve its genetic and phenotypic characterization through accurate molecular methodologies as well as *in vitro* and *in vivo* models, respectively. The final purpose is to increase our knowledge about the *T. gondii* genotypes circulating in Spanish relevant livestock species along with their virulence degree. In addition, the in-depth characterization of isolates gives the opportunity to the future study of new virulence factors that may explain the biological variability found among strains. To achieve this general aim, the following specific objectives were addressed:

Specific objective 1: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish sheep livestock and its genetic and phenotypic characterization

- **Sub-objective 1.1**: Obtaining of *Toxoplasma gondii* isolates from Spanish sheep livestock
- **Sub-objective 1.2**: Genetic characterization of *Toxoplasma gondii* isolates obtained from Spanish sheep livestock
- **Sub-objective 1.3**: Phenotypic characterization of *Toxoplasma gondii* isolates obtained from Spanish sheep livestock

Specific objective 2: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish Iberian pigs and its genetic and phenotypic characterization

- **Sub-objective 2.1**: Obtaining of *Toxoplasma gondii* isolates from Spanish Iberian pigs
- **Sub-objective 2.2**: Genetic characterization of *Toxoplasma gondii* isolates obtained from Spanish Iberian pigs
- **Sub-objective 2.3**: Phenotypic characterization of *Toxoplasma gondii* isolates obtained from Spanish Iberian pigs

CHAPTER IV

WORK PLAN AND METHODOLOGIES

CAPÍTULO IV

PLAN DE TRABAJO Y METODOLOGÍA

The description of the materials and methodologies that have been implemented for the development of each of the previously mentioned objectives are explained in detail in the research articles that are part of this Doctoral Thesis. However, according to the regulations for presenting a Doctoral Thesis through scientific publications, this section briefly presents the general work plan carried out to achieve those objectives. The **Figure 5** shows a schematic workflow to support this section.

It should be noted that animal procedures described below for the *T. gondii* isolation by bioassay in mice and evaluation of virulence degree (PROEX 274/16) were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU regulations (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize the suffering. As a humane endpoint, mice with a severe loss of body condition or nervous clinical signs were euthanized to limit unnecessary suffering.

<u>Specific objective 1</u>: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish sheep livestock and its genetic and phenotypic characterization

Sub-objective 1.1: Obtaining of *Toxoplasma gondii* isolates from Spanish sheep livestock

The goal of this sub-objective was to obtain a panel of ovine *T. gondii* isolates representative of a maximum geographical coverage and therefore, of the genetic diversity circulating in the Spanish territory. Due to the major role of *T. gondii* in reproductive failure in sheep, abortions cases and congenitally infected lambs were considered as a relevant source for *T. gondii* isolation. On the other hand, the high prevalence figures previously described in sheep in Spain encouraged us to consider the isolation of parasites from myocardial tissues from adult animals collected in authorized slaughterhouses. In both cases, isolation was performed by a bioassay in mice prior to propagation in cell culture but with certain differences between procedures (Regidor-Cerrillo et al., 2008; Dubey, 2010). Abortion-derived tissues (*i.e.*, foetal brains, brains from weak lambs that died shortly after birth, and placental tissues) were submitted from the University of León (ULE, Spain) after preliminary clinical and histopathological *Toxoplasma* diagnosis of different ovine abortion outbreaks occurred during 2015-2018 period. Definitive diagnosis was achieved by *T. gondii* DNA detection by single-tube nested PCR amplification of the specific *ITS1* region (Castaño et al., 2014). Selected *Toxoplasma* PCR-positive tissues were suspended in an antibiotics solution, homogenized, and then SC inoculated in Swiss/CD-1 mice (Regidor-Cerrillo et al., 2008). In the case of myocardial tissues from adult animals from slaughterhouses, after serological analysis (ELISA) of paired serum samples collected, those tissues associated with the highest antibody titres were selected for isolation procedures. Heart tissues were subjected to acid-pepsin artificial digestion prior to inoculation (Dubey, 2010). Mice were observed daily, clinical signs were scored, and *Toxoplasma* infection was confirmed in mice that died during the procedure (by tissue imprints of brains, lungs and/or lymph nodes examination for parasites presence) as well as in surviving mice at 30 dpi (days post-inoculation) by IFAT (Álvarez-García et al., 2003). After intraperitoneal subpassages in additional mice of freshly recovered brains from seropositive animals, peritoneal exudates were collected and subjected for *in vitro* cultivation and final isolation

of the parasite. Tachyzoites from infected cell cultures were harvested and cryopreserved in liquid nitrogen until further studies.

For further details, see the publication Fernández-Escobar et al. 2020. Parasites and Vectors (p. 65).

Sub-objective 1.2: Genetic characterization of *Toxoplasma gondii* isolates obtained from Spanish sheep livestock

The aim of this sub-objective was to determine the complete RFLP genotype of the ovine isolates obtained as well as define their alleles for the molecular marker *CS3* (presumably associated with virulence in mice). *Toxoplasma gondii* genotyping was performed by the widely used PCR-RFLP method based on *SAG1*, *SAG2* (5'- 3' *SAG2* ends, and alt. *SAG2*), *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1*, *Apico* and *CS3* markers (Pena et al., 2008; Su et al., 2010;). RFLP genotype numbers were assigned according to the ToxoDB database (https://toxodb.org/toxo/app). Genetic characterization was attempted also on clinical samples with an initial *ITS1* nested-PCR amplification positive result, thus increasing our knowledge about the genotypes circulating in the territory and avoiding discarding less proliferative strains during the isolation process.

Additionally, in awareness of the limited resolution of the RFLP methodology, we also conducted sequence-based genotyping by MLS typing analysis on *SAG3*, *GRA6* and *GRA7* well-known polymorphic loci (Bottós et al., 2009). Finally, in order to infer the *T. gondii* population structure circulating in Spanish sheep livestock, *SAG3*, *GRA6* and *GRA7* sequences obtained from all isolates and some reference strains were concatenated and aligned using MEGA X software (Kumar et al., 2018) to finally generate an unrooted phylogenetic tree.

For further details, see the publication Fernández-Escobar et al. 2020. Parasites and Vectors (p. 65).

Sub-objective 1.3: Phenotypic characterization of *Toxoplasma gondii* isolates obtained from Spanish sheep livestock

Virulence characterization of selected *T. gondii* ovine isolates was carried out through *in vivo* and *in vitro* models, as well as complementary molecular analyses. The isolates were selected according to three criteria: a) genotype, b) geographical origin, and c) clinical sample of origin (abortion-derived tissues or myocardial tissues from adult sheep).

- *In vivo* assays

First, cumulative mortality of the selected isolates was evaluated in a standardized *in vivo* mouse model as the gold standard method for *T. gondii* strains (Saraf et al., 2017). Doses from 10^5 to 1 tachyzoite(s) of each isolate were IP inoculated into five 8-week-old female Swiss/CD1 mice and clinical signs were recorded daily for 6 weeks. Cumulative mortality and morbidity rate were calculated based on the ratio of casualties or symptomatic mice to the total number of infected mice, respectively.

Secondly, additional groups of mice were IP-inoculated with 10^3 tachyzoites and sacrificed at 7 dpi or 30 dpi to study the acute and chronic phases of the infection, respectively. Selected organs (brain, eyes, lung, heart, liver, and kidney) were collected during necropsies for tissue tropism and parasite burden evaluations by *T. gondii* DNA detection (nPCR) and quantification (qPCR) as well as for histological lesions examination (fixation and haematoxylin and eosin staining). Further samples from *quadriceps femoris* muscle and tongue were also subjected for histological evaluation.

In all *in vivo* assays, serum samples from mice that were humanely euthanized, presented sudden death, or reached the end of the experiment at 7, 30 or 42 dpi, were collected to confirm the infection by IFAT; in early casualties, tissue imprints were also examined for parasites presence.

- *In vitro* assays

In *in vitro* experiments, the selection of an accurate cell culture type is of special relevance. In experimental *in vitro* infections, less restricted than animal *in vivo* models, it is of great value to choose cell lines derived from tissues of a host of interest, especially from a known infection target tissue. For this sub-objective, an ovine trophoblast cell line (AH-1) was chosen since it shares the ovine origin of the isolates and trophoblasts are target cells during congenital infection, with a proven immunomodulatory role (Wheelhouse et al., 2009). The cell line was kindly supplied by the Department of Veterinary Microbiology and Pathology of the Washington State University (Pullman, WA, USA). The invasion rate and proliferation kinetics in AH-1 cells were evaluated in some of the isolates with more contrasting results in the *in vivo* mouse model assays. The invasion rate in AH-1 cells was calculated at different time points determining the number of infection events (parasitophorous vacuoles or lysis plaques) derived from an infection at fixed MOI (multiplicity of infection) detected by immunofluorescence staining and counted by direct observation in an inverted fluorescence microscope. Proliferation kinetics in AH-1 cells were evaluated by quantifying the number of tachyzoites at specific times after the infection by means of qPCR. Proliferation kinetics of all isolates included were also studied by single immunostaining of fixed cultures using a confocal fluorescence inverted microscope (Jiménez-Pelayo et al., 2017; García-Sánchez et al., 2019).

- Molecular analyses on virulence markers

The allelic profile of the *CS3* marker and the virulence factors *ROP18* and *ROP5*, is considered as highly predictive of virulence degree in mice for *T. gondii* strains (Pena et al., 2008; Dubey et al., 2014; Shwab et al., 2016). The alleles for these genomic regions were studied by nested-PCR-DNA-sequencing to provide a possible correlation with the phenotypic *in vivo* and *in vitro* features studied. After sequencing, *in silico* digestion of each locus sequences by identification of restriction enzyme motifs was conducted.

For further details, see the publication Fernández-Escobar et al. 2021. Veterinary Research (p. 87).

<u>Specific objective 2</u>: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish Iberian pigs and its genetic and phenotypic characterization

Sub-objective 2.1: Obtaining of *Toxoplasma gondii* isolates from Spanish Iberian pigs

Given the relevance of Iberian pigs in Spanish livestock industry, the goal of this sub-objective was to obtain a panel of *T. gondii* isolates from Iberian pigs representative of a maximum geographical coverage and genetic diversity within Spain. Fattening Iberian pigs slaughtered for human consumption between December 2017 to June 2018 were considered for *T. gondii* isolation. Again, paired myocardial tissues and serum samples from adult animals were collected, and the procedures for parasite isolation were the same as previously reported for myocardial tissues from adult sheep (Sub-objective 1.1).

For further details, see the publication Fernández-Escobar et al. 2020. Frontiers in Veterinary Science (p. 105).

Sub-objective 2.2: Genetic characterization of *Toxoplasma gondii* isolates obtained from Spanish Iberian pigs

The purpose of this sub-objective was to determine the RFLP genotype of the isolates obtained from Iberian pigs as well as define their alleles for the virulence in mice molecular marker *CS3*. Once again, *T. gondii* genotyping was implemented by the PCR-RFLP method based on *SAG1*, *SAG2* (5'- 3' *SAG2* ends, and alt. *SAG2*), *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1*, *Apico* and *CS3* markers (Pena et al., 2008; Su et al., 2010). RFLP genotype numbers were assigned according to the ToxoDB database (https ://toxod b.org/toxo/).

For further details, see the publication Fernández-Escobar et al. 2020. Frontiers in Veterinary Science (p. 105).

Sub-objective 2.3: Phenotypic characterization of *Toxoplasma gondii* isolates obtained from Spanish Iberian pigs

Virulence characterization of selected *T. gondii* isolates from Iberian pigs was carried out through a standardized *in vivo* mouse model, following the same procedures as those indicated in Sub-objective 1.3.

For further details, see the publication Fernández-Escobar et al. 2020. Frontiers in Veterinary Science (p. 105).

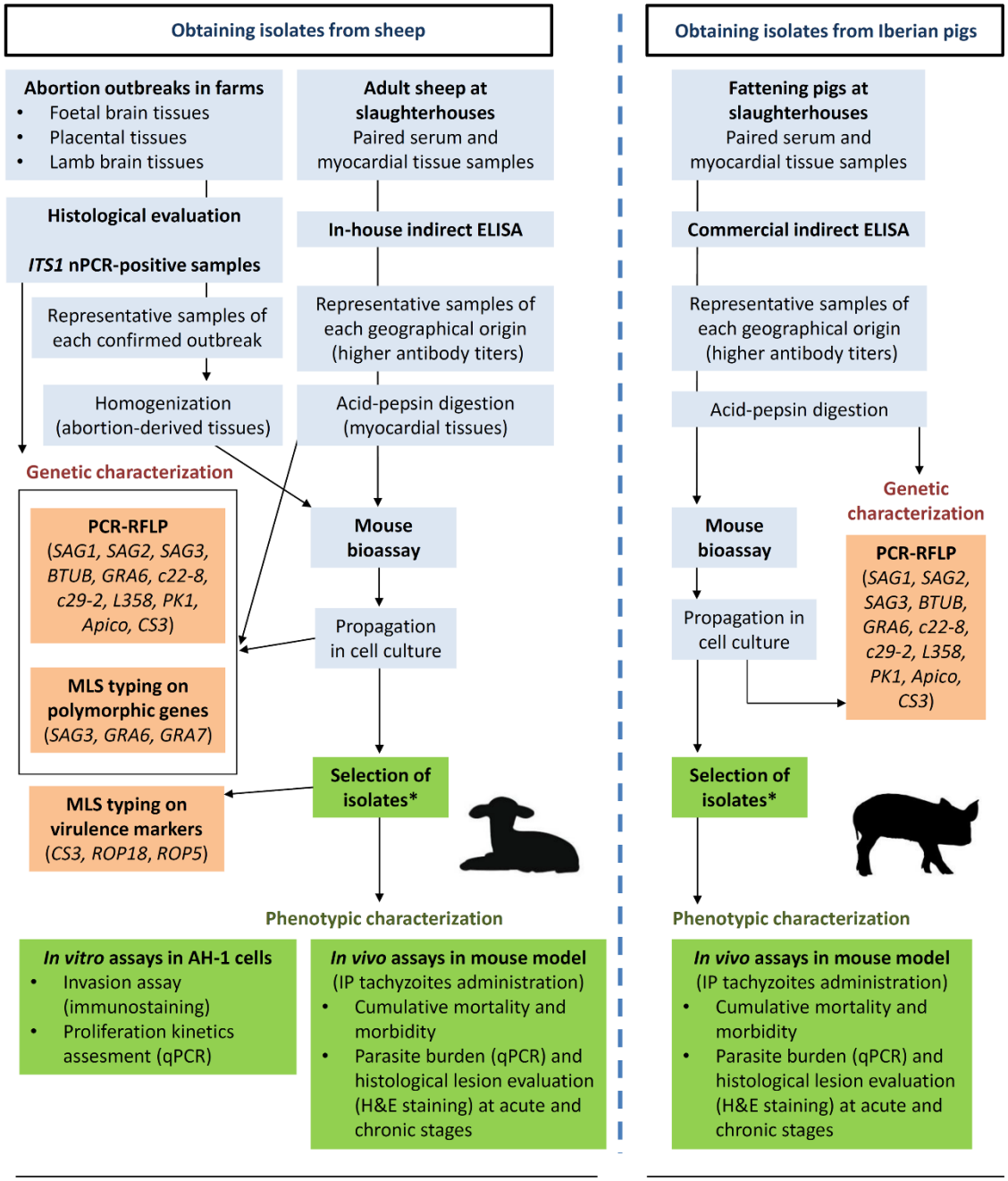

Figure 5. Schematic workflow with steps and methodologies implemented to achieve the different objectives of the present Doctoral Thesis. *, isolates were selected on the basis of PCR-RFLP genotype, geographic origin, and original clinical sample (abortion-derived or myocardial tissues), trying to maximize the diversity coverage.

CHAPTER V

RESULTS (PUBLICATIONS)

CAPÍTULO V

RESULTADOS (PUBLICACIONES)

<u>Specific objective 1</u>: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish sheep livestock and its genetic and phenotypic characterization

Sub-objective 1.1: Obtaining of *Toxoplasma gondii* isolates from Spanish sheep livestock
Sub-objective 1.2: Genetic characterization of *Toxoplasma gondii* isolates obtained from Spanish sheep livestock

The results that correspond to the first two sub-objectives defined within the specific objective 1 have been published in a scientific article presented below:

RESUMEN / SUMMARY: *Toxoplasma gondii* es una de las principales causas de aborto en pequeños rumiantes y presenta un carácter zoonótico de especial relevancia cuando se consume carne poco cocinada que contiene quistes. El objetivo del presente estudio fue investigar la diversidad genética de las cepas de *T. gondii* que circulan en el ganado ovino en España. Para ello, muestras seleccionadas procedentes de brotes de abortos por toxoplasmosis (n = 31) y de ovejas adultas (crónicamente infectadas) sacrificadas en matadero (n = 50) de diferentes regiones españolas, fueron sometidas a un bioensayo en ratón con el objetivo de aislar el parásito. Además, todas las muestras clínicas originales y los aislados resultantes fueron genotipados por el método PCR-RFLP basado en 11 marcadores moleculares, así como por secuenciación de fragmentos de los genes *SAG3*, *GRA6* y *GRA7*.

Como resultado, se obtuvieron 30 aislados de nueve regiones españolas: 10 aislados de muestras derivadas de abortos y 20 aislados de muestras de miocardio adulto. Se encontraron tres genotipos: ToxoDB #3 (variante PRU del genotipo clonal II) en el 90% (27/30) de los aislados, ToxoDB #2 (genotipo clonal III) en el 6.7% (2/30) y ToxoDB #1 (genotipo clonal II) en 3.3% (1/30). Cuando todas las muestras de tejido positivas a la presencia de ADN de *T. gondii* (n = 151) se sometieron directamente al genotipado por PCR-RFLP, se obtuvieron perfiles de restricción completos para el 33% de las muestras, y hasta el 98% de ellos pertenecían a la variante PRU del tipo clonal II. Un cerebro fetal mostró un patrón clonal de tipo II y cuatro muestras mostraron alelos inesperados de tipo I en el marcador *SAG3*, incluidos dos cerebros fetales que mostraron alelos I + II como eventos de coinfección. Los amplicones de *SAG3*, *GRA6* y *GRA7* obtenidos de los aislados y las muestras clínicas fueron secuenciaciados, lo que nos permitió confirmar los resultados de RFLP y detectar diferentes polimorfismos puntuales.

El presente estudio muestra la existencia de un genotipo PRU variante del tipo II clonal, (ToxoDB #3) predominante que infecta a ovejas en España, tanto en casos de aborto como en infecciones crónicas en adultos, coexistiendo con otros genotipos clonales (ToxoDB #1 y ToxoDB #2) mucho menos frecuentes, así como con cepas polimórficas, según reveló el genotipado de las muestras clínicas. El uso de la tipificación multilocus de secuencias ayudó a describir con mayor precisión la diversidad intragenotipo de *T. gondii*.

Reference: Fernández-Escobar M, Calero-Bernal R, Benavides J, Regidor-Cerrillo J, Guerrero-Molina MC, Gutiérrez-Expósito D, Collantes-Fernández C, Ortega-Mora LM. (2020). Isolation and genetic characterization of *Toxoplasma gondii* in Spanish sheep flocks. *Parasites & Vectors*. 13(1):396. doi: 10.1186/s13071-020-04275-z.
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JCR 2019 category, Journal rank/Ranked journals (Quartile): Parasitology, 9/38 (Q1).
Impact factor (2019): 2.824

Fernández-Escobar *et al. Parasites Vectors* (2020) 13:396
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Parasites & Vectors

RESEARCH Open Access

Isolation and genetic characterization of *Toxoplasma gondii* in Spanish sheep flocks

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Abstract

Background: *Toxoplasma gondii* is a major cause of abortion in small ruminants and presents a zoonotic risk when undercooked meat containing cysts is consumed. The aim of the present study was to investigate the genetic diversity among the *T. gondii* strains circulating in ovine livestock in Spain.

Methods: Selected samples collected from abortion outbreaks due to toxoplasmosis ($n = 31$) and from chronically infected adult sheep at slaughterhouses ($n = 50$) in different Spanish regions were bioassayed in mice, aiming at parasite isolation. In addition, all original clinical samples and the resulting isolates were genotyped by multi-nested PCR-RFLP analysis of 11 molecular markers and by PCR-DNA sequencing of portions of the *SAG3*, *GRA6* and *GRA7* genes.

Results: As a result, 30 isolates were obtained from 9 Spanish regions: 10 isolates from abortion-derived samples and 20 isolates from adult myocardial tissues. Overall, 3 genotypes were found: ToxoDB#3 (type II *PRU* variant) in 90% (27/30) of isolates, ToxoDB#2 (clonal type III) in 6.7% (2/30), and ToxoDB#1 (clonal type II) in 3.3% (1/30). When *T. gondii*-positive tissue samples ($n = 151$) were directly subjected to RFLP genotyping, complete restriction profiles were obtained for 33% of samples, and up to 98% of the specimens belonged to the type II *PRU* variant. A foetal brain showed a clonal type II pattern, and four specimens showed unexpected type I alleles at the *SAG3* marker, including two foetal brains that showed I + II alleles as co-infection events. Amplicons of *SAG3*, *GRA6* and *GRA7* obtained from isolates and clinical samples were subjected to sequencing, allowing us to confirm RFLP results and to detect different single-nucleotide polymorphisms.

Conclusions: The present study informed the existence of a predominant type II *PRU* variant genotype (ToxoDB#3) infecting domestic sheep in Spain, in both abortion cases and chronic infections in adults, coexisting with other clonal (ToxoDB#1 and ToxoDB#2), much less frequent genotypes, as well as polymorphic strains as revealed by clinical sample genotyping. The use of multilocus sequence typing aided in accurately estimating *T. gondii* intragenotype diversity.

Keywords: *Toxoplasma gondii*, Sheep, Abortion, Isolates, Genotyping, Sequencing, Population structure, Spain

Background

Toxoplasma gondii (Apicomplexa) is known as one of the main causes of ovine reproductive failure, causing significant economic losses to the sheep industry worldwide [1–3]. Among other factors, such as strain virulence and parasite stage at the time of infection [4, 5], clinical manifestations of ovine toxoplasmosis mostly depend on the pregnancy stage at which the primary infection occurs, ranging from early embryonic death with reabsorption to stillbirth or neonatal death, or even the birth of transplacentally infected lambs (congenital toxoplasmosis). In Europe, there is little information about *T. gondii* as the aetiological agent of ovine abortion outbreaks; nevertheless, similar rates

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of *T. gondii*-specific DNA have been detected in sheep abortion tissues submitted for diagnosis in distinct countries, i.e. in 10% of the ovine abortion-derived tissues from Ireland [6], 6–11% from UK [7], 11.1–18.1% from the Sardinia region, Italy [8, 9], 10.6% from Germany [10], and in 5.4–18.9% from Spain, as observed in previous reports [11–13].

The global seroprevalence of *T. gondii* in sheep flocks ranges between 3–98%, but the results are dependent on factors such as the age of the ewes or the management system [1]. In southern Spain, individual seroprevalence figures ranged between 41.2–49.3% in sheep flocks [14, 15], in agreement with rates found in other Mediterranean countries [3], giving the idea of a widespread prevalence.

Moreover, the role of *T. gondii* as a major pathogen in public health is well known, especially when raw or undercooked meat containing encysted bradyzoites is consumed [16, 17]. A risk assessment study estimated that the consumption of undercooked ovine meat is responsible for 14% of meat-related *T. gondii* infections in the Dutch population [18].

Vast research on *T. gondii* population structure, diversity, and geographical distribution is being conducted worldwide [19, 20]. Despite the importance of the ovine industry in Europe, information about *T. gondii* strains circulating in European ovine livestock is scarce (Table 1); while most ovine isolates and genotyping descriptions in Europe are clonal [21], some specific findings of novel genotypes [22] and non-clonal isolates [23], along with mixed infections [24], deserve attention. To date, no data are available from Spain.

This paper presents the genetic characterization of *T. gondii* ovine isolates and clinical samples obtained from abortion tissues and chronically infected adult animals, providing a picture of the genetic population of *T. gondii* infecting sheep in Spain.

Methods

Study design and sample collection

A workflow of the present study is shown in Fig. 1. Aiming to maximize the geographical extension covered within Spain and hypothesizing higher probabilities to describe genetic diversity among isolates, 2 types of tissue samples were collected for parasite isolation: (i) tissues derived from suspected *Toxoplasma*-related abortion outbreaks; and (ii) myocardial tissues from adult animals collected in authorized slaughterhouses. In this sense, between 2015 and 2018, foetal brains ($n = 182$), brains from weak lambs that died shortly after birth ($n = 18$), and placental/cotyledonary tissues ($n = 42$), were collected from 20 geographically distant farms of 22 different abortion cases (Table 2). Additionally, between February 2018 and July 2018, 342 paired serum and myocardial tissue samples were collected from adult animals slaughtered for human consumption at 2 different authorized slaughterhouses from Cáceres and Ciudad Real provinces (western and central Spain, respectively) (Table 3). The blood samples were collected with BD PLUS Serum tubes (Vacutainer; BD, Franklin Lakes, USA) at the bleeding step after the animals were euthanized, and half of the heart was taken during the evisceration process and individually stored refrigerated at 4 ℃ in labelled zip-lock plastic bags until analysis. Sampling covered a representative area within Spanish ovine farming, as samples were collected from 7 regions, representing 74.5% of the ovine census (16.6 million) in Spain [25].

Histological, molecular and serological diagnosis for sample selection

In abortion cases, brains from foetuses or dead lambs, and placental samples when available, were collected for histological, molecular, and mouse bioassay analyses. Initial screening for common protozoan, bacterial, and viral abortifacient agents was performed as reported elsewhere [26, 27]. Histological processing and evaluation were carried out following previous descriptions [12]. The cases were classified according to observed lesions as follows: (i) no significant lesions; (ii) lesions suggesting conditions other than toxoplasmosis; (iii) lesions compatible with toxoplasmosis (diffuse congestion and/or multifocal leukomalacia); and (iv) lesions consistent with toxoplasmosis (multifocal areas of necrosis at the placenta or glial foci with a central area of necrosis in the brain). Due to the low sensitivity and specificity of histological diagnosis, the selection of tissue samples for parasite isolation was carried out by *T. gondii* DNA detection by PCR. Genomic DNA was extracted from three different 50-mg pieces of each tissue using the Maxwell® 16 Mouse Tail DNA Purification Kit (Promega, Alcobendas, Spain), and *T. gondii* DNA detection was carried out by single-tube nested PCR amplification of the specific ITS1 region as previously described [28]. Within PCR-positive tissues, only representative cases with a lesser degree of autolysis of each confirmed outbreak were selected for the isolation assay ($n = 31$) to maximize the isolation success and geographic coverage of the study.

Regarding adult animals, *T. gondii*-specific IgG antibody levels in ovine serum samples were measured using an in-house indirect ELISA as previously described [28], considering the cut-off at 20 for ELISA IRPC (relative index per cent). Likewise, only those myocardial tissues associated with the highest antibody titres (> 60 ELISA

Table 1 Summary of studies reporting *T. gondii* genotypes circulating in ruminant livestock in Europe

Country	Host species	n	Type (%)				Method	References
			I	II	III	MRA		
Isolates								
France	Sheep	8	–	100	–	–	MS	[40]
	Sheep	46	–	97.8	2.2	–	RFLP-ML + MS	[21]
	Cattle	2	–	100	–	–	MS	[68]
Italy	Sheep	5	–	–	–	100	RFLP-ML	[23]
Portugal	Cattle	1	100	–	–	–	RFLP-ML + MS	[22]
Romania	Goat	2	–	100	–	–	MS	[69]
Serbia	Sheep	1	–	100	–	–	RFLP-ML	[70]
UK	Sheep	2	–	100	–	–	RFLP-*SAG2*	[47]
Clinical samples								
Ireland	Sheep (foetal tissues)	19	–	79	21	–	RFLP-ML	[6]
Italy	Goat (milk)	10	10	–	40	50	RFLP-ML	[71]
	Sheep (placental and foetal tissues)	21	–	100	–	–	RFLP-ML	[48]
	Sheep (milk)	1	100	–	–	–	RFLP-*SAG3*	[72]
	Cattle (skeletal muscle)	6	66.6	16.6	16.6	–	RFLP-ML	[51]
	Sheep (meat)	15	–	100	–	–	*B1*-Seq	[77]
	Goat (meat)	3	–	100	–	–		
Poland	Goat (milk)	25	–	–	100	–	RFLP-ML	[73]
Portugal	Sheep (myocardium)	6	–	100	–	33.3	RFLP-*SAG2*	[74]
	Goat (myocardium)	3	–	100	–	–		
	Cattle (myocardium)	3	–	100	–	–		
The Netherlands	Sheep (myocardium)	13	–	100	–	–	MS + *GRA6*-Seq	[46]
Slovakia	Goat (milk)	14	–	100	–	–	RFLP-*SAG2*	[75]
Switzerland	Sheep (diaphragm)	5	–	–	–	100	RFLP-ML	[24]
	Cattle (diaphragm)	9				100[a]		
	Sheep (diaphragm)	5	–	40	–	60	RFLP-ML	[76]
	Cattle (diaphragm)	9	–	–	–	100[a]		
UK	Sheep (placental tissues)	13	–	100	–	–	RFLP-*SAG2*	[47]
	Sheep (meat)	6	60	–	–	40	RFLP-*SAG2*	[45]
	Cattle (meat)	1	–	–	–	100		

[a] Incomplete markers resembling atypical or recombinant patterns

MRA, patterns showing mixed infections; recombinants or atypical; MS, microsatellites; ML, multilocus; Seq, sequencing

IRPC) were selected for the mouse bioassay ($n = 50$) (Table 3).

Bioassay in mice

Five to 15 g of brain tissue from abortion cases was suspended in a proportional volume (w/v) of PBS supplemented with penicillin (1000 IU/ml; Sigma-Aldrich, Madrid, Spain) and streptomycin (100 µg/ml; Sigma-Aldrich) [1], homogenized in a paddle blender (IUL-Maxicator, Masticator Classic 400 ml; Geneq, Quebec, Canada), centrifuged (1200×*g*, 10 min, 4 ℃), and then passed through a 20 G needle prior to subcutaneous inoculation into 2 or 3 female Swiss/CD1 mice (Janvier Labs, Laval, France) per tissue sample [29]. Additionally, hearts (portions of 50 g/each) from selected seropositive adult animals were subjected to acid-pepsin artificial digestion [1] prior to bioassay in 3 female Swiss/CD1 mice. The resulting inocula were also subjected to ITS1 nested PCR [28] to discern whether further genotyping analysis could be possible directly on these samples.

Mice were observed daily, and clinical signs were scored [30]. Tissue imprints of brains and lungs from mice that died were examined for tachyzoite or tissue cyst presence. At 30 dpi (days post-inoculation), surviving mice were bled, and serum samples were collected for anti-*T. gondii* IgG antibodies detection by an indirect fluorescent antibody test (IFAT) [31], using an anti-mouse IgG conjugated to FITC (Sigma-Aldrich) diluted

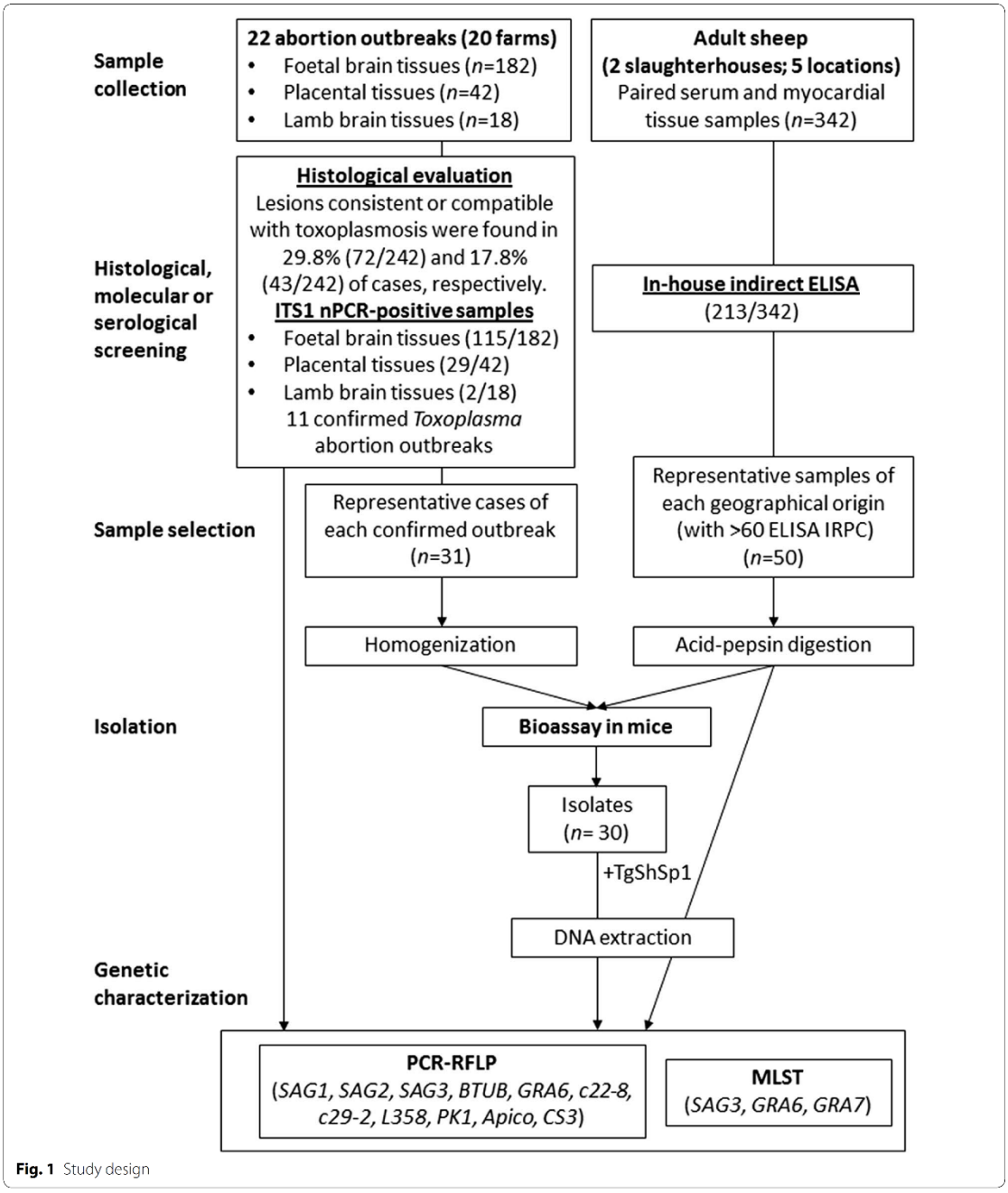

Fig. 1 Study design

1:64 in Evans Blue (Sigma-Aldrich) and considering the cut-off at 1:25. Seropositive mice were sacrificed at 42 dpi, and a fraction of freshly recovered brain tissue was homogenized in PBS supplemented with antibiotics by passing through tapered cross-section needles (20–25 G) to be intraperitoneally (IP) inoculated into two additional female Swiss/CD1 mice. At 7 dpi, peritoneal cavity

Table 2 Epidemiological data on toxoplasmosis-like abortion outbreaks in sheep flocks in Spain (2015–2018): etiology confirmation and T. gondii isolation

Sample ID	T. gondii-associated abortion outbreak ID	Municipality (province)	Description[a]	Period of time (lambing season)	Samples collected[b]			Isolate designation (original sample)	RFLP genotype ID# (ToxoDB)
					Foetal brain tissues	Placental tissues	Lamb brain tissues		
15/121	1	Fuentes de Valdepero (Palencia, North)	Assaf, 1450, 20%	2015/2016	3 (3, 2, 1)	2 (2, 2, 0)	–	TgShSp1 (15/121.4)[c]	#3
15/141	2	Artajona (Navarra, North)	Assaf, 3800, 10%	2015/2016	2 (2, 1, 1)	–	–	TgShSp2 (15/141.2)	#1
17/4, 17, 18, 19, 21, 24, 28	3	Autillo de Campos (Palencia, North)	Assaf, 2800, 4.3%	2016/2017	24 (22, 6, 6)	6 (6, 3, 1)	7 (1, 1, 0)	TgShSp3 (17/4.1); TgShSp4 (17/21.2); TgShSp5 (17/28.1); TgShSp6 (17/19.3)	#3
17/61	–	Villaconancio (Palencia, North)	Assaf, 600, 5%. Border disease virus	2016/2017	2 (0, 0, 1)	–	–	–	
17/5, 15	4[d]	Benavente (Zamora, North West)	Assaf, 500, 10%	2016/2017	3 (2, 2, 1)	3 (2, 0, 0)	–	–	
17/6, 20, 22, 23, 25, 27, 41	5[d]	Mayorga (Valladolid, North)	Assaf, 1600, 10%	2016/2017	11 (5, 2, 2)	4 (2, 0, 2)	–	–	
17/94, 17 2/7, 17 2/6, 17 2/8, 17/29, 17/32, 17/36, 17/52 17/62	6[d]	Villamañán (León, North-West)	Assaf, NA	2016/2017	14 (6, 4, 2)	7 (3, 0, 0)	5 (0, 0, 0)	–	
17/42	–	Alcarraz (Lérida, North-East)	Lacaune, 800, 25%	2016/2017	4 (0, 0, 0)	1 (0, 0, 0)	–	–	
17/49	–	Fuentes de Valdepero (Palencia, North East)	Assaf, 1600, 5%	2016/2017	2 (0, 0, 0)	1 (0, 0)	–	–	
17/220, 221, 222, 223, 224, 225, 18/1, 4, 10	7	Navas de Oro (Segovia, Centre)	Merino, 900, 83%	2017/2018	12 (10, 5, 2)	6 (6, 1, 0)	3 (1, 1, 0)	TgShSp7 (18/4.2)	#3
17/227	–	Santa Cruz de Mudela (Ciudad Real, Centre)	Manchega, 1200, 5%	2017/2018	3 (0,0,0)	–	–	–	
18/3	–	Benavente (Zamora, North–West)	Assaf, 500, 15%, Neospora caninum	2017/2018	1 (0, 0, 0)	1 (0, 0, 0)	–	–	

Table 2 (continued)

Sample ID	T. gondii-associated abortion outbreak ID	Municipality (province)	Description[a]	Period of time (lambing season)	Samples collected[b]			Isolate designation (original sample)	RFLP genotype ID# (ToxoDB)
					Foetal brain tissues	Placental tissues	Lamb brain tissues		
18/12	–	Benegiles (Zamora, North–West)	Assaf, 1049, 15.3%, Neospora caninum	2017/2018	2 (0, 1, 0)	–	–	–	
18/5, 17	–	Casas de Juan Núñez (Albacete, East)	Manchega, 3000, 40%	2017/2018	6 (0, 0, 0)	1 (0, 0, 0)	–	–	
18/6	–	Ledesma (Salamanca, West)	Castellana, 20, 25%, Neospora caninum	2017/2018	1(0, 1, 0)	1 (0, 0, 0)	–	–	
18/7	8	Catadau (Valencia, East)	Lacaune, 2400, 21%	2017/2018	2 (2, 2, 0)		–	TgShSp8 (18/7.2)	#3
18/14, 15, 16, 18, 20	9	Cuevas de Almudén (Teruel, East)	Lacaune, 700, 50%	2017/2018	62 (50, 29, 18)	6 (6, 5, 1)	3 (0,0,0)	TgShSp9 (18/18.1); TgShSp10 (18/15.1); TgShSp18 (18/16.1)	#3
18/23	–	Moral de la Reina (Valladolid, North)	Assaf, 3000, 15%	2017/2018	5 (0, 0, 0)	1 (0, 0, 0)	–	–	
18/36, 38	–	Valdetorres (Badajoz, West)	Merino, 450, 20%, Neospora caninum	2017/2018	3 (0, 1, 1)	–	–	–	
18/21	–	Autillo de Campos (Palencia, North)	Assaf, 3000, > 1%	2017/2018	5 (0, 0, 0)	–	–	–	
18/219, 228	10[d]	Villafrechos (Valladolid, North)	Assaf, 1100, 12%	2017/2018	4 (2, 2, 0)	–	–	–	
18/222, 226	11[d]	Aguilar de Campos (Valladolid, North)	Lacaune, 2500, 25%, Coxiella burnetii	2017/2018	11 (11, 2, 4)	2 (2, 0, 0)	–	–	
Total	11				182 (115, 59, 3, 9)	42 (29, 11, 4)	18 (2, 2, 0)		

[a] Breed, flock size, % of abortion, other pathogens detected. Screening for common protozoal, bacterial and viral abortifacient agents was performed as reported in references [26] and [27]

[b] PCR-positive cases, cases with consistent lesions, cases with compatible lesions

[c] Isolation reported in reference [32]

[d] Bioassay in mice was not successful or was not carried out due to previous tissue freezing

na, data not available

Table 3 Summarized data on adult sheep myocardial tissue sample collection in authorized slaughterhouses in central and western Spain (2018)

Animal origin (province)	Breeding area	Age	Breed	No. of serum samples		ELISA IRPC	Isolate ID
				Samples analysed	ELISA-positive (%)		
Plasencia (Cáceres)	West	Adult (4–5 years-old)	Merino	100	39.0 (39/100)	85.0	TgShSp11
						76.9	TgShSp12
						81.1	TgShSp13
						81.6	TgShSp14
						66.3	TgShSp15
						88.1	TgShSp19
						85.1	TgShSp20
						92.0	TgShSp21
						66.0	TgShSp22
						74.7	TgShSp28
Alburquerque (Badajoz)	South–West	Adult (4–5 years-old)	Merino	100	95.0 (95/100)	107.2	TgShSp16
						115.2	TgShSp17
						113.6	TgShSp23
						86.7	TgShSp27
						122.6	TgShSp31
Sisante (Cuenca)	Centre	Adult (4–5 years-old)	Manchega × Lacaune	42	47.6 (20/42)	75.1	TgShSp26
						115.5	TgShSp30
Valdepeñas (Ciudad Real)	Centre	Adult (4–5 years-old)	Manchega × Lacaune	50	40.0 (20/50)	89.0	TgShSp24
						82.5	TgShSp25
Puertollano (Ciudad Real)	Centre	Adult (4–5 years-old)	Manchega × Lacaune	50	78.0 (39/50)	107.4	TgShSp29
Total	–	–	–	342	62.3 (213/342)	–	–

flushes were aseptically collected from mice and used for *in vitro* culture.

In vitro cultivation

Peritoneal exudates of infected Swiss/CD1 mice were seeded into African green monkey kidney-derived cells (MARC-145 line) and maintained by serial passages. Cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with foetal bovine serum (FBS) (Gibco), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml) (Lonza Group, Basel, Switzerland) at 37 ℃ and 5% CO_2 in 75 or 25 cm^2 tissue culture flasks. Tachyzoites from successfully grown cultures were harvested from the medium for DNA isolation, and infected cells were suspended in FBS supplemented with 10% of DMSO (dimethyl sulfoxide; Sigma-Aldrich) and cryopreserved in liquid nitrogen for further studies as described previously [1].

Genetic characterization of *T. gondii*

Toxoplasma gondii DNA was extracted from cell-culture-derived tachyzoites of all 30 isolates obtained, along with the isolate TgShSp1 [32]. Strain typing was performed by the widely used PCR-restriction fragment length polymorphism (RFLP) method based on *SAG1*, *SAG2* (5'-3' *SAG2*, and alt. *SAG2*), *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1* and *Apico* markers [33]. An additional RFLP marker, *CS3*, was included in the present study due to its proven link with the virulence of *T. gondii* strains in mice [34]. Reference strains of *T. gondii* were also incorporated in genotyping, including clonal type I (TgRH), clonal type II (TgMe49) and clonal type III (TgNED). Genotyping was also directly applied to DNA extracted from all brain and placental tissues in which *T. gondii* had been previously detected ($n=133$) and from the *T. gondii* PCR-positive digests of sheep myocardial tissues inoculated into mice ($n=18$). RFLP genotype numbers were assigned according to the ToxoDB database (https://toxodb.org/toxo/).

Multilocus sequence typing (MLST) analysis

We conducted PCR sequencing of 3 polymorphic genes, *SAG3*, *GRA6* and *GRA7*, on all 31 isolates and clinical samples with previous successful nested PCR amplification of each marker (*SAG3*, $n=123$; *GRA6*, $n=108$; *GRA7*, $n=99$) to provide sequence-based genotyping. Gene amplification of *SAG3* and *GRA6* resulted from

the above-described Mn-PCR-RFLP method, and *GRA7* amplifications were obtained by nested PCR using specific primer pairs [35]. PCR products were sent to the Center for Genomic Technologies of the Complutense University of Madrid (Spain) for direct sequencing. Briefly, amplicons were sequenced in both directions with the same internal primer pair used for amplification employing a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and a 3730 × l DNA Sequence Analyser (Applied Biosystems). Sequencing was successful for 121 out of 123 *SAG3*-PCR positives, 77 out of 108 *GRA6*-PCR positives, and all 99 *GRA7*-PCR positives. The resulting sequences were imported, read, edited manually if necessary, and analysed using BioEdit software, version 7.0.5.3 [36]. Generated DNA consensus sequences were aligned to appropriate reference sequences using MEGA X software (http://www.megasoftware.net/) [37], and compared with sequences retrieved from the National Center for Biotechnology Information (NCBI) database through the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analyses

By using DNA sequence-based phylogenetic analyses, we evaluated the population structure of *T. gondii* isolates obtained; clonal reference strains (TgRH, TgMe49 and TgNED) were included for comparison. Consensus *SAG3*, *GRA6* and *GRA7* sequences from isolates and reference strains were concatenated and aligned using MEGA X software [37] to generate an unrooted phylogenetic tree. The evolutionary history was inferred using the neighbor-joining method [38]. The evolutionary distances were computed using the maximum composite likelihood method [39].

Results

Parasite detection and isolation

Two hundred forty-two tissue samples (182 foetal brains, 42 placentae and 18 lamb brains) from 22 suspected *Toxoplasma*-related abortion outbreaks among 20 farms distributed all over Spain were analysed by histological evaluation and nested PCR assay (Table 2). Histological lesions consistent or compatible with toxoplasmosis were found in 29.8% (72/242) and 17.8% (43/242), respectively, of the studied cases. No lesions suggesting other conditions were found, although multifocal necrotic glial foci and protozoan tissue cysts, lesions classified as characteristic of *Toxoplasma* infection, in two cases these were later confirmed to be caused by *Neospora caninum*. *Toxoplasma gondii*-specific DNA was detected in 60.3% (146/242) of samples; indeed, 63.2% (115/182) of foetal brains, 69.0% (29/42) of placental samples, and 11.1% (2/18) of lamb brains were positive for *T. gondii* DNA. Such findings allowed us to confirm *T. gondii* as the aetiological agent in 11 out of 22 abortion outbreaks in sheep farms (description is summarized in Table 2). Ten isolates (TgShSp2 to TgShSp10 and TgShSp18) were obtained from 31 bioassayed foetal brains (representing 5 different abortion outbreaks, Table 2).

Furthermore, *T. gondii*-specific IgG antibodies were detected in 62.3% (213/342) of adult sheep serum samples collected in slaughterhouses; 50 selected samples with the highest ELISA IRPC titres (ranging from 60.5 to 122.6; Table 3) were subjected to bioassay, and 20 isolates (TgShSp11 to 17, TgShSp19 to 31) were obtained (Table 3).

The bioassay success rate was established in 32.3% (10/31) of abortion cases and in 40% (20/50) of chronically infected adult tissues. Regarding samples from abortion outbreaks that occurred in Zamora (#4), Valladolid (# 5, #10 and #11) and León (# 6) provinces, bioassays in mice were not successful or were not carried out due to previous freezing of the tissues (Table 2).

PCR-RFLP genotyping

Cell-culture-derived tachyzoites from all 31 isolates (including TgShSp1) were successfully typed, revealing 3 different genotypes: ToxoDB#3 (90.3%; 28/31 isolates); ToxoDB#2 (6.5%; 2/31); and ToxoDB#1 (3.2%; 1/31). Although ToxoDB#3 was the most frequently found genotype, and ToxoDB#2 was detected only in chronically infected adult animals, no specific dominance of any RFLP genotype appears to be involved in abortion cases or chronic infections (Additional file 1: Table S1). The *CS3* marker, a gene with a suggested high predictive value for virulence in mice [34], resulted in the type II allele in all isolates but 2 (TgShSp24 and 25), exhibiting a type III allele.

When PCR-RFLP assays were applied to *T. gondii* DNA-positive brain ($n=108$) and placental tissues ($n=25$) obtained from abortion outbreaks (Additional file 2: Table S2), and to myocardial sample digests ($n=18$) (Additional file 3: Table S3), more complexity was observed, revealing co-infection events and suggesting the possible selection of certain strains during bioassay experiments. Amplifications yielded complete RFLP profiles for approximately 33% of specimens, with up to 98% belonging to the type II *PRU* variant (ToxoDB#3). Although incomplete RFLP profiles were obtained in some samples, allelic variations were detected in the *SAG3* marker (Additional file 2: Table S2, Additional file 3: Table S3). Infection with multiple *T. gondii* strains in the same foetus was detected in 2 brain tissues

collected in outbreak #3 (Table 2), which occurred in 2017 in Palencia Province (North Spain) (ID#17/21.1 and #17/21.2), due to the coexistence of type II and type I *SAG3* alleles in the same tissue. Apart from that, a type I allele was also detected in another foetal brain tissue from the same outbreak (ID#17/28.1) and in a myocardial sample (digest) from an adult sheep from Badajoz Province (Southwest Spain) (ID: BA18 G#34).

MLST genotyping

PCR-DNA sequencing-based genotyping considering 3 polymorphic genes, *SAG3*, *GRA6* and *GRA7*, revealed that most isolates and samples showed complete sequence homology with either TgMe49 (clonal type II) or TgNED sequences (clonal type III), supporting the RFLP results in the case of the *SAG3* and *GRA6* markers (Additional file 1: Table S1, Additional file 2: Table S2, Additional file 3: Table S3).

The *SAG3* sequence alignment of the samples (and isolates) that showed a type II allele identified a single-nucleotide polymorphism (SNP), G1691T, that divides our clonal type II (ToxoDB#1) and type II *PRU* variant (ToxoDB#3) isolates and samples into two well-defined groups. The first group had 100% homology with the TgMe49 reference sequence included and others deposited in GenBank, such as JX218226 (IIa *SAG3* allele, MT361125), and the other group (G1691T) showed 100% identity with ovine (KU599412; KU599407) or caprine (KU599396) isolate sequences deposited (IIb *SAG3* allele, MT361126) and leads to an amino acid change at codon 368 from Met to Ile. Concerning the incidence of each *SAG3* type II allele, all outbreaks described were homogeneous, presenting one allele spread over all specimens; of note, only outbreak #6 occurring in León Province during 2017 presented foetuses infected by parasites showing alleles IIa and IIb. There appears to be a higher incidence of the *SAG3* IIa allele in those outbreaks occurring in 2017, while the IIb allele seems to be more frequent in outbreaks during 2018. Regarding sample ID#17/28.1, which showed a type I allele by PCR-RFLP confirmed by PCR sequencing, a double peak was detected at position 1113 of the gene sequence, indicating a co-infection event. Between both alleles detected, one of them showed 100% homology with the TgRH reference strain sequence included and others deposited in GenBank (JX218225; AF340227) (Ia *SAG3* allele, MT358429), but the other presented a SNP (T1113C) not shared by any other sequence reported previously (Ib *SAG3* allele, MT361124) and resulted in a silent mutation. When using the BLAST tool to compare resulting consensus *SAG3* sequences with those publicly available in the GenBank database obtained from sheep or goats, it was noted that the IIb *SAG3* allele detected along part of our samples is also present in some French ovine isolates (GenBank: KU599412, KU599411 and KU599407) coexisting with IIa *SAG3* alleles (GenBank: KU599409, KU599410). The same occurred with some Ethiopian ovine and caprine isolates deposited (GenBank: KU599394, KU599396, KU599399 and KU599400), showing one allele or the other. This fact also illustrated other instances of type I and III alleles present in sheep and lamb meat samples analysed in Iraq (GenBank: MK801822- MK801830).

GRA6 marker sequencing also sustained RFLP findings; nevertheless, a double peak at position 1013 of the gene was detected in a foetal brain tissue collected in an abortion outbreak (#9, Table 2) that occurred in 2018 in Teruel Province (East Spain) (ID#18/14.5; PCR-RFLP type II allele), indicating co-infection by two strains. Between both alleles detected, one of them presented 100% homology with the TgMe49 sequence included and others deposited in GenBank (AF239285) (IIa *GRA6* allele, MT370491), but the other one showed a SNP (C1013T) not shared by any other sequence deposited previously in that database (IIb *GRA6* allele, MT370489) and was located at the 5' UTR fragment.

Finally, *GRA7* gene sequencing enabled us to test the sequence homology of our isolates and original clinical samples with the clonal reference strains included. Concerning the isolates obtained, the analysis showed 100% homology with the TgMe49 (clonal type II) sequence included in all cases except for the TgShSp24 and TgShSp25 sequences, which were found to be identical to the TgNED (clonal type III) sequence included. Besides, the *GRA7* sequence obtained from DNA amplified from a foetal brain collected in the above-mentioned outbreak (#9, Table 2) that occurred in Teruel Province (East Spain) (ID#18/15.21), possessed a double peak at position 2688 of the gene sequence, indicating that a co-infection was also present in this tissue. Between both alleles detected, one of them presented 100% homology with the TgMe49 sequence included and others deposited in GenBank (DQ459445) (IIa *GRA7* allele, MT361127), but the other carried a SNP (C2688T) not shared by any other sequence available (IIb *GRA7* allele, MT361128), causing an amino acid change at codon 188 from Ala to Val.

Phylogenetic analyses

The population structure of *T. gondii* isolates obtained was evaluated by DNA sequence-based phylogenetic analyses. A phylogenetic tree was constructed based on concatenated *SAG3*, *GRA6* and *GRA7* sequences from isolates obtained, in addition to those from the clonal reference strains included (TgRH, TgMe49 and TgNED) (Fig. 2). Predictably, TgShSp24 and TgShSp25 isolates (type III alleles for the three markers studied) were

situated next to the TgNED strain. On the other hand, the rest of the isolates (type II alleles for the three markers) formed two well-defined clusters obeying the presence of the SNP (G1691T) described previously at the *SAG3* locus.

Discussion

Toxoplasma gondii has been recognized as a major cause of reproductive failure. Here, 11 toxoplasmosis-related ovine abortion outbreaks occurring in the 2015, 2016 and 2017 lambing seasons are reported. In addition to the clinical and economic interest derived from abortion outbreaks, the high seroprevalence in sheep highlights the potential public health risk posed by the consumption of lamb meat containing viable tissue cysts. In the present study, 62.3% (213/342) of blood samples collected from sheep slaughterhouses in western and central Spain were positive for *T. gondii* IgG antibodies, in agreement with the values in other European reports [3].

To the best of our knowledge, the present survey, along with two previous French investigations [21, 40], might be the only European studies comprehensive enough to analyse *T. gondii* population genetic diversity circulating through sheep flocks, and this is the first Spanish report of this nature. There is scarce knowledge regarding the genetic diversity of the *T. gondii* population in Spain. Pioneering studies were focused on genotyping human clinical samples [41] and positive tissues from wild big game species [42]; both studies corroborated a predominance of genotype II but also presented a significant prevalence of other clonal and recombinant types. To date, only two reports of *T. gondii* parasite isolation have been carried out in Spain. The first one was focused on stray cats, and typing based only on the *SAG2* locus [43] showed that 26% ($n=12$) of isolates were type I and 74% ($n=34$) of isolates were type II, with an absence of type III. In a second report [32], the isolate TgShSp1 (ToxoDB#3) was obtained from an ovine abortion case and was also included in the present study for further in-depth genetic analysis. We were able to isolate *T. gondii* from five additional ovine abortion outbreaks that occurred among widely geographically distributed Spanish farms during 2015–2018, as well as from chronically infected adult sheep. Overall, 30 isolates were obtained that, along with the isolate TgShSp1 [32], represented a significant cross-section of the *T. gondii* Spanish population infecting sheep, covering a wide part of the country's territory.

Genetic characterization based on PCR-RFLP classified most isolates (90.3%; 28/31) as the type II *PRU* variant (ToxoDB#3). This fact is consistent with traditional literature referring to the predominance of *T. gondii* type II alleles among European sheep flocks [44] (summarized in Table 1). As observed in the present study, no specific genotypes have been reported in the literature in association with chronically infected adult animals (e.g. commercial meat products) [21, 40, 45, 46] or causing abortion [47, 48]. Until recently, most *T. gondii* clonal types had been recognized as infecting livestock, pets and wild animals in Europe [43, 49], but this might be biased due to the use of only *SAG2* genetic markers or a few of them for typing assays; currently, more comprehensive studies in terms of the sample size, number of molecular markers, and interactions between livestock and wildlife species have revealed an unexpectedly higher presence of polymorphic strains [50, 51], similar to direct genotyping from clinical samples.

As previously stated, in a context in which a high occurrence of *Toxoplasma* would suggest multiple exposures to the parasite during the life time of the animal [52], bioassay experiments might induce a selection of certain strains at the expense of others, resulting in an underestimation of co-infection events and, as a consequence, intraspecific diversity. Co-infection events were observed in our study in two abortion outbreaks (#3 and #9) that occurred in 2017 in Palencia Province (North Spain) and 2018 in Teruel Province (East Spain), with not only type II but also type I alleles at the *SAG3* marker detected in different foetal brain tissues (samples #17/21.1 and #17/21.2). Mixed infections have been described previously not only in ovine and porcine livestock [24, 45, 53] but also in European wildlife species [42, 54]. The type I allele at the *SAG3* marker was also detected alone in another foetal brain tissue (ID#17/28.1) from outbreak #3 and in the myocardium from an adult animal (BA18 G#34) bred in Badajoz Province (Southwest Spain), calling attention to the extension of this type I allele through livestock, as in other European studies [23, 45, 51]. Considering that the bioassay of samples #17/21.2 (Ia and IIa alleles detected) and #17/28.1 (Ia and Ib alleles present) resulted in TgShSp4 and TgShSp5 isolation (only IIa allele found), a selection of certain strains is evident during isolation experiments. It should be noted that the greatest genetic variability was detected in abortion outbreaks #3 and #9, coinciding with those from which more samples were collected, demonstrating that sampling effort is an important factor.

Phylogenetic analyses of strongly variable loci coding for virulence factors such as surface and secretory antigens, often under significant selective pressure, have been widely used to infer possible genetic population structure models, evolutionary relationships between *T. gondii* populations, reservoirs, and transmission patterns, among other factors [20, 55, 56]. Our results suggest that Spanish and French *T. gondii* populations could be genetically related based on limited *SAG3* sequences

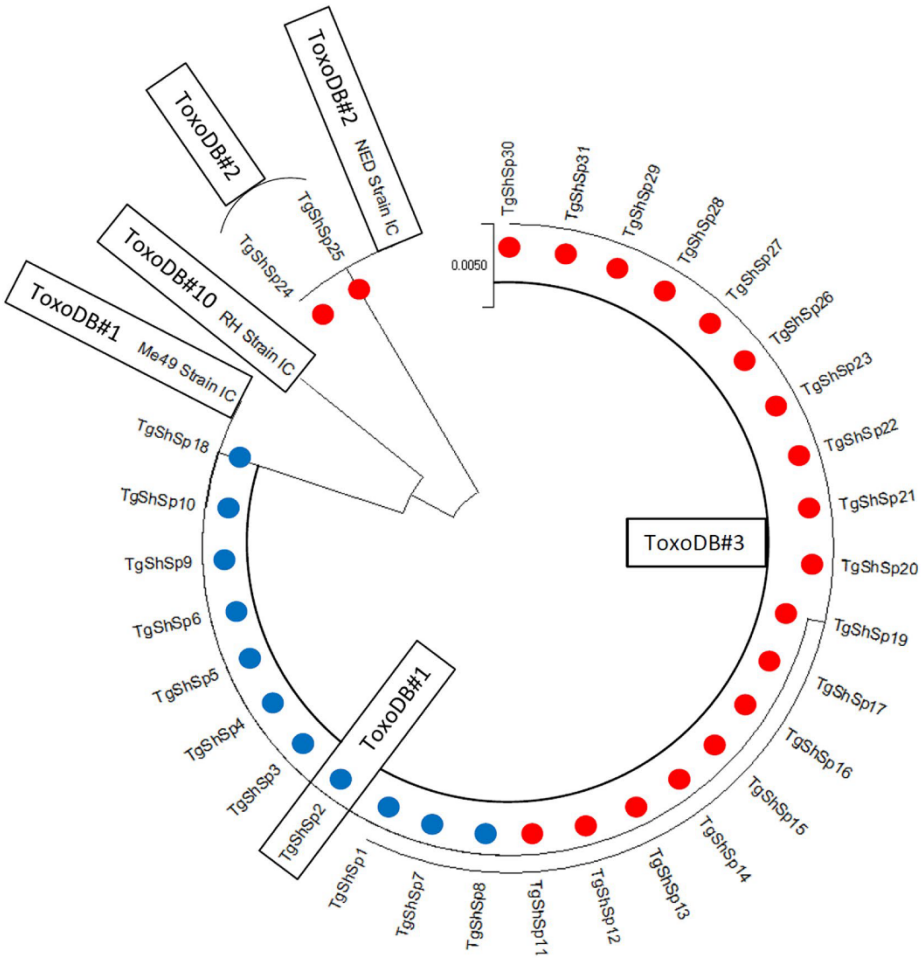

Fig. 2 Phylogenetic tree constructed using concatenated consensus *SAG3*, *GRA6* and *GRA7* sequences of *Toxoplasma gondii* isolates obtained from abortion-derived tissues (blue dots) and myocardium from adult sheep (red dots) in Spain and T. gondii reference strains used along genotyping methods as internal controls (IC) (RH strain IC, Me49 strain IC and NED strain IC)

of sheep origin deposited in the GenBank database. Both sets of sequences clustered in two groups determined by the specific SNP (G1691T) described here. This may suggest common evolutionary forces or most likely common origins in livestock from both countries [56] due to a historical and intense trade exchange of sheep from Spain to France and *vice versa*. Sequences from two Ethiopian goat isolates deposited in GenBank also presented such dichotomy, possibly implying a further extension of the mutation.

The *CS3* gene has been described previously as a marker highly predictive of *T. gondii* isolates mortality in mice [34]. Bioassay results suggest a low degree of virulence for isolates obtained here, since none of the mice infected during the isolation process presented acute symptoms or died of toxoplasmosis. Our *CS3* typing results disagree with those of previous studies carried out with Brazilian and Chinese isolates of different host origins that report high mortality rates (normally above 80%) associated with type I or II alleles for the *CS3* gene and low (3.7–9.3%) or null rates with type III alleles [34, 57–60]. Contradictory results were already exposed within avirulent Brazilian isolates presenting type I [61] or type II [62] alleles for the *CS3* locus. Thus, the fact that all strains included in the studies mentioned above are polymorphic, none of them with a European or North American origin ("clonal" regions), suggests the need for further investigations to unravel the role of the gene in

Toxoplasma virulence and clear differences between distant biogeographical global areas. Considering the known proximity of the *CS3* gene to demonstrated virulence factors such as *ROP18* and *ROP5* in the *Toxoplasma* genome (chromosome VIIa) [63–67], a linked expression with still unknown implications might be possible; therefore, research on the expression of both factors would be relevant in future studies of isolates characterization.

In conclusion, our results show that a large majority of isolates circulating around sheep farms fall within three genotypes (ToxoDB#3, 2 and 1), with some infrequent SNPs, in agreement with low genetic variability in Europe. The differential clinical outcomes observed in abortion cases draw attention to the necessity of analysing the genetic and phenotypic diversity among *Toxoplasma* parasites in Europe, especially aiming to (i) predict epidemiological changes, (ii) identify virulence factors, and (iii) design effective vaccines against field strains. Thus, increasing the effort in isolation and genotyping will provide interesting information on the epidemiology of *T. gondii* and the paradigm of One Health parasites infecting humans, livestock, and wildlife in Europe.

Conclusions

To the best of our knowledge, the present survey constitutes the first study aiming to describe the genetic population of *T. gondii* circulating in sheep flocks in Spain. Genetic characterization of 31 strains isolated from abortion cases and chronically infected adult animals showed low genetic variability, with a predominant type II *PRU* variant genotype (ToxoDB#3) coexisting with other clonal (ToxoDB#2 and #1), much less frequent genotypes. Furthermore, when directly examining the clinical samples and inocula, the genetic richness increases, allowing the identification of other genetic variants. The present results support the hypothesis of the existence of polymorphic and overlapping strains within ovine livestock in Spain and point out the necessity of increased genotyping and sampling efforts to accurately estimate *T. gondii* intraspecific genetic diversity.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04275-z.

Additional file 1: Table S1. Genotyping allele profile obtained by PCR-RFLP and PCR-sequencing on *T. gondii* isolates.

Additional file 2: Table S2. Genotyping allele profile obtained by PCR-RFLP and PCR-sequencing on *T. gondii* DNA-positive clinical samples collected from abortion outbreaks.

Additional file 3: Table S3. Genotyping allele profile obtained by PCR-RFLP and PCR-sequencing on *T. gondii* DNA-positive adult sheep myocardium digests.

Abbreviations

DMEM: Dulbecco's modified Eagle's medium; DMSO: dimethyl sulfoxide; dpi: days post-inoculation; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; IFAT: indirect fluorescent antibody test; IP: intraperitoneally; IRPC: relative index per cent; MLST: multilocus sequence typing; PBS: phosphate-buffered saline; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; SNP: single nucleotide polymorphisms.

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Authors' contributions

MF, RC, EC and LMO conceived and designed the laboratory tests, MF, RC, MCG, JR, DG, and JB performed experiments, MF, RC, DG, JR, and EC analysed the data and LO, EC and JB contributed reagents/materials/analysis tools. MF, RC, JR, JB, EC and LMO drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its Additional files 1, 2, 3. The sequences generated in the present study were submitted to the GenBank database under the following accession numbers: *SAG3* sequences (MT358429, MT361124-MT361126); *GRA6* sequences (MT370489, MT370491); and *GRA7* sequences (MT361127, MT361128). Histological samples are available from the authors upon reasonable request.

Ethics approval and consent to participate

Animal procedures for the bioassay in mice were approved by the Animal Welfare Committee of the Community of Madrid, Spain (PROEX 274/16, section 2.1.3), following proceedings described in Spanish and EU legislation (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering. As a humane endpoint, mice exhibiting significant weight loss or nervous clinical signs were culled to limit unnecessary suffering.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Supplementary information

Additional file 1: Table S1. Genotyping allele profile obtained by PCR-RFLP and PCR-sequencing on *T. gondii* isolates.

			PCR-RFLP alleles[a]													PCR-Seq alleles		
ID# Isolate	**Sample, host (location) abortion outbreak #**	**Mice bioassay (no. infected/no. inoculated)**	**SAG1**	**3' -SAG2**	**5' -SAG2**	**Alt. SAG2**	**SAG3**	**BTUB**	**GRA6**	**c22-8**	**C29-2**	**L358**	**PK1**	**Apico**	**CS3**	**SAG3[b]**	**GRA6[c]**	**GRA7[d]**
RH	CNS, human (EEUU)	-	I	I/III	I/II	I	I	I	I	I	I	I	I	I	-	I	I	I
Me-49	Muscle, ovine (EEUU)	-	II/III	II	I/II	II	II	II	II	II	II	II	II	II	-	IIa	IIa	IIa
NED	Placental tissues, human (France)	-	II/III	I/III	III	III	III	III	III	III	III	III	III	III	-	III	III	III
TgShSp1	Foetal brain, ovine (Palencia, Spain) #1	(1/1)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
TgShSp2	Foetal brain, ovine (Navarra, Spain) #2	(2/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	II	II	IIa	IIa	IIa
TgShSp3-6	Foetal brain, ovine (Palencia, Spain) #3	(1/2); (2/2); 1/2); (1/2)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
TgShSp7	Foetal brain, ovine (Segovia, Spain) #7	(3/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
TgShSp8	Foetal brain, ovine (Valencia, Spain) #8	(2/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
TgShSp9, 10, 18	Foetal brain, ovine (Teruel, Spain) #9	(3/3); (3/3); (1/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
TgShSp11, 14,15,19,20, 21, 28	Myocardium, ovine (Cáceres, Spain)	(3/3);(3/3); (3/3); (2/3); (3/3); (2/3); (1/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
TgShSp12, 13, 22	Myocardium, ovine (Cáceres, Spain)	(3/3);(3/3); (1/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
TgShSp16, 17, 23, 27, 31	Myocardium, ovine (Badajoz, Spain)	(2/3);(3/3); (3/3); (2/3); (1/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
TgShSp24, 25	Myocardium, ovine (Ciudad Real, Spain)	(1/3); (1/3)	II/III	I/III	III	III	III	III	III	III	III	III	III	III	III	III	III	III
TgShSp26, 30	Myocardium, ovine (Cuenca, Spain)	(1/3); (1/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
TgShSp29	Myocardium, ovine (Ciudad Real, Spain)	(3/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa

[a]I, II or III refers to the archetypal alleles from a Type I, II or III, for each molecular marker [33].
[b]I=100% homology with GenBank accession no. AF340227 sequence; IIa=100% homology with GenBank accession no. JX218226 or MT361125 sequences; IIb=G1691T, GenBank accession no. MT361126; III=100% homology with GenBank accession no. LN714502 sequence.
[c]I=100% homology with GenBank accession no. AF239283 sequence; IIa=100% homology with GenBank accession no. AF239285 or MT370491 sequences; III=100% homology with GenBank accession no. AF239286 sequence.
[d]I=100% homology with GenBank accession no. DQ459443 sequence; IIa=100% homology with GenBank accession no. DQ459445 or MT361127 sequence; III=100% homology with GenBank accession no. DQ459455 sequence.

Additional file 2: Table S2. Genotyping allele profile obtained by PCR-RFLP and PCR-sequencing on *T. gondii* DNA positive clinical samples collected from abortion outbreaks.

ID# Sample	Location, year, abortion outbreak #	PCR-RFLP alleles[a]													PCR-Seq alleles		
		SAG1	3' -SAG2	5' -SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	C29-2	L358	PK1	Apico	CS3	SAG3[b]	GRA6[c]	GRA7[d]
15/121.1	Fuentes de Valdepero (Palencia) 2015 #1	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
15/121.2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	IIa
15/121.3		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	-	-
15/121.4		-	-	-	-	II	-	-	-	-	-	II	-	-	IIb	-	-
15/121.5		II/III	II	I/II	II	II	-	II	II	II	-	II	I	II	IIb	-	IIa
15/141	Artajona (Navarra) 2015 #2	II/III	II	II	II	II	II	II	II	II	II	II	II	II	IIa	IIa	IIa
17/4.1a	Autillo de Campos (Palencia) 2017 #3	II/III	II	I/II	-	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
17/4.1b		II/III	II	I/II	II	II	II	II	II	II	II	-	I	II	IIa	IIa	
17/4.2		II/III	II	I/II	II	II	-	II	II	II	II	-	I	II	IIa	IIa	IIa
17/4.3		II/III	II	I/II	-	II	II	II	-	-	II	-	I	II	IIa	IIa	-
17/4.5		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	-	IIa
17/4.6		II/III	II	I/II	II	II	II	-	II	II	-	II	I	-	IIa	-	IIa
17/17.1		II/III	II	I/II	-	II	II	-	II	-	-	-	I	-	IIa	-	-
17/17.2		II/III	II	I/II	-	II	-	-	II	II	II	-	I	-	IIa	-	IIa
17/18.1		II/III	II	I/II	-	II	-	II	-	-	-	-	I	II	IIa	-	IIa
17/18.2		II/III	II	I/II	II	II	-	II	II	II	II	II	I	II	IIa	-	-
17/18.2Pla		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
17/19.1		II/III	II	I/II	-	II	-	II	-	-	-	-	I	II	IIa	-	-
17/19.1Pla		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
17/19.2		II/III	II	I/II	-	-	-	-	II	-	II	-	I	II	-	-	-
17/19.3		-	-	-	-	II	II	II	II	II	II	II	I	-	IIa	-	IIa
17/19.3Pla		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
17/21.1		II/III	II	I/II	II	I+II	II	II	-	II	II	II	I	II	Ia+IIa	-	IIa
17/21.2		-	II	I/II	II	I+II	-	II	II	II	II	II	I	-	Ia+IIa	IIa	IIa
17/21.3		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	-	IIa
17/21.1Pla		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
17/24.1		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	-
17/24.2		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
17/24.3		II/III	II	I/II	-	II	II	II	-	II	-	II	I	II	IIa	-	-
17/28.1		-	II	I/II	II	I	II	II	-	-	II	-	I	II	Ia+Ib	-	-
17/28.2		II/III	II	I/II	-	II	II	II	-	-	-	-	I	II	IIa	-	-
17/15.1	Benavente (Zamora) 2017 #4	II/III	II	I/II	-	II	-	-	II	-	-	-	I	II	IIa	-	IIa
17/15.1Pla		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	-	-
17/20.1	Mayorga (Valladolid) 2017 #5	II/III	II	I/II	-	II	-	-	-	-	-	-	I	II	IIa	-	-
17/20.1Pla		II/III	II	I/II	-	II	II	-	-	-	II	-	I	-	IIa	-	IIa
17/20.2		II/III	II	I/II	-	II	-	II	-	-	-	-	I	II	IIa	-	-
17/24.4		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIa	-	IIa
17/24.4Pla		-	II	I/II	-	-	-	-	-	II	-	II	I	-	-	-	-
17/29.1	Villamañán (León) 2017 #6	II/III	II	I/II	-	II	-	-	II	-	II	-	I	II	IIb	-	-
17/29.1Pla		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
17/32.2		II/III	II	I/II	-	II	II	-	II	-	-	-	I	II	IIa	-	-
17/33		II/III	II	I/II	-	II	-	-	-	II	II	-	I	-	-	-	-
17/33Pla		II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	IIb	-	IIa
17/220.1	Navas de Oro (Segovia) 2017/18 #7	II/III	II	II	II	II	II	II	II	II	II	II	-	II	IIb	IIa	IIa
17/221.1		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
17/222.1		II/III	II	-	-	II	-	II	II	II	II	II	I	II	IIb	IIa	IIa
17/220.2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
17/221.2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
17/222.2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa

17/223.1		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
17/224.1		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
17/225.1		-	-	II	II	II	II	II	II	-	II	-	I	II	IIb	-	IIa
17/224.2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/4.1		-	II	-	-	-	-	II	-	II	-	-	I	II	-	IIa	-
18/4.2		II/III	II	II	II	II	II	II	II	II	II	II	-	II	IIb	IIa	IIa
18/4.3		II/III	II	-	-	II	-	II	-	-	II	II	I	-	IIb	IIa	IIa
18/4.4		-	-	II	II	-	-	II	II	-	II	II	I	II	-	-	IIa
18/4.5		II/III	-	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/4.8		-	-	II	II	II	II	II	II	-	II	-	I	II	IIb	-	-
18/10.C2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/7.1	Catadau (Valencia) 2018 #8	II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/7.2		-	II	II	II	II	-	II	II	II	II	II	I	-	IIb	IIa	IIa
18/14.1		-	II	-	-	II	-	-	II	-	II	II	I	II	IIa	-	IIa
18/14.2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/14.3		II/III	II	II	II	II	-	II	-	II	II	II	I	II	IIa	IIa	IIa
18/14.4		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/14.5		-	-	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa+IIb	IIa
18/14.6		II/III	II	II	II	II	II	II	II	II	II	II	-	II	IIa	IIa	IIa
18/14.7		-	-	-	-	-	-	-	-	II	-	-	I	-	-	-	-
18/14.8		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/14.9		-	-	-	-	-	II	II	-	-	-	-	-	-	-	IIa	-
18/14.10		II/III	II	-	-	II	-	-	-	-	-	-	I	-	IIa	-	IIa
18/14.11		II/III	II	-	-	II	II	II	II	II	II	-	I	II	IIa	-	IIa
18/14.12		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/14.13		-	-	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/14.14		-	-	-	-	-	-	-	II	-	-	-	I	-	-	-	-
18/14.15		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/14.16		II/III	II	-	II	-	II	II	-	II	II	II	I	II	-	-	-
18/14.17		-	-	-	-	II	-	-	-	-	-	-	-	-	IIa	-	-
18/14.18		-	-	-	-	II	-	-	-	-	-	-	-	-	IIa	-	-
18/14.19		-	-	-	-	II	-	-	-	II	-	-	-	II	IIa	-	IIa
18/14.20		II/III	-	I/II	II	-	II	II	-	-	II	-	I	-	-	-	IIa
18/14.21		-	-	-	-	II	II	-	-	-	II	-	I	II	IIa	-	IIa
18/14.22		II/III	II	II	II	II	II	-	II	-	-	II	I	-	IIa	-	IIa
18/15.1	Cuevas de Almudén (Teruel) 2018 #9	II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/15.2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/15.3		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/15.4		II/III	II	II	II	II	II	II	II	II	II	II	-	II	IIa	IIa	IIa
18/15.5		-	-	-	-	-	-	II	II	II	-	-	I	-	-	-	-
18/15.6		II/III	-	II	II	II	II	II	II	II	II	-	I	II	IIa	-	-
18/15.7		II/III	II	II	II	II	II	-	II	II	II	II	I	II	IIa	-	IIa
18/15.8		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/15.9		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/15.10		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	-	IIa
18/15.11		-	II	-	-	II	-	II	-	II	II	-	I	-	IIa	-	-
18/15.12		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/15.13		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/15.14		II/III	-	II	II	II	II	II	-	II	II	-	I	II	IIa	IIa	IIa
18/15.16		II/III	II	II	II	II	II	II	II	II	II	II	I	-	IIa	IIa	IIa
18/15.18		II/III	II	II	II	II	II	II	II	II	II	II	-	II	IIa	IIa	IIa
18/15.19		II/III	-	-	-	-	-	II	II	-	-	-	I	II	-	-	IIa
18/15.20		II/III	II	II	II	II	II	II	II	II	II	II	-	II	IIa	-	IIa
18/15.21		-	II	II	II	II	II	II	-	II	II	II	-	-	IIa	IIa	IIa+IIb
18/15.23		-	-	II	II	II	II	-	II	-	II	-	I	-	IIa	-	-
18/15.24		-	II	II	II	II	-	-	II	-	-	-	I	II	IIa	-	-
18/16.1		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/16.2		-	-	-	-	II	-	II	-	-	-	-	I	II	IIa	IIa	-
18/18.1		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/18.3		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/18.5		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa

18/18.6		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/18.7		II/III	II	II	II	II	II	II	II	II	II	-	I	II	IIa	IIa	IIa
18/18.8		II/III	-	-	-	-	II	II	II	II	II	II	I	II	-	IIa	-
18/18.10		II/III	II	II	II	II	II	II	II	II	II	II	-	II	IIa	IIa	IIa
18/18.12		-	II	II	II	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/18.14		II/III	II	-	-	II	II	II	II	II	II	II	I	II	IIa	IIa	IIa
18/18.15		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIa	-	IIa
18/18.17		-	II	-	-	II	-	II	-	-	II	-	I	-	IIa	-	-
18/228.1	Villafrechos (Valladolid) 2018 #10	II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/228.2		-	-	-	-	II	II	-	-	-	II	-	I	II	IIb	-	-
18/226.1	Aguilar de Campos (Valladolid) 2018 #11	II/III	II	-	-	II	II	II	II	II	II	-	I	II	IIb	IIa	IIa
18/226.2		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/226.3		-	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/226.4		II/III	II	II	II	II	II	II	II	II	II	-	I	II	IIb	IIa	IIa
18/226.5		II/III	II	II	II	II	II	II	II	II	II	-	I	II	IIb	IIa	IIa
18/226.6		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/226.7		II/III	II	-	-	-	II	II	II	-	-	II	I	II	-	IIa	IIa
18/226.8		II/III	II	II	II	-	II	-	II	II	-	II	I	II	-	-	IIa
18/226.9		-	II	-	-	II	II	-	-	II	-	II	I	II	IIb	-	IIa
18/226.10		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa
18/226.11		-	II	-	-	II	II	II	II	II	-	-	I	II	IIb	-	IIa
18/226.10Pla		II/III	II	II	II	II	II	II	II	-	II	II	-	II	IIb	IIa	IIa
18/226.11Pla		II/III	II	II	II	II	II	II	II	II	II	II	I	II	IIb	IIa	IIa

[a]I, II or III refers to the archetypal allele from a Type I, II or III, for each molecular marker [33].
[b]Ia=100% homology with GenBank accession no. AF340227 or MT358429 sequences; Ib=T1113C, GenBank accession no. MT361124; IIa=100% homology with GenBank accession no. JX218226 or MT361125 sequences; IIb=G1691T, GenBank accession no. MT361126.
[c]IIa=100% homology with GenBank accession no. AF239285 or MT370491 sequences; IIb= C1013T, GenBank accession no. MT370489.
[d]IIa=100% homology with GenBank accession no. DQ459445 or MT361127 sequences; IIb= C2688T, GenBank accession no. MT361128.

Additional file 3: Table S3. Genotyping allele profile obtained by PCR-RFLP and PCR-sequencing on *T. gondii* DNA positive adult sheep myocardium digests.

		PCR-RFLP alleles[a]													PCR-Seq alleles		
ID# Sample	**Location, year**	**SAG1**	**3' -SAG2**	**5' -SAG2**	**Alt. SAG2**	**SAG3**	**BTUB**	**GRA6**	**c22-8**	**C29-2**	**L358**	**PK1**	**Apico**	**CS3**	**SAG3[b]**	**GRA6[c]**	**GRA7[d]**
CC18 G#4	Plasencia (Cáceres) 2018	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-
CC18 G#5		-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-
CC18 G#6		-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-
CC18 G#7		II/III	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-
CC18 G#9		II/III	-	-	-	II	II	II	II	-	-	II	I	II	IIa	IIa	-
CC18 G#15		-	II	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BA18 G#28	Alburquerque (Badajoz) 2018	II/III	II	II	II	II	II	II	II	II	-	II	I	II	IIb	IIa	-
BA18 G#34		II/III	-	-	-	I	-	-	-	-	II	-	I	-	Ia	-	-
BA18 G#35		-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-
BA18 G#39		II/III	II	II	II	II	II	II	II	-	-	-	I	II	IIb	IIa	IIa
BA18 G#44		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CU18 G#55	Sisante (Cuenca) 2018	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CR18 G#56	Valdepeñas (Ciudad Real) 2018	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CR18 G#57		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CR18 G#58		-	-	-	-	-	II	-	-	-	-	-	-	-	-	-	-
CR18 G#59		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CR18 G#60	Puertollano (Ciudad Real)	II/III	II	II	II	II	II	-	II	II	II	II	I	II	-	-	IIa
CR18 G#61		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]I, II or III refers to the archetypal allele from a Type I, II or III, for each molecular marker [33].

[b]Ia=100% homology with GenBank accession no. AF340227 or MT358429 sequences; IIa=100% homology with GenBank accession no. JX218226 or MT361125 sequences; IIb=G1691T, GenBank accession no. MT361126.

[c]IIa=100% homology with GenBank accession no. AF239285 or MT370491 sequences.

[d]IIa=100% homology with GenBank accession no. DQ459445 or MT361127 sequences.

<u>Specific objective 1</u>: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish sheep livestock and its genetic and phenotypic characterization

Sub-objective 1.3: Phenotypic characterization of *Toxoplasma gondii* isolates obtained from Spanish sheep livestock

The results that correspond to the third sub-objective defined within the specific objective 1 were published in a scientific article presented below:

RESUMEN / SUMMARY: *Toxoplasma gondii* es un patógeno zoonótico relevante con una alta diversidad genética, una epidemiología compleja y manifestaciones clínicas variables en animales y humanos. En medicina veterinaria, este parásito apicomplejo se considera uno de los principales agentes infecciosos responsables del fallo reproductivo en pequeños rumiantes a nivel mundial. El objetivo de este estudio fue caracterizar fenotípicamente 10 aislados de *T. gondii* obtenidos recientemente de ovejas españolas en un modelo de ratón normalizado y en una línea celular de trofoblasto ovino (AH-1) como células diana de la infección. El panel de aislados se seleccionó con respecto a parámetros como la diversidad genética (genotipos II [ToxoDB #1 y #3] y III [#2]), la procedencia geográfica y el tipo de muestra de origen (encéfalos de fetos abortados o miocardio de ovejas adultas).

Se realizaron evaluaciones *in vivo* de morbilidad, mortalidad, carga parasitaria y lesiones histopatológicas. Se observaron variaciones importantes entre los aislados, aunque todos se clasificaron como "no virulentos" (<30% de mortalidad acumulada). Los aislados TgShSp16 (#3) y TgShSp24 (#2) presentaron mayores grados de virulencia. Se encontraron diferencias significativas en términos de tasas de invasión y de proliferación a las 72 horas post-inoculación *in vitro* entre los aislados TgShSp1 y TgShSp24, que exhibieron las tasas más baja y más alta, respectivamente. El estudio de los perfiles alélicos de los loci *CS3*, *ROP18* y *ROP5* reveló sólo alelos de tipo III en los aislados de genotipo ToxoDB #2 y alelos de tipo II en los aislados de genotipo #1 o #3 incluidos. Concluimos que existen importantes diferencias intra e intergenotipo en cuestión de virulencia entre los aislados españoles de *T. gondii*, que no pueden inferirse mediante la caracterización genética utilizando los marcadores moleculares habituales.

Reference: Fernández-Escobar M, Calero-Bernal R, Regidor-Cerrillo J, Vallejo R, Benavides J, Collantes-Fernández E, Ortega-Mora LM. (2021). *In vivo* and *in vitro* models show unexpected degrees of virulence among *Toxoplasma gondii* type II and III isolates from sheep. *Veterinary Research*. 52(1):82. doi: 10.1186/s13567-021-00953-7.
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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

In vivo and in vitro models show unexpected degrees of virulence among *Toxoplasma gondii* type II and III isolates from sheep

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Abstract

Toxoplasma gondii is an important zoonotic agent with high genetic diversity, complex epidemiology, and variable clinical outcomes in animals and humans. In veterinary medicine, this apicomplexan parasite is considered one of the main infectious agents responsible for reproductive failure in small ruminants worldwide. The aim of this study was to phenotypically characterize 10 Spanish *T. gondii* isolates recently obtained from sheep in a normalized mouse model and in an ovine trophoblast cell line (AH-1) as infection target cells. The panel of isolates met selection criteria regarding such parameters as genetic diversity [types II (ToxoDB #1 and #3) and III (#2)], geographical location, and sample of origin (aborted foetal brain tissues or adult sheep myocardium). Evaluations of in vivo mortality, morbidity, parasite burden and histopathology were performed. Important variations between isolates were observed, although all isolates were classified as "nonvirulent" (< 30% cumulative mortality). The isolates TgShSp16 (#3) and TgShSp24 (#2) presented higher degrees of virulence. Significant differences were found in terms of in vitro invasion rates and tachyzoite yield at 72 h post-inoculation (hpi) between TgShSp1 and TgShSp24 isolates, which exhibited the lowest and highest rates, respectively. The study of the *CS3*, *ROP18* and *ROP5* loci allelic profiles revealed only type III alleles in ToxoDB #2 isolates and type II alleles in the #1 and #3 isolates included. We concluded that there are relevant intra- and inter-genotype virulence differences in Spanish *T. gondii* isolates, which could not be inferred by genetic characterization using currently described molecular markers.

Keywords: *Toxoplasma gondii*, Genotype, Murine model, Ovine trophoblast, Virulence degree, Virulence factors

Introduction

The cosmopolitan apicomplexan parasite *Toxoplasma gondii* can infect almost all homoeothermic species [1]. It is estimated that approximately one third of the global human population is infected by this obligate intracellular protist, and its high prevalence values in primary livestock species support its consideration as an important risk to food safety [2]. *Toxoplasma gondii* infection is commonly subclinical in immunocompetent individuals; however, it may cause important disorders in immunocompromised and pregnant hosts [1]. In this regard, *T. gondii* is one of the main causes of reproductive failure in small ruminants and is responsible for approximately 10% of ovine abortions in Europe [3], thereby implying notable economic losses caused to the sheep industry worldwide.

Toxoplasma gondii possesses significant genetic and phenotypic diversity that has been proposed to be responsible for variations in clinical presentation. Initially, the *T. gondii* global population was assumed to be structured in three clonal lineages associated to virulence

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in a murine model (highly virulent type I, moderately virulent type II, and nonvirulent type III) [4–7]. Nonetheless, the studies were strongly biased by the fact that isolates included were mainly from human patients and domestic animals originating from France and the USA, along with the implementation of monolocus typing strategies promoting the misidentification of atypical and recombinant strains circulating [8]. Two decades of scientific effort in isolation and both molecular and phenotypic characterization of the parasite led to the discovery of a much more complex reality involving a population structure with at least 16 haplogroups worldwide [9–11] and a virulence degree classification under debate [12]. Regarding strains that circulate in European and Spanish sheep livestock, there are several genotyping studies that demonstrate the total predominance of type II strains, which coexist with small percentages of type III and recombinant strains [13–15].

In vivo murine models have been traditionally used to evaluate the virulence degree of *Toxoplasma* isolates by calculating the cumulative mortality rate [16]. On the other hand, in vitro culture models have also been shown to be highly suitable and informative for phenotypic characterization of apicomplexan parasite strains [17–20]. Regarding *T. gondii*, studies employing host target-cell lines such as those derived from central nervous system or placental tissues [21–24] are of special interest. However, most studies have been carried out with laboratory-adapted isolates that are nonrepresentative of *Toxoplasma* population-wide biological diversity.

There is growing evidence of different outcomes when the same strains infect different hosts [25–27]. In this sense, alternative virulence approaches, including allelic combination characterization of demonstrated virulence factors such as *ROP18* and *ROP5* [28–31] or virulence molecular markers such as *CS3* [32], have been tested recently, producing promising results concerning allelic variation linked to virulence.

The present study aimed to characterize the virulence degree of a panel of *T. gondii* isolates recently obtained from naturally infected Spanish sheep through an in vivo murine model (including cumulative mortality and morbidity rates, parasite burdens and pathological lesions evaluation), along with in vitro invasion and proliferation assays in an ovine trophoblast cell line (AH-1). In addition, *CS3*, *ROP18* and *ROP5* allelic profile characterization of all isolates was carried out.

Materials and methods

Ethic statement

Animal procedures for the *T. gondii* strains virulence degree evaluation in mice (PROEX 274/16) were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU regulations (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering. As a humane endpoint, mice with a severe loss of body condition or nervous clinical signs were euthanized to limit unnecessary suffering.

Mice

Seven-week-old female Swiss/CD1 mice were obtained from a commercial supplier (Janvier Labs, Le Genest-Saint-Isle, France). The animals were free from common viral, parasitic, and bacterial pathogens according to the results of routine screening analyses performed by the manufacturer. Mice were housed with ad libitum access to food and water in a controlled environment with 12-h light and 12-h dark cycles, and the experimental procedures were performed at 8 weeks of age.

Parasites and cell cultures

A panel of 10 *T. gondii* isolates previously obtained from sheep [15] was selected for phenotypic characterization according to three criteria: (a) genetic diversity, limited to the three predominant PCR–RFLP genotypes present in Spain (ToxoDB #1, #2 or #3); (b) geographical location within Spanish territory; and (c) clinical sample of origin (abortion-derived tissues or myocardial tissues from chronically infected adult sheep) (Table 1). The *T. gondii* isolates included in this study were subjected to a

Table 1 Panel of 10 *Toxoplasma gondii* Spanish ovine isolates [15] selected for the in vitro and/or in vivo assays on the basis of PCR-RFLP genotype (ToxoDB #1, #2 or #3), geographic origin, and original clinical sample (ovine foetal brain or adult ovine myocardium)

Isolate ID	Genotype # (ToxoDB)	Geographic origin	Original clinical sample
TgShSp1	#3	Palencia, central Spain	Ovine foetal brain
TgShSp2	#1	Navarra, northern Spain	Ovine foetal brain
TgShSp3	#3	Palencia, central Spain	Ovine foetal brain
TgShSp7	#3	Segovia, central Spain	Ovine foetal brain
TgShSp8	#3	Valencia, eastern Spain	Ovine foetal brain
TgShSp10	#3	Teruel, central Spain	Ovine foetal brain
TgShSp11	#3	Cáceres, western Spain	Adult ovine myocardium
TgShSp16	#3	Badajoz, western Spain	Adult ovine myocardium
TgShSp24	#2	Ciudad Real, central Spain	Adult ovine myocardium
TgShSp30	#3	Badajoz, western Spain	Adult ovine myocardium

restricted number (from 8 to 12) of lytic cycles completed in cell culture or passages, to preserve their in vivo biological behaviour and avoid adaptation to the cell culture. Parasites were maintained by serial passages in Vero cells (ATCC CCL-81). Briefly, cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with foetal bovine serum (FBS) (Gibco), penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (Lonza Group, Basel, Switzerland), at 37 °C and 5% CO_2 in 75 or 25 cm^2 tissue culture flasks. Tachyzoites used for in vivo and in vitro assays were recovered from cultures of Vero cells at low passages, when the majority of the parasites were still intracellular, and purified by filtration through a 3-µm polycarbonate filter (IpPORE®, IT4IP, Louvain-la-Neuve, Belgium) as previously described [16]. The quantity and viability of tachyzoites were determined by Trypan blue exclusion, followed by direct counting in a Neubauer chamber.

For in vitro assays, an immortalized trophoblast cell line (AH-1) originally obtained from primary cultures of ovine placenta was used [33]. The cell line was kindly supplied by the Department of Veterinary Microbiology and Pathology of Washington State University (Pullman, WA, USA). AH-1 cells were cultured under conditions similar to those used for Vero cells.

Assays of virulence in mice

Two in vivo experiments were conducted to evaluate cumulative mortality and morbidity rates at 42 days post-inoculation (dpi) (Section "Assay A") and the parasite burden and histological lesions shown by the isolates in mouse organs during the acute and chronic stages of the infection (Section "Assay B").

Assay A

Serial dilutions in phosphate buffered saline (PBS) were performed to obtain doses from 10^5 to 1 tachyzoite(s) of each isolate per 200 µL. Each dose was intraperitoneally (IP) inoculated into five 8-week-old female Swiss/CD1 mice. Five control mice were inoculated with 200 µL of PBS. Mice were monitored twice daily for 6 weeks, and clinical signs were recorded. The cumulative morbidity rate was evaluated by establishing clinical sign scoring adapted from reference [34]. The cumulative mortality rate was calculated based on the ratio of casualties to the total number of infected mice [16]. Serum samples from mice that were humanely euthanized, presented sudden death, or reached the end of the experiment at 6 weeks post-inoculation were collected and stored at −20 °C until serological procedures for *T. gondii* antibody detection using an indirect fluorescent antibody test (IFAT) to confirm infection.

Assay B

An additional group of 10 mice per isolate was IP-inoculated with 10^3 tachyzoites. Five animals were sacrificed at 7 dpi, and the remaining five mice were sacrificed at 30 dpi to study the acute and chronic phases of the infection, respectively. Selected organs were collected during necropsies for *T. gondii* DNA detection and quantification. Briefly, the mice were bled, and the right cerebral hemisphere, the right eye, the right lung, half of the heart, a piece of a liver lobe, and the right kidney from each mouse were transferred immediately following euthanasia into clean 1.5 mL tubes and stored at −80 °C until DNA extraction. The left cerebral hemisphere, the left lung, half of the heart, the left kidney, and a piece of a liver lobe, the tongue, and the *quadriceps femoris* muscle were fixed in 10% buffered formalin and processed for conventional histological examination. After staining with haematoxylin/eosin, lesions in the samples were subjectively categorized from 0 (no lesion) to 4 (the most severe grade within observed lesions). Serum samples were also collected and stored at −20 °C until analysis.

Toxoplasma gondii antibody detection by IFAT and DNA extraction, detection and quantification in assay B were implemented exactly as in reference [12]. In both assays A and B, mouse *Toxoplasma* infections were confirmed by IFAT (titer > 1:25) or by brain or lung tissue imprints.

In vitro assays in ovine trophoblast target cells

A limited number of six isolates (TgShSp1, TgShSp2, TgShSp3, TgShSp11, TgShSp16 and TgShSp24) were selected for in vitro phenotypic characterization according to the same criteria used in the in vivo experiments but also considering the more contrasting in vivo results. Phenotypic characterization was carried out in AH-1 ovine trophoblast cells [33] since trophoblasts are target cells in congenital *Toxoplasma* infection and mediate the innate immune response [35].

Invasion assays

Parasite invasion rate (pInvR) determination was attempted as described previously [36]. Briefly, AH-1 cells were seeded at 1×10^5 cells per well into 24-well culture plates. In total, 200 purified tachyzoites were added per well. Cultures were washed three times with PBS at different time points (4 or 8 hpi) to remove unadhered/non-invading tachyzoites. Unwashed cultures were also included in the study. All plates were fixed for 15 min with cold methanol at 56 hpi and the number of infection events (parasitophorous vacuoles or lysis plaques) per well was determined by applying single immunofluorescence staining (see Section "Immunofluorescence

staining") directly over the wells and counting by direct observation under an inverted fluorescence microscope. Experiments were assayed in triplicate, and three independent experiments were carried out.

The parasite invasion rate at 4 and 8 hpi and the total parasite invasion rate at 56 hpi ($pInvR_{4h}$, $pInvR_{8h}$, and $pInvR_{T}$, respectively) were determined as the number of infection events observed per well in cell monolayers washed at the different time points (4 hpi, 8 hpi or unwashed) divided by two to estimate the percentage of invading tachyzoites. This assay allowed us to determine the time at which each isolate reached the maximum peak of invasion.

Immunofluorescence staining

Fixed cultures were permeabilized using a solution of 0.25% Triton 100X in PBS 3% BSA (Bovine Serum Albumin Fraction V; Roche, Germany) (30 min, 37 °C). After washing with PBS (× 3), wells were treated with 3% BSA in PBS for 30 min at room temperature (RT) to block nonspecific antibody binding. Then, parasites were stained using positive murine serum samples from previous experimental infections (1:200 in a 0.3% BSA/PBS solution) [12, 15] as a primary antibody (1 h, 37 °C) and a 1:1000 dilution (in PBS) of goat anti-mouse IgG conjugated to Alexa Fluor® 488 (green, Thermo Fisher Scientific, Waltham, MA, USA) as a secondary antibody (1 h, RT). The nuclei were stained by washing the cells with a solution of 1:10 000 DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Invitrogen™) in PBS.

Proliferation kinetics assays

The proliferation kinetic of each of the isolates in the AH-1 cell line were determined by quantifying the number of tachyzoites at specific times after inoculation (8, 24, 32, 48, 56, 72, 80 and 96 hpi) by real-time PCR (qPCR). Cells were cultured and infected as indicated above, but in this case, a multiplicity of infection (MOI) of 4 was used. Cultures were washed at 8 hpi (time post-inoculation at which the parasite reached > 50% invasion rate, as determined in the previous experiment) and subsequently maintained at 37 °C in a 5% CO_2 atmosphere. At the selected time points and after removing the supernatant, samples were collected by adding 150 µL of lysis buffer and 20 µL of proteinase K (Qiagen, Hilden, Germany) to each well, transferred into PCR clean 1.5 mL tubes and frozen at −80 °C prior to DNA extraction. In parallel, replicates of cell cultures grown on coverslips were identically infected and fixed at the same time points selected for DNA sample collection. Fixed cultures were labelled by single immunostaining (as described above for the invasion assays) to microscopically study the proliferation kinetics of all isolates included using a confocal fluorescence inverted microscope (CBM-SO Microscopy Services, Madrid, Spain). Experiments were assayed in triplicate, and three independent experiments were carried out.

DNA extraction and qPCR parasite quantification

Genomic DNA was extracted from the collected samples using the DNeasy® Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Parasite quantification was carried out by qPCR using primer pairs for the 529-bp repetitive element of *T. gondii* [37]. DNA samples were adjusted to 20 ng/µL, and reactions were performed in a final volume of 25 µL using GoTaq® qPCR Master Mix (Promega, Alcobendas, Madrid, Spain), 10 pmol of each primer and 100 ng of DNA in an Applied Biosystems 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification was performed by a standard protocol (10 min at 95 °C, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min). The number of *T. gondii* tachyzoites was calculated by interpolating the average Ct values on a standard curve equivalent to $1 \times 10^{5} - 1 \times 10^{-1}$ tachyzoites generated by tenfold serial dilutions of parasite DNA in a solution of ovine genomic DNA at 20 ng/µL. Parasite proliferation was expressed as the parasite number/ng of DNA. Standard curves for *T. gondii* showed an average slope always close to −3.3 and an $R^2 > 0.98$.

Proliferation kinetics and tachyzoite yield (TY) determination

The parasite proliferation kinetics of each *T. gondii* isolate were studied by plotting the values of tachyzoites/ng of total DNA reached, which was determined by qPCR, against the specific collection time points. The tachyzoite yield at 72 hpi (TY_{72h}) was defined as the average number of tachyzoites/ng DNA quantified by qPCR at that time point for each isolate.

Molecular analyses of predictive markers for virulence in mice

The *CS3* marker [32] and the virulence factors *ROP18* and *ROP5* [28, 30], which are suggested to have high predictive value for the virulence degree in mice, were studied, aiming to provide a correlation between the allelic profile and unexpected differences in phenotypic features observed. Briefly, the methodology was based on nested PCR-DNA sequencing of each marker and the details are summarized in Additional file 1. DNA samples of strains representative of the three canonical clonal lineages were used as references to note TgRH (type I, ToxoDB #10), TgMe49 (type II, #1), and TgNED (type III, #2).

The yielded amplicons were directly subjected to Sanger sequencing in both directions using the internal primers described in Additional file 1. The sequencing

procedures were carried out as shown in reference [15] at the Center for Genomic Technologies of the Complutense University of Madrid (Spain). The resulting sequences were imported, read, edited manually if necessary, and analysed using BioEdit software (version 7.0.5.3) [38]. Necessary alignments were performed using Clustal Omega software [39]. Finally, in silico digestion of each locus sequences by identification of restriction enzyme motifs was conducted by the NEBCutter 2.0 program [40]. Specific restriction enzymes are indicated in Additional file 1.

Data statistical analysis

One-way ANOVA followed by Tukey's multiple range tests were employed to compare the parasite burden assessed for each isolate within each organ and the time of infection (7 or 30 dpi) studied. The Kruskal–Wallis test was employed for comparisons among the pInvRs shown for the different isolates within each time point (4, 8, and 56 hpi), among the parasite proliferation values (no of parasites/ng DNA) reached by each isolate at the time points included in the proliferation kinetics experiments and finally used to compare the TY_{72h} reached by each isolate. When statistically significant differences were found with the Kruskal–Wallis test, Dunn's multiple-comparison test was applied to examine all the possible pairwise comparisons. The significance for these analyses was established at $p < 0.05$. GraphPad Prism 6 v.6.01 (San Diego, CA, USA) software was used to perform all statistical analyses and graphical illustrations.

Results

In vivo characterization in a murine model

Cumulative mortality and morbidity rates (assay A)

The cumulative mortality rate was 4.7% for TgShSp10, 8.0% for TgShSp11, 20.8% for TgShSp16, 18.2% for TgShSp24, and 0% for the rest of the isolates, as shown in the survival curves (Figure 1A). Therefore, all isolates must be classified as nonvirulent (cumulative mortality < 30%) according to the criteria established in [41]. Regarding the cumulated morbidity rate, the maximum clinical sign score reached in each inoculated mouse is shown in Figures 1B and C. Isolates TgShSp7, TgShSp10, TgShSp11, TgShSp16 and TgShSp24 were noted to cause

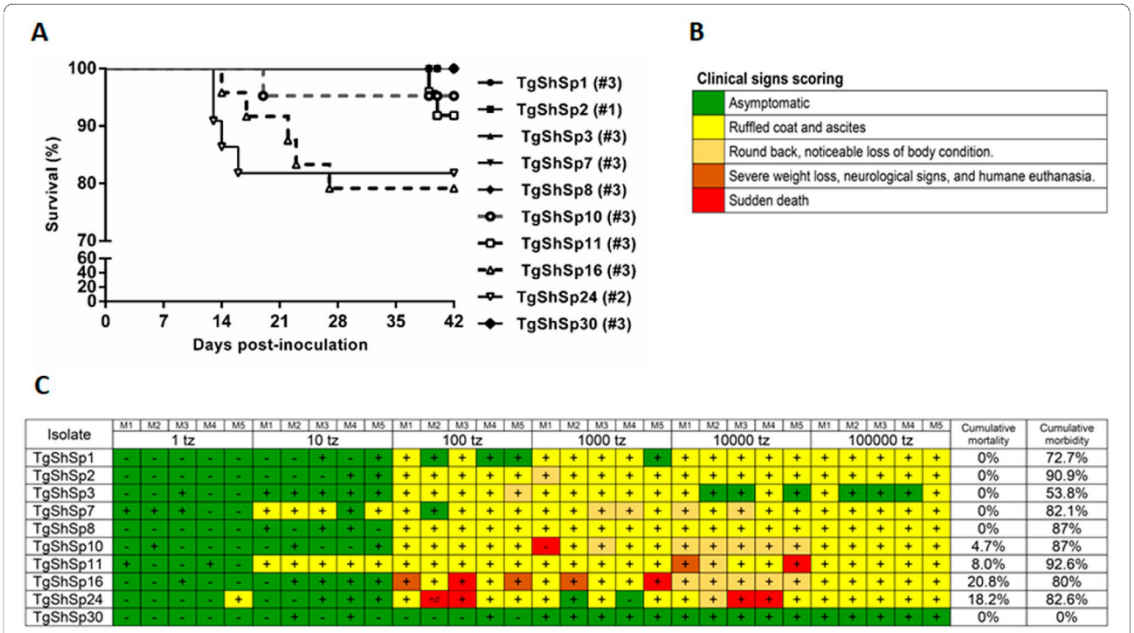


Figure 1 Cumulative mortality and morbidity parameters in the evaluation of virulence degree in *Toxoplasma gondii* isolates. A Kaplan–Meier survival curves of *Toxoplasma gondii* isolates from sheep. ToxoDB# genotype of each isolate is indicated. **B** Clinical sign scoring used in the evaluation of morbidity. **C** Summary of the cumulative mortality and morbidity parameters in the evaluation of virulence degree. The gradation of clinical signs observed in mice inoculated with each isolate is shown. Coloured boxes represent the most severe clinical sign observed in each individual mouse challenged with each dose according to the clinical sign scoring included below. +: mice with a *Toxoplasma*-infection confirmed by IFAT or tissue imprints of brain or lung. −: mice without a confirmed *Toxoplasma*-infection. nd: not done. Cumulative mortality and morbidity rates are indicated. The dosages 10^5, 10^4,10^3, 10^2, and 10^1 are included for cumulative mortality rate calculation, whereas all dosages were considered for cumulative morbidity rate calculation.

a rounded back and noticeable loss of body condition in a higher proportion compared to the other isolates, even causing severe weight loss and nervous signs and consequently humane euthanasia in the case of TgShSp11 and TgShSp16. TgShSp16 and TgShSp24 stood out in terms of spontaneous death (2/25 and 4/23, respectively). On the other hand, the TgShSp1, TgShSp3, TgShSp8 and TgShSp30 isolates triggered only mild clinical signs in a lower proportion of infected mice, specifically including a ruffled coat and ascites; besides, the clinical signs completely receded quickly.

Tissue tropism, parasite burden and histopathological lesions (assay B)

The parasite burden reached by each isolate in the different tissues studied is shown in Figure 2. None of the mice scheduled for sacrifice at 7 dpi seroconverted, whereas those scheduled to be sacrificed at 30 dpi all seroconverted except for one mouse in each group infected with the isolates TgShSp2, TgShSp24 and TgShSp30, which in consequence were not considered for the calculation of the parasite loads. In the case of the lung, the parasite burden during the acute phase was low in general (unpublished observations) and higher during the chronic phase for all isolates except TgShSp7, TgShSp10 and TgShSp16, with the TgShSp24 isolate showing the highest mean value (Figure 2A). Regarding the parasite burden in acute tropism tissues such us liver and kidney, values were considered negligible in mice sacrificed at 30 dpi (unpublished observations). For both organs at 7 dpi, all mean parasite burden values were low except those reached by TgShSp16 in kidney tissues with 100% of animals having parasite DNA present (5/5) (Figure 2B). Concerning the brain at 30 dpi (Figure 2C), TgShSp16 and TgShSp24 isolates drew attention for having the highest median values with notably high parasite loads (726.5 and 410.3 zoites/mg of brain tissue, respectively). The mean parasite burden reached in the brain by TgShSp16 was between 10 and 100 times higher than of the rest of the isolates ($p < 0.0001$), and twice that of TgShSp24 (no statistically significant differences; $p > 0.05$). On the other hand, the TgShSp8 and TgShSp30 isolates stood out for

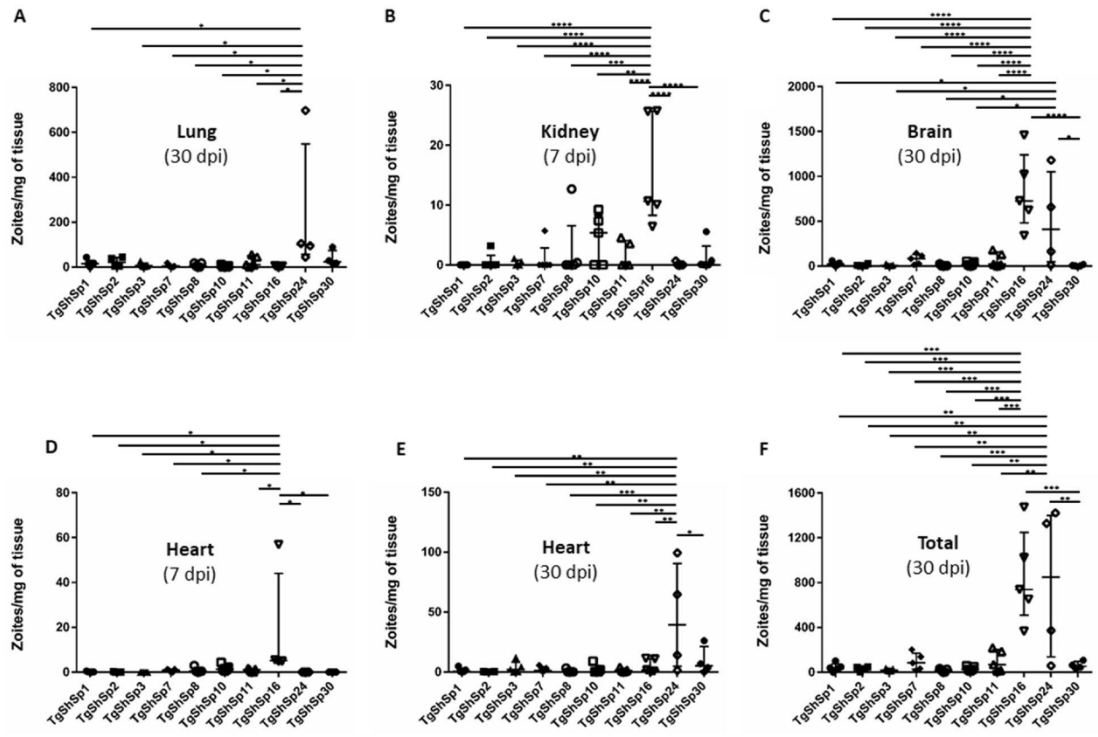


Figure 2 Parasite loads (zoites/mg of tissue) found in different mouse organs. A Brain at 30 dpi; **B** lung at 30 dpi; **C** heart at 7 dpi; **D** heart at 30 dpi; **E** kidney at 7 dpi; and **F** total parasite burden per mouse at 30 dpi. Median and interquartile values are represented. One-way ANOVA followed by Tukey's test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

having the lowest median parasite loads, closely followed by TgShSp2 and TgShSp3. With respect to heart tissues, all isolates showed a higher mean burden at 30 dpi than at 7 dpi, with the exception of the TgShSp16 isolate, whose parasite load during acute infection was significantly higher than that of the rest of the isolates except TgShSp10 (Figures 2D and E). Notably, the TgShSp24 mean parasite burden reached at 30 dpi was many times the average burden reached by the rest of the strains at this infection stage, supposing statistically significant differences (Figure 2E). Ocular tissue showed great variability in DNA detection and quantification, making it difficult to interpret the results (unpublished observations). However, the TgShSp24 isolate caused 100% of infected animals to exhibit parasite DNA in the eye (4/4) and a median value of 5.6 parasites/mg of tissue on average during the chronic phase. Likewise, strain TgShSp16 resulted in 60% of infected animals with parasite DNA in ocular tissues (3/5) and a median value of 8.2 parasites/mg of tissue at the same time point. Overall, the total parasite burden reached in mice infected with TgShSp16 and TgShSp24 at 30 dpi was significantly higher than that of the rest of the isolates (Figure 2F). On the other hand, TgShSp8 and TgShSp3 produced the lowest total parasite burdens.

Histological lesions were mainly observed in the brain, liver, and lung, where multifocal aggregates of mononuclear inflammatory cells were detected. The intensity of the inflammatory infiltrate ranged from clusters of scant lymphocytes (grade 1) to aggregates of numerous lymphocytes, macrophages and plasma cells, frequently in relation to blood vessels (grade 4). Lesions were notably more frequent in tissues collected at 30 dpi than at 7 dpi. No lesions were found in the kidney. In the liver, nonspecific inflammatory lesions were observed in mice infected with all isolates studied at 30 dpi, but they were observed more often and with greater severity in the case of mice infected with TgShSp1, TgShSp11 and TgShSp24 isolates. Lesions in the brain were distinguished by glial foci and perivascular infiltration of inflammatory cells mainly present in a chronic phase of the infection. Mice infected by TgShSp16 and TgShSp24 isolates stood out from the rest of mice due to the severity of the brain lesions (especially TgShSp24-infected tissues, which showed a case of grade 4 lesions) and tissue cyst-like structures presence in the unique case of TgShSp16-infected brains (three animals) (Figure 3A). Mice infected with TgShSp2, TgShSp3, and TgShSp30 isolates showed minimal or no presence of brain lesions at 30 dpi. In the case of lung tissues, mice inoculated with the TgShSp24 isolate were highlighted again for presenting increasingly severe foci of inflammation (two animals had grade 3 lesions, and two animals had grade 4 lesions) (Figure 3B).

Histological lesions detected in heart tissues were less abundant, with 0–20% of mice affected in the case of TgShSp1, TgShSp2, TgShSp3, TgShSp7, TgShSp8, TgShSp10, TgShSp11 and TgShSp30 infections, which contrasted with the figures of 60% in the case of TgShSp16 and 80% in the case of TgShSp24-infected mice, noting in even higher severity in the latter case (two animals had grade 1 lesions, and two animals had grade 2 lesions). Portions of quadriceps and tongue tissues, as skeletal muscle instances, were also evaluated for inflammation signs. Lesions in the quadriceps were only found in mice infected with TgShSp7, TgShSp16, TgShSp24 and TgShSp30 isolates at 30 dpi, with those from TgShSp24-inoculated mice highlighted due to much higher significance (one animal had grade 2 lesions, and two animals had grade 3 lesions). Inflammation foci in tongue tissues were even scarcer and were only detected in mice inoculated with TgShSp1, TgShSp7, TgShSp24 y TgShSp30 at 30 dpi, again with more severe degree in

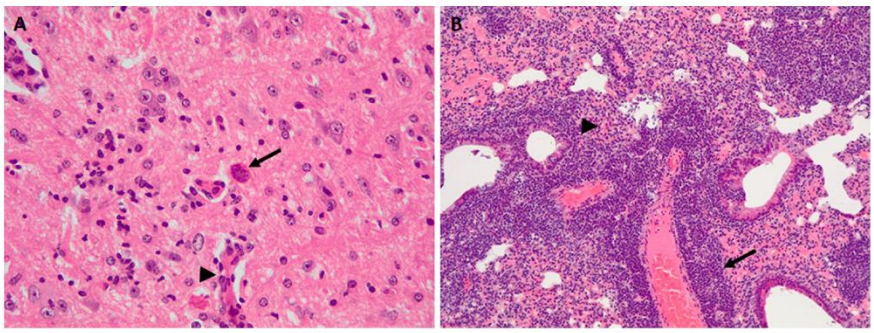


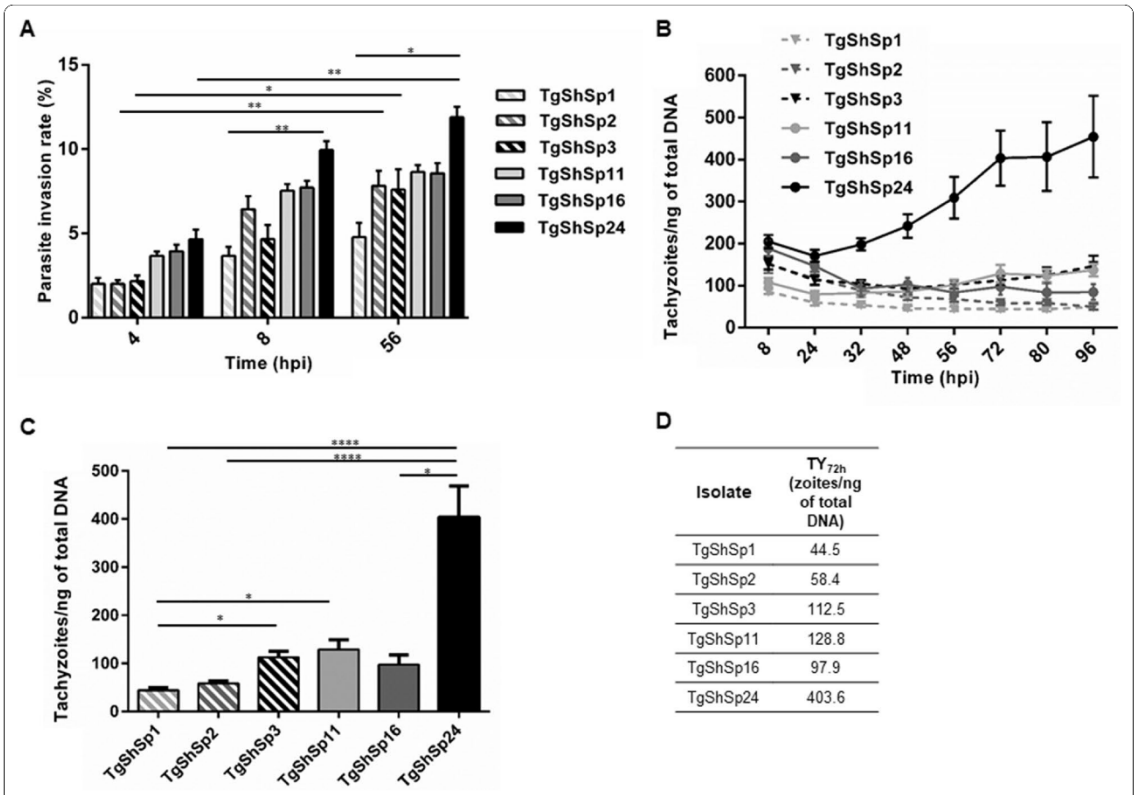
Figure 3 Histological lesions observed in *Toxoplasma gondii* infected mice. A Brain. Mouse infected with the TgShSp16 strain. Note, the tissue cyst-like structure (arrow) and non-purulent vasculitis (arrowhead). **B** Lung. Mouse infected with the TgShSp24 strain. Note, the perivascular infiltration of non-purulent inflammatory cells (arrow) and focal thickening of the alveolar wall (arrowhead).

the case of TgShSp24-infected animals (two animals had grade 2 lesions). The histopathological evaluation data were in strong agreement with the results of the parasite load quantification.

In vitro characterization in an ovine trophoblast target cell

The parasite invasion rates at 4 and 8 hpi, and the total parasite invasion rate at 56 hpi ($pInvR_{4h}$, $pInvR_{8h}$, and $pInvR_T$) of the TgShSp1, TgShSp2, TgShSp3, TgShSp11, TgShSp16 and TgShSp24 isolates in the AH-1 cell line are shown in Figure 4A. The percentage of invading tachyzoites varied significantly between 4 and 56 hpi for TgShSp2, TgShSp3 and TgShSp24 but not for the rest of the isolates tested. TgShSp1 and TgShSp24 were the isolates with lower and higher parasite invasion rates, respectively, with significant differences between them at 8 hpi (3.7% and 10%, respectively; $p < 0.01$) and at 56 hpi (4.8% and 11.9%; $p < 0.05$).

The parasite proliferation kinetics of each *T. gondii* isolate are plotted in Figure 4B. In terms of tachyzoites/ng of total DNA produced, there were significant differences between TgShSp1 and TgShSp24 isolates from 32 to 96 hpi and between TgShSp2 and TgShSp24 from 56 hpi until the end of the experiment. Since 72 hpi appeared to be the point at which the isolate TgShSp24 (the only isolate with growth kinetics that fit the exponential growth equation) completed a lytic cycle, we decided to estimate the tachyzoite yield at that time point (TY_{72h})



Isolate	TY_{72h} (zoites/ng of total DNA)
TgShSp1	44.5
TgShSp2	58.4
TgShSp3	112.5
TgShSp11	128.8
TgShSp16	97.9
TgShSp24	403.6

Figure 4 Differential growth of *Toxoplasma gondii* isolates in ovine trophoblast AH-1 cells. A Parasite invasion rate at 4 and 8 hpi and total parasite invasion rate at 56 hpi ($pInvR_{4h}$, $pInvR_{8h}$, and $pInvR_T$, respectively) of TgShSp1, TgShSp2, TgShSp3, TgShSp11, TgShSp16 and TgShSp24 isolates infecting AH-1 cells. Parasite infection rates were defined as the percentage of invading tachyzoites (number of infection events per well) at the different time points for each isolate. Each column and error bar represent the mean and the SD of 3 replicates from 3 independent assays at the indicated sampling times. **B** Proliferation kinetics of each of the isolates in AH-1 cells. The number of tachyzoites/ng of total DNA reached at specific times after infection (8, 24, 32, 48, 56, 72, 80 and 96 hpi) by each isolate is represented. TgShSp1 vs. TgShSp24 at 32 h ($p < 0.01$), 48 h ($p = 0.0001$), 56 h ($p < 0.0001$), 72 h ($p < 0.0001$), 80 h ($p < 0.0001$), and 96 h ($p < 0.001$); TgShSp2 vs. TgShSp24 at 56 h ($p < 0.05$), 72 h ($p < 0.001$), 80 h ($p < 0.01$), and 96 h ($p < 0.001$). **C**, **D** Tachyzoite yield at 72 hpi (TY_{72h}) reached by each isolate. TY_{72h} mean values for each isolate are tabulated. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

and compared it between isolates (Figure 4C). Significant differences ($p < 0.0001$) were detected in the number of tachyzoites/ng of total DNA reached at 72 hpi between TgShSp1 and TgShSp24 and between TgShSp2 and TgShSp24. Additionally, slight differences ($p < 0.05$) were found between TgShSp1 and both TgShSp3 and TgShSp11 isolates and finally between TgShSp16 and TgShSp24 (specific $\bar{x}$ TY_{72h} values for each isolate are presented in Figure 4D).

Microscopic examination of infected cell cultures subjected to immunostaining at different time points showed that the multiplication of the isolates studied began between 8 and 24 hpi (Figure 5). Notable differences in the parasitophorous vacuole sizes between isolates were observed from 24 hpi onwards, with much larger vacuoles in TgShSp24-infected cells than in the rest of the infections. Furthermore, differences in the number of infection events were found between isolates. TgShSp1 and TgShSp2 appeared to successfully infect in a lower proportion than the TgShSp3, TgShSp11 and TgShSp16 isolates. Likewise, the TgShSp24 isolate achieved an even higher number of infections with much faster replication of tachyzoites inside each parasitophorous vacuole. Between 56 and 72 hpi, the rupture of host cells and egress of tachyzoites were observed in all cases (Figure 5).

Allelic profile characterization of *CS3*, *ROP18* and *ROP5* loci

We amplified, sequenced, and virtually digested the *CS3*, *ROP18* and *ROP5* loci of the 10 isolates included in the assays to examine the possible correlation between the allelic profile and virulence in mice [28, 30, 32]. The *CS3* marker presented a type II allele in all isolates with the ToxoDB #1 or 3 genotype, and the type III allele was only detected in the TgShSp24 isolate (ToxoDB #2). *CS3*

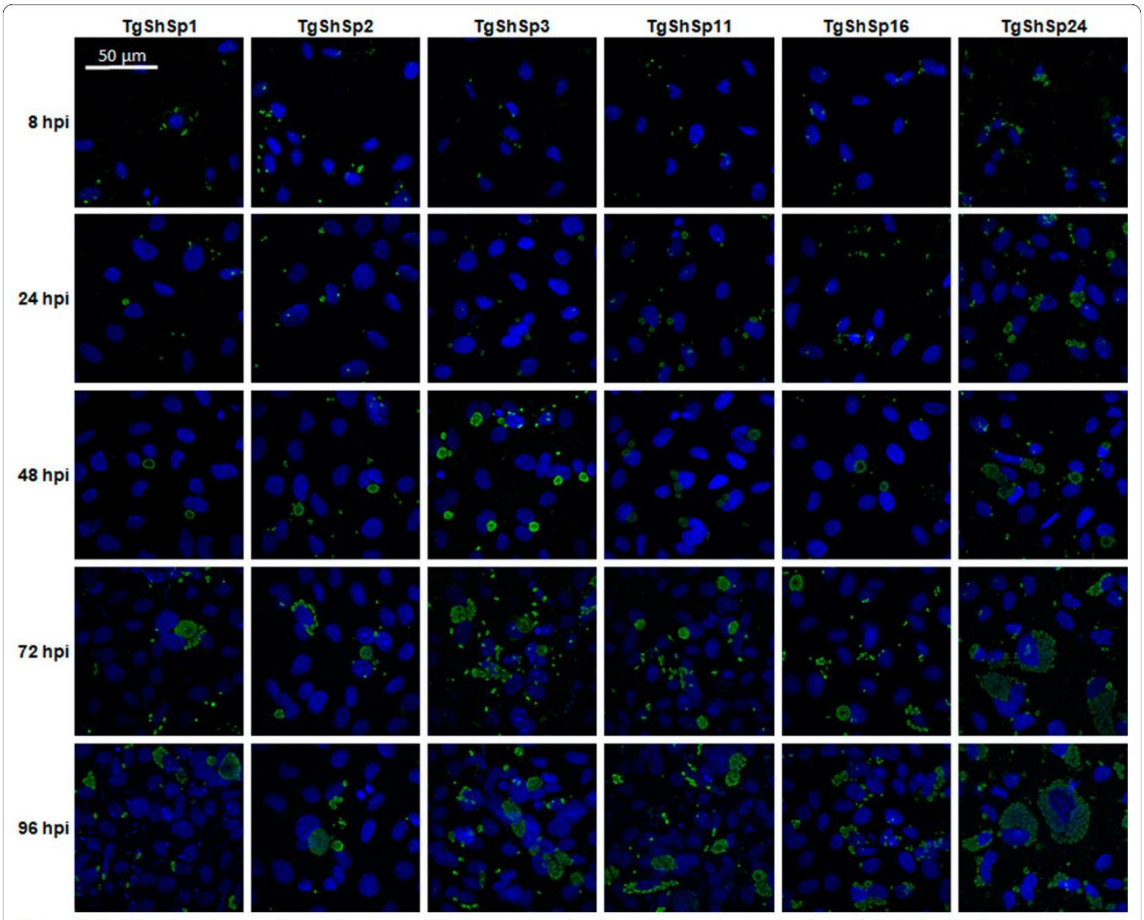


Figure 5 Proliferation kinetics of *Toxoplasma gondii* isolates determined by microscopic examination of infected cell cultures subjected to immunostaining. Representative images at 8, 24, 48, 72, and 96 hpi were selected. The scale bar (50 µm) applies to all micrographs.

sequences from the TgShSp1 and TgShSp24 isolates were deposited in GenBank as instances of each allele detected (MW727456-7). Regarding the *ROP18* gene, the upstream promoter insertion sequence (UPS) of the archetypal type III allele was amplified only in the case of the TgShSp24 isolate ("nonvirulent" allele 3), while the rest of the isolates presented allele 2 ("virulent" allele) according to DEL fragment amplification and sequencing [42]. Similarly, the *ROP5* locus was found to have allele 2 ("nonvirulent" allele) in all type II strains but allele 3 ("virulent" allele) in the TgShSp24 isolate [42]. The *CS3*, *ROP18* and *ROP5* allelic profiles of the 10 isolates included are summarized in Table 2.

Discussion

The virulence degree of *Toxoplasma gondii* strains has been conventionally determined according to the cumulative mortality rate in outbred laboratory mice. In this regard, *T. gondii* clonal lineages I, II and III have been traditionally classified as highly virulent (100% lethality, $LD_{100}=1$; type I), intermediate virulent (99–30%, $LD_{50}\geq 1000$; type II) and nonvirulent (< 30%, $LD_{50}>10^5$; type III) [4, 41]. However, this simplistic criterion may hide important differences not only in lethality but also in the severity of the clinical outcome [45]. Along with cumulative mortality rates, the virulence degree has also been evaluated by morbidity, parasite burdens and pathological lesions detected in different tissues (e.g., central nervous system) [12], and by other nonlethal infection parameters, such as weight loss, anti-*T. gondii* IgG antibodies and haptoglobin levels in serum, cystogenic capacity, or even animal behavioural changes [31, 45–48]. Variations in specific virulence features have already been demonstrated among strains presenting different genotypes, as well as within some belonging to the same genetic type determined by RFLP-based methods [8, 31, 45, 49, 50]. As in the case of the use of laboratory mice for virulence evaluation in a standardized manner (reviewed in [16]) to obtain comparable results, the use of archetypal strains long-term maintained under laboratory conditions is accepted but not representative of the vast biological diversity of the *Toxoplasma* population. It is well known how maintenance under cell culture conditions during successive passages involves strong phenotypic changes in laboratory-adapted strains [6, 26]. In view of the above situation, we present a comprehensive study of the virulence degree in mice of a panel of Spanish *T. gondii* isolates recently obtained from sheep based on lethal and nonlethal parameters. It should be highlighted that the isolates included in the present experiments belong to the most prevalent genotypes in Spanish farm animals [12, 15] and present a low number of cell culture passages, avoiding adaptation to in vitro laboratory conditions.

In the present study, a panel of isolates was subjected to in vivo virulence assays. Of the 10 isolates evaluated, eight had been classified as type II-PRU variants according to 11 RFLP markers, while examples of clonal type II (TgShSp2) and clonal type III (TgShSp24) were also included [15]. All isolates were classified as nonvirulent (cumulative mortality < 30%; $LD_{50}>10^5$) according to the traditional criterion. Here, most of the type II isolates (ToxoDB #1 and 3) presented cumulative mortalities of 0 or close to 0, except TgShSp16, which stood out

Table 2 Genotyping of *Toxoplasma gondii* isolates with virulence in mice-related loci of *CS3*, *ROP18* and *ROP5*

Strain ID	ToxoDB#	*CS3*	*RO18*	*ROP5*	Virulence[a]	Cumulative mortality in mice (%)	References
TgRH	10	I	1	1	Vir	100	[1, 15, 43]; this study
TgMe49	1	II	2	2	Int	40	[6, 15, 30]; this study
TgNED	2	III	3	3	Unknown	Unknown	[1, 15, 44]; this study
TgShSp1	3	II	2	2	Non	0	This study
TgShSp2	1	II	2	2	Non	0	This study
TgShSp3	3	II	2	2	Non	0	This study
TgShSp7	3	II	2	2	Non	0	This study
TgShSp8	3	II	2	2	Non	0	This study
TgShSp10	3	II	2	2	Non	4.7	This study
TgShSp11	3	II	2	2	Non	8	This study
TgShSp16	3	II	2	2	Non	20.8	This study
TgShSp24	2	III	3	3	Non	18.2	This study
TgShSp30	3	II	2	2	Non	0	This study

[a] *Toxoplasma gondii* strains are classified according to cumulative mortality in mice into highly virulent (Vir, 100% mortality); intermediately virulent (Int, 99–30%), and nonvirulent (Non, < 30%) [41].

with a ratio of 21%. This isolate presented the highest mean parasite burden in the brain at 30 dpi, which was between 10 and 100 times higher than that of the rest of the type II isolates ($p < 0.0001$), and it was the only case in which tissue cyst-like structures were found in the brain during histopathological analysis of this tissue. Comparable results were only found in the case of the isolate TgShSp24 (ToxoDB #2), which also presented an almost 20% cumulative mortality and similar parasite load values in the brain. The TgShSp16 and TgShSp24 isolates reached similar total parasite burden values at 30 dpi, ranging between 8 and 55 times higher than those of the rest of the isolates. Concretely, the brain was the organ that most contributed to these differences. Overall, histopathological analysis outlined the enhanced ability to disseminate of both TgShSp16 and TgShSp24 isolates, especially that of TgShSp24. Regarding type II isolates, our results are similar to those reported in reference [31], in which the isolate TgCkStk12 (ToxoDB #1) presented 0% mortality and negligible parasite burdens in mouse tissues, while the Moredun M4 isolate (ToxoDB #3) presented 20% mortality and intermediate parasite burden values in murine tissues evaluated. In another report [45], a group of 16 type II Danish isolates assessed showed how the strains that caused more severe loss in mice bodyweight also induced the highest serum haptoglobin and specific antibodies response in the acute phase of the infection but, likewise, significant differences were found between isolates. Although type II *T. gondii* strains confirmed their low virulence in mice in mortality assays, notable differences in infection dynamics were described between isolates recently obtained from ovine tissues.

Additionally, we used the AH-1 ovine trophoblast cell line, which is a target cell during transplacental *Toxoplasma* invasion in ovine gestation, to study the in vitro invasion rate and proliferation kinetics of selected isolates. This cell line was previously used to demonstrate the role of trophoblasts in the initiation and propagation of placental inflammation during ovine enzootic abortion (*Chlamydia abortus*) [35]. The present results showed significant differences between the clonal type III isolate (TgShSp24) and the other 5 type II isolates (clonal and PRU variant) included. TgShSp24 presented the highest invasion rate in AH-1 cells and reached a tachyzoite production (TY_{72h}) nine to three times higher than that of the rest of the isolates. However, between type II isolates, there were also important differences. The outstanding TgShSp1 isolate (ToxoDB #3) had the lowest invasion rate and tachyzoite production, closely followed by TgShSp2 (ToxoDB #1) and TgShSp16 (ToxoDB #3), which had slightly higher invasion rates than TgShSp1 but quite similar low TY_{72h} values. TgShSp3 and TgShSp11 were somewhere between regarding both parameters. Tachyzoite production resulted in clear concordance with microscopic monitoring of the infection, with the TgShSp24 isolate developing notably larger parasitophorous vacuoles and more frequent infection events than the rest of the isolates, and the same evident differences between type II isolates. Numerous previous studies have evaluated the in vitro proliferation kinetics of *Toxoplasma* strains in known infection-target tissues (e.g., central nervous system, muscular, or placental tissues, and immune system cells), normally with the goal of testing a drug treatment or the effect of the disruption of a potential virulence effector, or of studying cellular antiparasitic immune response mechanisms [22, 51–56], almost always involving laboratory strains belonging to type I or II (mostly RH and Me49, respectively). Only a few studies have tested in vitro differential phenotypic characteristics between nonlaboratory strains. A report [57] described similar experiments to those performed herein, evaluating invasion, multiplication and cyst formation rates in an HFF (human foreskin fibroblasts) cell line of a set of four type II strains isolated from human congenital infections. Hence, although there were no relevant differences in terms of multiplication rates, invasion and cyst formation rates varied between isolates included. Another research [58] found differences in terms of in vitro growth in a human acute monocytic leukaemia THP-1 cell line between *T. gondii* type II strains; furthermore, it provided evidence of significantly lower proliferation rates in type II strains than in those belonging to the type III genotype. The present study was pioneering in the use of ovine trophoblast cells for virulence evaluation of recently obtained *T. gondii* isolates from natural sheep infections.

Considering both in vivo and in vitro assays, most type II isolates (ToxoDB #1 and #3) possessed nonvirulent characteristics, except for the TgShSp16 isolate (#3), which showed a 21% cumulative mortality rate and an especially relevant enhanced ability to disseminate in vivo to organs such as the brain, despite low-intermediate in vitro invasion and proliferation rates in AH-1 cells. The type III TgShSp24 isolate presented the most virulent profile among the strains evaluated. This finding contradicts former classifications of *T. gondii* isolates that regarded type III as the least virulent in mice strains among the three major linages [4, 41]. Increasing evidence of this inconsistency can be found in recent literature [12, 25, 30]. In a recent study, a clonal type III isolate obtained from an Iberian domestic pig had nearly 90% mortality in Swiss mice in an identical virulence assay [12]. Similarly, in a Japanese study, 100% lethality in mice inoculated with doses of 10^2-cyst of a type III isolate obtained from a cat was reported [25]. The apparently broken linkage between virulence and genotype

demonstrates the limitations of RFLP-genotyping and the need to investigate new *T. gondii* strain virulence markers.

The *CS3* locus has been described as a highly predictive marker of *T. gondii* strains lethality in mice, with several studies in which Brazilian and Chinese isolates exhibiting high mortality rates (normally above 80%) also presented type I or II alleles for the *CS3* locus, while nonvirulent isolates (mainly 0% mortality) showed type III alleles [32, 59, 60]. Hence, our *CS3* typing results completely disagree with those above-mentioned investigations due to the presence of alleles II among the type II isolates assessed (0–21% mortality) and alleles III in the case of the type III isolate (18%). Contradictory results had been also reported in the literature [12, 61], suggesting the need for further research to unravel the definitive role of the locus in *Toxoplasma* virulence.

As an intracellular pathogen, during infection, *T. gondii* governs the cellular immune response through the mobilization of several virulence factors secreted by different specialized organelles. Concretely, a wide list of GRA, ROP and MIC effectors have been described. Quantitative trait locus (QTL) mapping analyses of the virulence of F1 progeny derived from sexual recombination experiments of representative strains of the three *T. gondii* archetypal genotypes resulted in the identification of ROP18 and ROP5 as key determinants of acute virulence in mice [62, 63]. Previous studies concluded that the allelic combination of *ROP18*/*ROP5* is highly predictive of virulence in mice across globally distributed *T. gondii* isolates [28, 30]. Here, we determined that all type II isolates included in virulence assays presented the *ROP18*/*ROP5* allelic combination of 2/2, regardless of the mortality rate reached. The allelic combination of 2/2 has been associated with 0% lethality in mice, with the exception of laboratory strains Me49 and ARI (40 and 60%, respectively), reflecting the influence of long-term laboratory conditions on parasite behaviour [28, 30]. In addition, the TgShSp24 isolate *ROP18*/*ROP5* allelic combination was 3/3, the most unspecific profile due to its association with levels of mortality strongly varying from 100 to 0% [12, 30, 31, 64]. While there appears to be a correlation between the *ROP18*/*ROP5* allelic combination observed in the isolates evaluated and their virulence degree to some extent, additional genetic factors might be also involved.

Recent investigations [19, 64, 65] also accomplished in vivo and in vitro virulence assays of nonlaboratory *Toxoplasma* strains, along with allelic profile characterization of *ROP18* and *ROP5*, among other relevant loci. The first investigation [64] tested the lethality in Swiss-Webster mice, as well as in vitro growth and plaque formation in Vero cells, of four recombinant strains obtained from different Serbian hosts. Similarly, Japanese researchers [65] carried out lethality assessments in CD1 mice, as well as in vitro invasion and cyst formation assays in HFF cells, of different partly genotyped Japanese strains. Finally, the cumulative mortality and morbidity rates in BALB/c mice, along with the growth rate and spontaneous cyst formation ability in HFF cells and primary mouse peritoneal macrophages, of the recently obtained TgCatJpObi1 isolate (genotype #4) was studied in [19]. Our results are not directly comparable with these studies due to the clonal genetic character of our selected strains and the different methodologies implemented; however, it could be said that the Japanese isolates TgCatJpObi1 (#4) [19] and TgCatJpOk3 (haplogroup 2) [65] are phenotypically similar to most of the Spanish type II isolates, with nonvirulent in vivo and in vitro phenotypes and presenting the same *ROP18*/*ROP5* allelic combination of 2/2.

In this study, we showed some new examples of inter- and intra-genotype phenotypic variation in in vivo and in vitro virulence features between recently obtained isolates. Thus, we were able to demonstrate that current widely used genetic characterization methods are not entirely appropriate to predict virulence of *T. gondii* field isolates, drawing attention to the need to implement genetic tools that may allow us to obtain much more detailed, precise, and complete genetic information (such as whole-genome sequencing methods) which in turn may serve to explain the biological variability found.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-021-00953-7.

Additional file 1*CS3*, *ROP5* and *ROP18* loci genotyping procedures. *CS3*, *ROP5* and *ROP18* loci genotyping was based on nested PCR of each locus. First, each locus was individually pre-amplified using the corresponding external primers and subsequently amplified by nested PCR using the internal primer pairs and the external PCR products as DNA template. For *ROP18*, one set of three external primers and two sets of internal primers were used. One set of internal primers aimed to amplify a repetitive sequence (DEL) in the promoters of the archetypal type I and II alleles, and the other to amplify the upstream promoter insertion sequence (UPS) exclusive to the archetypal type III allele. The nested PCR products were subjected to Sanger sequencing in both directions using the internal primers. Finally, in silico digestion of each locus sequences by specific restriction enzymes indicated was conducted by the NEBCutter V2.0 program [40]. No restriction enzyme digestion was required to distinguish alleles of the UPS sequence, as product is only generated for the type III allele. DNA samples of strains representative of the three archetypal lineages were used for comparisons, to note TgRH (type I, ToxoDB #10), TgMe49 (type II, #1), and TgNED (type III, #2).

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Authors' contributions
MF-E, RC-B, JR-C, EC-F, and LO-M conceived and designed the laboratory tests. MF-F, RC-B, RV, and JB performed experiments. MF-F, RC-B, RV, JR-C, and EC-F analysed the data. LO-M, EC-F, and JB contributed reagents/materials/analysis tools. MF-E, RC-B, JR-C, JB, EC-F, and LO-M drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Data supporting the conclusions of this study are included within the article and the Additional file 1. The *CS3* sequences generated in the present study were submitted to the GenBank database under the accession numbers MW727456-7. Histological samples are available from the authors upon reasonable request.

Declarations

Ethics approval and consent to participate
The animal study was reviewed and approved by Animal Welfare Committee of the Community of Madrid (PROEX 274/16).

Competing interests
The authors declare that they have no competing interests.

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Supplementary Information

Additional file 1: Table S1. *CS3*, *ROP5* and *ROP18* loci genotyping procedures.

Marker	External primers (5' - 3')	Tm (°C)	Internal primers (5' - 3')	Tm (°C)	NEB Restriction enzymes	Reference
CS3	CS3-Fext_GTGTATCTCCGAGGGGGTCT CS3-Rext_TGTGACTTCTTCTTCGCATCGAC	55	CS3-Fint_AGCGGATTTTCCAACACTGTC CS3-Rint_CTGCTGCATTCACAAACTCC	60	N1aIII + MboI	[32]
ROP5	ROP5-Fext_GGACAGACGCAGGCTTTTAC ROP5-Rext_TCAAACGTCCTGACACTTCG	55	ROP5-Fint_TGTGGCAGTTCAGTCTCAGC ROP5-Rint_TCGAAGTTGAGGAACCGTCT	55	BfaI	[28, 30]
ROP18	ROP18-DelFext_CTCGTCGACCACACACAGCTAA (*) ROP18-UPSFext_TTTTATCGACATCCCGCTTC ROP18-UPSRext_GAGTGCTTTCTGTCGCTCCT	56 55.2	ROP18-DelFint_AGTTCCCTTCCCTGGTGTCT ($) ROP18-DelRint2_CACCGCAAGACAGGCTGTCTTC ($) ROP18-UPSFint_CACAGCATGAGCTTAAGAGTTG (&) ROP18-UPSRint2_ACAAACTGGACTGGGGTGAG (&)	60 54.5	ScrFI + MfeI No enzyme treatment	[28, 30]

(*) ROP18-UPSRext is also used here as reverse primer; primer ROP18-DelRext does not exist.
($) Amplify the ROP18-Del fragment (Type III has no PCR products. Others have PCR products for RFLP analysis).
(&) Amplify the ROP18-UPS fragment (Type I and II have no PCR products. Type III has positive PCR products for RFLP analysis).

***CS3*, *ROP5* and *ROP18* loci genotyping procedures.** *CS3*, *ROP5* and *ROP18* loci genotyping was based on nested PCR of each locus. First, each locus was individually pre-amplified using the corresponding external primers and subsequently amplified by nested PCR using the internal primer pairs and the external PCR products as DNA template. For *ROP18*, one set of three external primers and two sets of internal primers were used. One set of internal primers aimed to amplify a repetitive sequence (DEL) in the promoters of the archetypal type I and II alleles, and the other to amplify the upstream promoter insertion sequence (UPS) exclusive to the archetypal type III allele. The nested PCR products were subjected to Sanger sequencing in both directions using the internal primers. Finally, in silico digestion of each locus sequences by specific restriction enzymes indicated was conducted by the NEBCutter V2.0 program [40]. No restriction enzyme digestion was required to distinguish alleles of the UPS sequence, as product is only generated for the type III allele. DNA samples of strains representative of the three archetypal lineages were used for comparisons, to note TgRH (type I, ToxoDB #10), TgMe49 (type II, #1), and TgNED (type III, #2).

Specific objective 2: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish Iberian pigs and its genetic and phenotypic characterization

> **Sub-objective 2.1**: Obtaining of *Toxoplasma gondii* isolates from Spanish Iberian pigs
> **Sub-objective 2.2**: Genetic characterization of *Toxoplasma gondii* isolates obtained from Iberian pigs
> **Sub-objective 2.3**: Phenotypic characterization of *Toxoplasma gondii* isolates obtained from Iberian pigs

The results that correspond to the three sub-objectives defined within the specific objective 2 have been published in a scientific article presented below:

RESUMEN / SUMMARY: El presente estudio tuvo como objetivo aislar y realizar la caracterización molecular y fenotípica de cepas de *Toxoplasma gondii* que infectan a cerdos ibéricos criados en condiciones de extensividad y destinados al consumo humano. Para ello se recogieron a nivel de matadero muestras de sangre y tejido cardíaco de 361 cerdos de engorde procedentes de 10 explotaciones distintas seleccionadas en las principales zonas de producción porcina ibérica. Los sueros se analizaron para detectar anticuerpos frente a *T. gondii* utilizando un kit comercial de ELISA indirecto, y se llevó a cabo un bioensayo en ratón con aquellos tejidos correspondientes a individuos seropositivos con los títulos de anticuerpos más altos representativos de ubicaciones geográficas diferentes. Setenta y nueve (21,9%) de los 361 animales presentaron anticuerpos frente a *T. gondii*, quince muestras de tejido miocárdico fueron inoculadas en ratón y finalmente se obtuvieron cinco aislados (TgPigSp1 a TgPigSp5).
Los aislados se caracterizaron mediante el método PCR-RFLP basado en 11 marcadores genéticos; tres aislados presentaban un genotipo ToxoDB #3 (3/5) y dos aislados un genotipo ToxoDB #2 (2/5). Los aislados TgPigSp1 y TgPigSp4 se seleccionaron para caracterizar su virulencia en ratón como ejemplo de cada genotipo RFLP encontrado. El aislado TgPigSp1 (genotipo #2) fue virulento en ratones, presentando unas tasas de mortalidad (87,5%) y morbilidad (100%) acumuladas notables. El aislado TgPigSp4 (#3) no fue virulento (0% de mortalidad) y desencadenó signos clínicos leves en el 42,1% de los ratones seropositivos. Se analizó el tropismo y las cargas parasitarias en diferentes órganos alcanzadas por ambos aislados; los datos revelaron diferencias significativas, incluida una carga parasitaria sustancialmente mayor en el pulmón durante la fase aguda de la infección en ratones infectados con el aislado TgPigSp1 frente a los infectados con el aislado TgPigSp4 (carga parasitaria media de 7,6 frente a 0 parásitos/mg de tejido, respectivamente; $p < 0,05$). Además, los grados de gravedad de las lesiones histopatológicas detectadas parecían estar relacionados con una mayor carga de parásitos. Teniendo en cuenta la tasa de mortalidad acumulada y la carga parasitaria inesperadamente altas asociadas con el genotipo clonal III, que tradicionalmente se considera no virulento en ratón, se enfatiza la necesidad de aumentar los esfuerzos en la caracterización de las cepas de *T. gondii* que circulan en cualquier huésped en Europa.

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Isolation, Genotyping, and Mouse Virulence Characterization of *Toxoplasma gondii* From Free Ranging Iberian Pigs

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The present study aimed to isolate and perform molecular and phenotypic characterization of *Toxoplasma gondii* strains infecting Iberian pigs bred under semi-free conditions and destined for human consumption. Blood and heart tissue samples from 361 fattening pigs from 10 various herds selected in the main areas of Iberian pig production were collected at a slaughterhouse; the sera were tested for anti-*T. gondii* antibodies using a commercial indirect ELISA kit, and a mouse bioassay was carried out using heart muscle of seropositive individual representatives from each geographical location. Seventy-nine (21.9%) of the 361 animals tested positive for anti-*T. gondii* antibodies according to the serology test. Fifteen samples of myocardial tissue were subjected to bioassay and 5 isolates (TgPigSp1 to TgPigSp5) were obtained. The isolates were characterized by using 11 PCR-RFLP genetic markers; three isolates had a ToxoDB #3 genotype (3/5) and two isolates had a ToxoDB #2 genotype (2/5). The TgPigSp1 and TgPigSp4 isolates were selected for virulence in mice characterization as instances of each different RFLP-genotype found. The TgPigSp1 isolate (#2 genotype) was virulent in mice with notable cumulative mortality (87.5%) and morbidity rates (100%); the TgPigSp4 (#3) was nonvirulent and triggered mild clinical signs in 42.1% of seropositive mice. Infection dynamics and organ distribution of both isolates were analyzed; the data revealed significant differences, including substantially higher parasite load in the lung during the acute phase of infection, in mice infected with TgPigSp1 than in the case of TgPigSp4 (median parasite load 7.6 vs. 0 zoites/mg, respectively; $p < 0.05$). Furthermore, degrees of severity of detected histopathological lesions appeared to be related to higher parasite burdens. Taking into account the unexpectedly high mortality rate and parasite load associated with the clonal genotype III, which is traditionally considered nonvirulent in mice, the need for further investigation and characterization of the *T. gondii* strains circulating in any host in Europe is emphasized.

Keywords: *Toxoplasma gondii*, Iberian pigs, isolates, genotypes, virulence

INTRODUCTION

The eukaryotic parasite *Toxoplasma gondii* (Apicomplexa) can infect virtually all warm-blooded animals and constitutes a specific risk for food safety in the European Union (1–3); additionally, it is considered the second causal agent of foodborne illness in the USA (4). Generally, human infections are mainly acquired after ingestion of raw or undercooked meat containing viable *T. gondii* tissue cysts (5). Infections are dramatically associated with reproductive failure in pregnant women, neurological signs in immunocompromised patients, and ocular disease in otherwise healthy humans. Thus, control of *T. gondii* presence in meat destined for consumption is of major interest (2, 6). After chicken, pork meat is the most frequently consumed protein source in western countries, and Spain is the top producer of pork within the EU (7).

Black Iberian pigs (*Sus scrofa*) constitute a traditional and well-adapted pig breed whose production is linked to highly valuable meat products, especially cured ham and sausages. These animals are usually reared in extensive systems in southwestern areas of the Iberian Peninsula (covering Portugal and Spain), within a favorable ecosystem, called Dehesa, composed mostly of acorn Mediterranean forest with a high natural biodiversity, which is ideal for swine breeding (8) in sympatry with ruminant livestock and a number of other wild animals, such as Cervidae and wild boar.

Natural infections in pigs are usually asymptomatic. In Iberian pigs raised in Spain, anti-*T. gondii* antibodies have been detected with a frequency from 9.5 to 58.2% (9, 10); extensive management systems and facilities with outdoor access are associated with higher seroprevalence (11, 12); thus, parasite isolation and characterization of the circulating strains are of major interest. Currently, only a few studies reported the genotypes and virulence degrees of *T. gondii* strains infecting domestic pigs in Europe; thus, the aim of the present study was to isolate and characterize *T. gondii* strains in domestic Iberian pigs bred in the semifree systems in the traditional raising area of Dehesa located in the southwestern part of the Iberian Peninsula.

MATERIALS AND METHODS

Ethical Statement

Animal procedures for the *T. gondii* isolation by bioassay in mice and evaluation of virulence degree (PROEX 274/16) were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU regulations (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize the suffering. As a humane endpoint, mice with a severe loss of body condition or nervous clinical signs were sacrificed to limit unnecessary suffering.

Mice

Seven-week-old female Swiss/CD1 mice were obtained from a commercial supplier (Janviers Labs, Le Genest-Saint-Isle, France). The animals were free from common viral, parasite, and bacterial pathogens according to the results of the routine screening analyses performed by the manufacturer. Mice were housed with *ad libitum* access to food and water in a controlled environment with 12-h light and 12-h dark cycles, and the experimental procedures were performed at 8 weeks of age.

Sample Collection and Serological Diagnosis for Tissue Selection

A total of 361 paired blood and myocardial tissue samples were collected from December, 2017 to June, 2018 from fattening Iberian pigs slaughtered for human consumption at an authorized slaughterhouse in Salamanca Province (western Spain) (**Table 1**). The blood samples were collected using BD PLUS serum tubes (Vacutainer; BD, Franklin Lakes, USA) at the bleeding step after animal stunning (Council Regulation (EC) No 1099/2009), and the apical part of the heart was sampled during the evisceration process; the samples were stored individually at 4°C until analysis. The serum was obtained after blood centrifugation and stored frozen (−20°C) until serological testing.

Toxoplasma gondii-specific IgG antibody levels in swine serum samples were measured using a commercial ELISA kit (PrioCHECK® Toxoplasma Ab SR, Prionics Schlieren-Zurich, Switzerland) (cut-off at 20 for percentage of positivity -ELISA PP-). Only myocardial tissues associated with the highest ELISA PP values and representative of the widespread locations (farms of origin) (**Table 1**) were selected for isolation by mouse bioassay ($n = 15$).

Bioassay in Mice and *in vitro* Cultivation

Portions of the heart muscle (50 g/each) from selected seropositive animals were subjected to acid-pepsin artificial digestion (13) prior to subcutaneous inoculation into 3 female Swiss/CD1 mice (Janvier-Labs, Le Genest-Saint-Isle, France). Mice were observed daily, and clinical signs were scored. Procedures of confirmation of mouse infection and *in vitro* isolation in cell culture were carried out as described in reference (14).

Genetic Characterization of *T. gondii*

Genomic DNA was extracted from the cell culture-derived *T. gondii* tachyzoites of all five isolates obtained using the Maxwell® 16 mouse tail DNA purification kit (Promega, Alcobendas, Spain). Genotyping was performed by the PCR-restriction fragment length polymorphisms (RFLP) method using 11 markers as described by earlier (15, 16). Clonal type reference strains of *T. gondii* were also included in the genotyping (TgRH, type I; TgMe49, type II; and TgNED type III). RFLP-genotype numbers were assigned according to the ToxoDB database (https://toxodb.org/toxo/).

Assays of Virulence in Mice

Two *in vivo* experiments were set for the evaluation of cumulative morbidity and mortality rates at 42 dpi (days post-inoculation) (assay A), and the tropism and burden reached by the isolates in mouse tissues during the acute and chronic stages of the infection (assay B).

TABLE 1 | Summarized data on geographical area of origin, number of samples collected, ELISA results and isolate obtaining from Iberian fattening pigs.

Location of origin (province)	Breeding area within Spain	No. of serum samples analyzed	% of positive serum samples	ELISA PP	Isolate ID
Navas de la Concepción (Sevilla)	South	50	14.0% (7/50)	133.3	TgPigSp1
				132.0	–
				62.1	–
Jerez de los Caballeros (Badajoz)	Southwestern	68	45.6% (31/68)	111.5	TgPigSp2
				112.0	–
				116.2	–
				110.1	–
				109.3	–
Fuente del Maestre (Badajoz)	Southwestern	34	67.6% (23/34)	111.9	TgPigSp3
				116.6	TgPigSp4
				113.8	TgPigSp5
Constantina (Sevilla)	South	20	15.0% (3/20)	55.7	–
				41.5	–
Badajoz (Badajoz)	Southwestern	14	57.1% (8/14)	–	–
San Nicolás del Puerto (Sevilla)	South	20	10.0% (2/20)	–	–
Arroyomolinos de Montánchez (Cáceres)	West	50	6.0% (3/50)	30.9	–
Monesterio (Badajoz)	Southwestern	40	0% (0/40)	–	–
Valdelosa (Salamanca)	West	14	14.3% (2/14)	30.3	–
Fuente Obejuna (Córdoba)	South	51	0% (0/51)	–	–
Total	–	361	21.9% (79/361)	–	–

Parasites and Inocula Preparation

The isolates TgPigSp1 and TgPigSp4 were selected as the instances of each RFLP-genotype detected in the study. Tachyzoites used for *in vivo* assays were harvested at low passages (p7 and p8, respectively) from cultures of Vero cells, when the majority of the parasites were still intracellular, and purified by filtration through a 3-μm polycarbonate filter (IpPORE®, IT4IP, Louvain-la-Neuve, Belgium) as described previously (17). The quantity and viability of tachyzoites were determined by Trypan blue exclusion followed by direct counting in a Neubauer chamber. Serial dilutions in PBS were performed to obtain the doses from 10^5 to 1 tachyzoite(s) of each isolate per 200 μl.

Assay A

Each dose was intraperitoneally (IP) inoculated into five 8-week-old female Swiss/CD1 mice. Five control mice were inoculated with 200 μl of PBS. Mice were monitored twice daily for 6 weeks and clinical signs were recorded. Cumulative morbidity rate was evaluated establishing a clinical sign scoring adapted from reference (18). Cumulative mortality rate was calculated based on the ratio of casualties to the total number of infected mice (17). Serum samples from mice, which were humanely euthanized, presented sudden death, or reached the end of the experiment at 6 weeks post-inoculation, were collected and stored at −20°C until anti-*T. gondii* antibodies detection by IFAT to confirm the infection. Alternative procedures to evidence animal infection were carried out as described in reference (14).

Assay B

An additional group of 10 mice per isolate was IP-inoculated with 10^3 tachyzoites; five animals were sacrificed at 7 dpi and the remaining five mice were sacrificed at 30 dpi to mimic the acute and chronic phases of the infection, respectively. Selected organs were collected during necropsies for *T. gondii* DNA detection and quantification. Briefly, mice were bled and the right cerebral hemisphere, the right eye, the right lung, half of the heart, a piece of a liver lobe, and the right kidney from each mouse were transferred immediately following euthanasia to clean 1.5 mL tubes and stored at −80°C until DNA extraction. Samples from the brain, lung, heart, liver, kidney, quadriceps femoris muscle, and tongue were fixed in 10% buffered formalin and processed for conventional histological examination. After staining with haematoxylin/eosin, lesions in the samples were subjectively categorized from 0 (no lesion) to 3 (the most severe grade within observed lesions). Serum samples were also collected and stored at −20°C until analysis.

Indirect Fluorescent Antibody Test (IFAT)

Detection of anti-*T. gondii* IgG antibodies in the sera was carried out by indirect fluorescent antibody test (IFAT) (19), using an anti-mouse IgG conjugated to FITC (Sigma-Aldrich) diluted 1:64 in Evans blue (Sigma-Aldrich); the cut-off was set at 1:25. Serum samples from previously known positive and negative animals were included as the controls. Tachyzoites of the Me49 strain were used as the coating antigen.

TABLE 2 | PCR-RFLP genotyping of *Toxoplasma gondii* isolates from Iberian pigs.

Isolate ID	Sample, host (location)	ELISA PP	Mice bioassay (no. infected/no. inoculated)	PCR-RFLP alleles[a]											RFLP-ToxoDB genotype #
				SAG1	3′-SAG2	5′-SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	C29-2	L358	PK1	Apico	CS3	
RH	CNS, human (EEUU)	–	–	I	I/III	I/II	I	I	I	I	I	I	I	I	I	–	10
Me49	Muscle, sheep (EEUU)	–	–	II/III	II	I/II	II	II	II	II	II	II	II	II	II	–	1
NED	Placental tissues, human (France)	–	–	II/III	I/III	III	III	III	III	III	III	III	III	III	III	–	2
TgPigSp1	Myocardium, pig (Sevilla, Spain)	133.3	(1/3)	II/III	I/III	III	III	III	III	III	III	III	III	III	III	III	2
TgPigSp2	Myocardium, pig (Badajoz, Spain)	111.5	(2/3)	II/III	I/III	III	III	III	III	III	III	III	III	III	III	III	2
TgPigSp3	Myocardium, pig (Badajoz, Spain)	111.9	(1/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	3
TgPigSp4	Myocardium, pig (Badajoz, Spain)	116.6	(3/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	I	II	3
TgPigSp5	Myocardium, pig (Badajoz, Spain)	113.8	(3/3)	II/III	II	I/II	II	II	II	II	II	II	II	II	II	II	3

[a] *I, II or III refers to the archetypal alleles from a Type I, II or III, for each molecular marker (15).*

DNA Extraction, Detection, and Quantification

For parasites quantification, genomic DNA was extracted from 50 to 80 mg of selected tissues using a Maxwell® 16 mouse tail DNA purification kit (Promega). DNA samples were adjusted to 20 ng/µl. Screening for the *T. gondii* DNA presence was carried out by single-tube nested PCR (nPCR) amplification of the specific *ITS-1* region as described previously (20). In samples with confirmed *T. gondii* presence according to nPCR, parasites were quantified by qPCR using primer pairs for the 529-bp repeat element of *T. gondii* (21). Reactions were performed in a final volume of 25 µl using GoTaq® qPCR master mix (Promega), 10 pmol of each primer and 100 ng of DNA in an Applied Biosystems 7500 FAST real-time PCR system (Applied Biosystems, Foster City, CA, USA). Amplification was performed according to a standard protocol (10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min). The number of *T. gondii* tachyzoites was calculated by interpolating the average Ct values on a standard curve equivalent to 10^{-1} to 10^{5} tachyzoites generated by 10-fold serial dilutions of parasite DNA in a solution of mouse genomic DNA at 20 ng/µl. Parasite load was expressed as parasite number/mg of mouse tissue. Standard curves for *T. gondii* had a consistent average slope close to −3.3 with a $R^2 > 0.98$.

In addition, due to the unexpected mortality rates observed, we conducted nested PCR for *CS3* locus amplification (16) over *Toxoplasma*-positive DNA samples from lung tissues of mice infected by TgPigSp1 ($n = 3$) and TgPigSp4 ($n = 4$) isolates (sacrificed at 7 or 30 dpi, respectively) for inocula genotype confirmation. PCR products were subjected to Sanger sequencing and obtained sequences were analyzed as described previously (14). *CS3* sequences alignment to previously obtained TgPigSp1 (MW132600), TgPigSp4 (MW132601) and other reference strains (TgRH, MW151245; TgMe49, MW151246; TgNED, MW151247) sequences was carried out using the Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical Analyses

Variations in parasite burden in the target tissues between the inoculated mice groups were analyzed by pairwise comparisons using the Mann-Whitney U test. Lethal outcome in mice was analyzed by the Kaplan–Meier survival method, and the Mantel-Cox log-rank test was used to compare the resulting survival curves. The significance for these analyses was established at $p < 0.05$. GraphPad Prism 6 v.6.01 (San Diego, CA, USA) software was used to perform all statistical analyses and graphical illustrations.

RESULTS

Serological Screening in Iberian Pigs and Parasite Isolation

Toxoplasma gondii-specific IgG antibodies were detected in 21.9% (79/361) of serum samples collected from pigs raised in 10 various locations (**Table 1**); 15 myocardial tissues of representative animals of various origins with the highest ELISA PP values were subjected to bioassay; subsequently, five isolates (TgPigSp1 to TgPigSp5) were obtained (**Table 2**). The bioassay

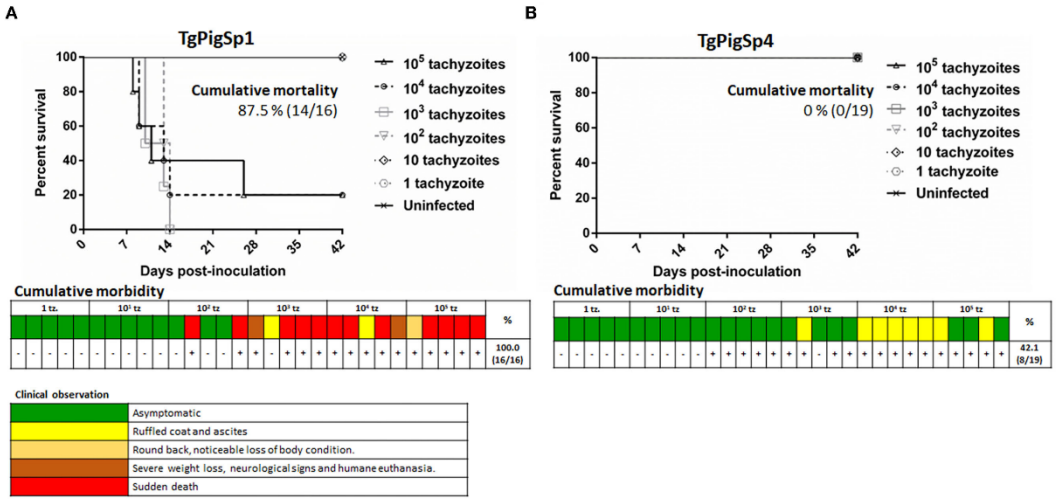


FIGURE 1 | Cumulative mortality and morbidity parameters in the evaluation of virulence degree in two *Toxoplasma gondii* isolates from Iberian pigs. Survival curves and gradation of clinical signs observed in mice infected/inoculated with TgPigSp1 **(A)** and TgPigSp4 **(B)** isolates are shown. Colored boxes represent the most severe clinical sign observed in each individual mouse challenged with each dose according to clinical signs scoring included down below. +: mice with a confirmed *Toxoplasma*-infection by IFAT or tissue imprints of brain or lung. -: mice without a confirmed *Toxoplasma*-infection. Cumulative mortality rates are indicated.

success rate was 33.3% (5/15). The isolation rate appeared to increase at higher ELISA PP values; parasites were isolated from 0 of 6 pigs with PP values < 110 and from 5 of 9 pigs with PP values > 110.

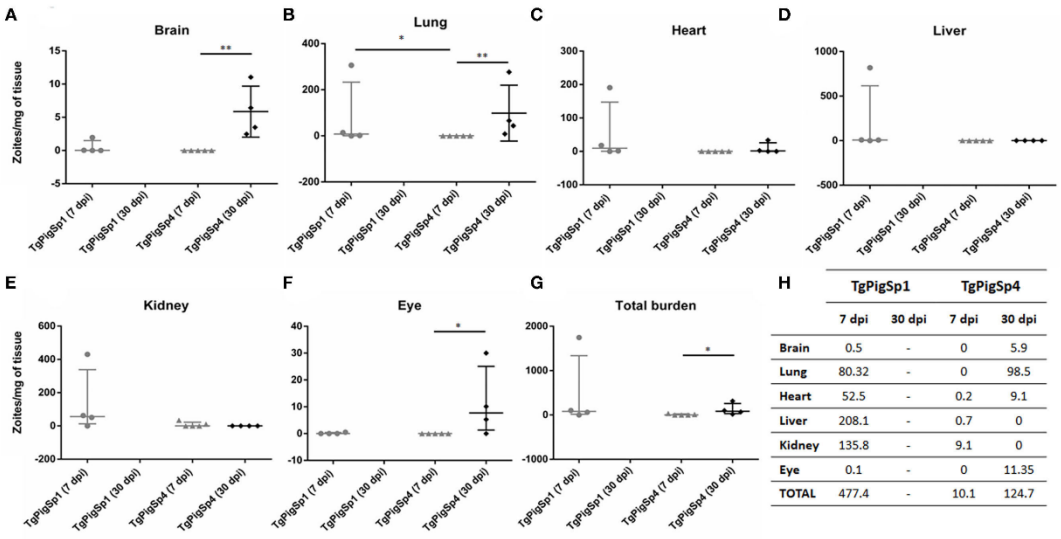
Genetic Characterization

Cell culture-derived tachyzoites from all five isolates were successfully characterized as having two different genotypes: ToxoDB #3 (3/5 isolates) and ToxoDB #2 (2/5). The *CS3* marker was previously reported to have a high predictive value on virulence in mice (16), and was present in type II alleles in all isolates with the ToxoDB #3 genotype; type III alleles were detected in all isolates with the ToxoDB #2 genotype (**Table 2**).

Pathogenicity Characterization

In assay A, the cumulative mortality rate was 87.5% for TgPigSp1 and 0% for the TgPigSp4 isolates, as shown in the survival curves (**Figure 1A**). Clear and statistically significant differences were observed between the survival curves of the two isolates at the doses starting from 10^2 tachyzoites/mouse ($p < 0.01$). Regarding morbidity rate, maximum clinical signs score reached by each animal is shown in **Figure 1**. In detail, the TgPigSp1 isolate caused clinical signs in 100% (16/16) of infected mice at a very acute phase of the infection at the doses starting from 10^2, rapidly inducing rounded back and noticeable loss of body conditions in the majority of mice (93.8%, 15/16); severe weight loss and development of neurological signs (with consequential humane euthanasia) (12.5%, 2/16) were observed with frequent cases of sudden death (75%, 12/16) (**Figure 1A**). All sudden deaths or humane euthanasia episodes occurred before 15 dpi, except an animal that died at 26 dpi. On the other hand, the TgPigSp4 isolate triggered clinical signs in 42.1% (8/19) of infected mice, specifically including ruffled coat and ascites in all cases during a very acute phase of the infection; however, the clinical signs receded completely at 13 dpi (**Figure 1B**).

Parasite burden reached by both isolates in the different organs studied at 7 and 30 dpi (assay B) is shown in **Figure 2**. All mice from the negative control group were seronegative and no *Toxoplasma*-DNA was detected by PCR in their tissue samples. *CS3* sequences amplified from *Toxoplasma*-positive DNA samples from lung tissues of infected mice were aligned to previously obtained sequences from TgPigSp1 (MW132600), TgPigSp4 (MW132601) and other reference strains (TgRH, MW151245; TgMe49, MW151246; TgNED, MW151247). 100% identity was found between *CS3* sequences got from TgPigSp1-infected tissues, that from the original isolate and TgNED sequence; likewise, all *CS3* sequences from TgPigSp4-infected tissues were identical to TgPigSp4 isolate original one and to TgMe49 sequence, confirming the correct genotype of each inoculum. Only 2 out of 5 mice infected by TgPigSp1 isolate and scheduled for sacrifice at 30 dpi survived until that point; however, they were not seroconverted and did not have parasite DNA in their tissues. This fact revealed a low success rate of infection achieved, probably due to variations inherent to proceedings (e.g., slight differences in inocula or animal individual susceptibility), and supported the high mortality rate exposed previously in the case of TgPigSp1, which made not possible to compare the parasite burdens reached by each isolate



H

	TgPigSp1		TgPigSp4	
	7 dpi	30 dpi	7 dpi	30 dpi
Brain	0.5	-	0	5.9
Lung	80.32	-	0	98.5
Heart	52.5	-	0.2	9.1
Liver	208.1	-	0.7	0
Kidney	135.8	-	9.1	0
Eye	0.1	-	0	11.35
TOTAL	477.4	-	10.1	124.7

FIGURE 2 | Parasite load (zoites/mg of tissue) found in six different murine organs **(A–F)** and total parasite burden per mouse **(G)** at 7- or 30-days post inoculation with 1,000 *Toxoplasma gondii* tachyzoites of TgPigSp1 and TgPigSp4 isolates. Median and interquartile values are represented. U-Mann-Whitney test; $^{*}p < 0.05$; $^{**}p < 0.01$. **(H)** Parasite burden (zoites/mg of tissue) mean values reached in each organ studied at 7 and 30 dpi by TgPigSp1 and TgPigSp4 isolates were tabulated.

at 30 dpi. At 7 dpi, all tested organs had higher average parasite burden in mice infected with TgPigSp1 than that in mice infected with TgPigSp4 isolate (**Figure 2H**); nevertheless, due to high variance of the data, differences were significant only between the parasite loads in the lung tissues, where TgPigSp1 infection had a median parasite load of 7.6 zoites/mg, whereas no *T. gondii* DNA was detected in mice infected with TgPigSp4 ($p < 0.05$) (**Figure 2B**). In the eye, at the acute stage, *T. gondii*-DNA was detected only in a mouse infected with TgPigSp1 (0.5 zoites/mg). Usually, *T. gondii* eye infection is not bilateral; detection by nPCR was performed only in the right eye of each animal thus limiting the frequency of detection. Statistically significant differences were detected in mice infected with TgPigSp4 at 7 vs. 30 dpi in terms of parasite burden in the brain (median 0 vs. 5 zoites/mg; $p < 0.01$), lung (median 0 vs. 54.7 zoites/mg; $p < 0.01$) and ocular tissues (median 0 vs. 7.7 zoites/mg; $p < 0.05$); no significant differences were detected in the case of heart, liver, and kidney tissues. Overall, total parasite burden reached in mice infected with TgPigSp4 was significantly increased from 7 to 30 dpi (median 0.7 vs. 83.3 zoites/mg; $p < 0.05$).

In all histologically evaluated organs, lesions were mainly observed in the brain, liver, and lung, where multifocal aggregates of mononuclear inflammatory cells were detected (**Figure 3**). No lesions were found in the kidney, quadriceps femoris muscle or tongue tissue. Similar to the parasite load assessment, due to the high mortality rate caused by TgPigSp1, it was not possible to compare the tissue lesion patterns triggered by each isolate at 30 dpi. Organs of three seropositive mice scheduled to be sacrificed at 30 dpi that experienced early sudden dead or were humanely culled, were included for histopathological evaluation to provide further evidence of TgPigSp1 infection course; however, the results were not useful for comparison. In the liver of mice infected with TgPigSp1 lesions were subjectively more severe at an early phase of infection (taking into account the tissue samples from mice sacrificed at 7 dpi and samples from mice who died early at 11–14 dpi (four mice in total) than of those infected with TgPigSp4 (only one animal had grade 3 lesions at 7 dpi). Moreover, the only mouse with heart lesions (i.e., multifocal nonpurulent myocarditis, **Figure 3**) was infected with TgPigSp1 and died at 14 dpi. On the other hand, the lesions in the brain were distinguished by glial foci and perivascular infiltration of inflammatory cells, and were detected in two mice infected with TgPigSp4; this phenomenon can be explained by development of chronic infection induced by TgPigSp4 isolate unlike acute infection caused by TgPigSp1. Regarding the lung, only 2 mice (one infected by each isolate) had histopathological lesions at 7 dpi; the lesions were more notable in the case of the TgPigSp1 infection. Furthermore, a TgPigSp1-infected animal with evidence of myocarditis (died at 14 dpi) also presented inflammatory lesions in the lung. Finally, 4 out of 5 mice inoculated with TgPigSp4 isolate had multifocal aggregates of mononuclear cells in the lung at 30 dpi.

DISCUSSION

The present study aimed to isolate, genotype, and evaluate the virulence degree in a normalized mouse model of the *T. gondii* strains infecting Iberian pigs. Seroprevalence values observed in

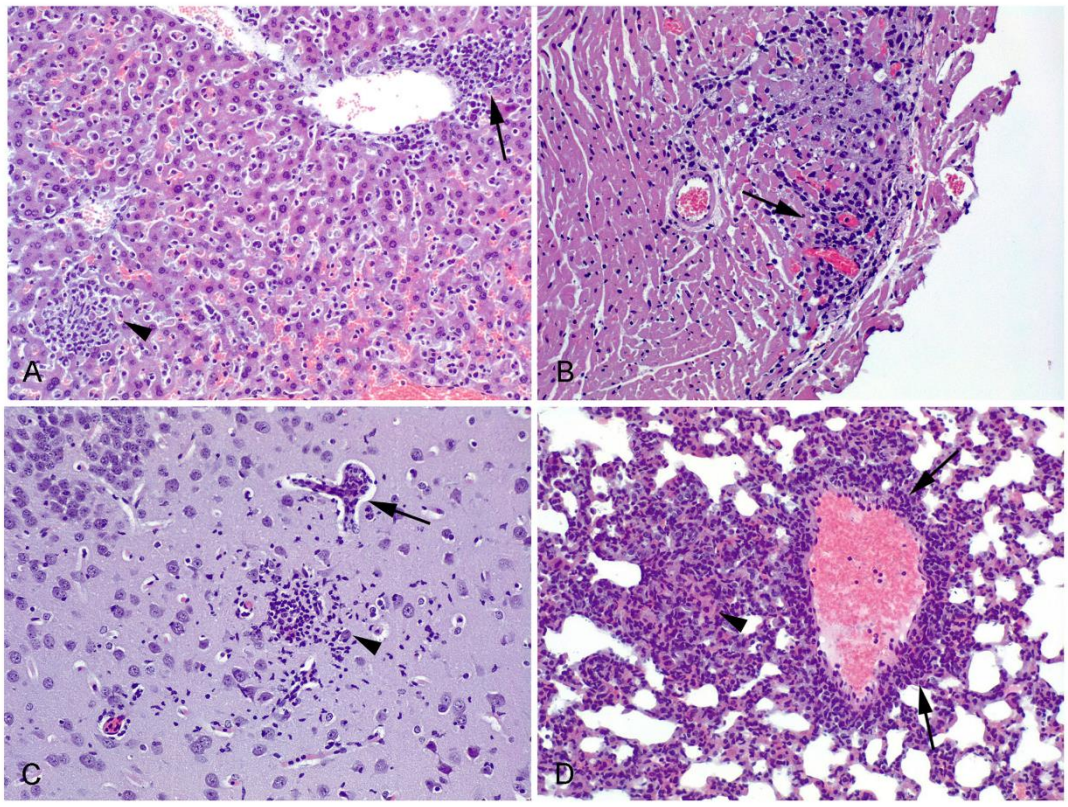


FIGURE 3 | Histological lesions observed in *Toxoplasma gondii* infected mice. HE. 20 x. **(A)** Liver. Mouse infected with TgPigSp4, 7 dpi. Aggregation of mononuclear inflammatory cells at the hepatic parenchyma (arrowhead) and in relation with a central vein (arrow). **(B)** Heart. Mouse infected with TgPigSp1, 14 dpi. Foci of non-purulent myocarditis (arrow). **(C)** Brain. Mouse infected with TgPigSp4, 30 dpi. There is a glial focus at the neuropile (arrowhead) close to a small vessel showing non-purulent vasculitis (arrow). **(D)** Lung. Mouse infected with TgPigSp4, 30 dpi. Perivascular infiltration of nonpurulent inflammatory cells (arrows). A focal thickening of the alveolar wall, caused by the infiltration of inflammatory cells, could also be observed (arrowhead).

the present study are in agreement with the previous data (9.5–58.2%) reported in the same host and breeding systems in Spain (9, 10, 22). Age and management systems are important factors related to an increase in the seroprevalence levels (11), which makes free-ranging fattening Iberian pigs a perfect model for *T. gondii* isolation and for evaluation of consumer risk when trends in organic pig production are concerned with natural breeding and welfare. The observed higher success of parasite isolation from the animals with higher ELISA titers should be considered in the future surveys.

Genetic characterization based on PCR-RFLP identified 2 genotypes, ToxoDB #3 (type II PRU-variant) and #2 (clonal type III), predominantly identified in the European domestic livestock (reviewed in (14)), wildlife (23) and humans (24–26). In Europe, the available data on genotyping of *T. gondii* in infected pigs is scarce; recently, an study (27) summarized the major facts, including the presence of all three clonal lineage alleles with an apparent predominance of type II; however, it should be noted that in most of studies, only one or a few molecular markers was used thus limiting the resolution of the results. A unique study using the same 11 molecular markers reported unexpected combinations of I, II and III alleles in 11 tissue samples from organic pigs in Italy (28). Notable frequency of type III alleles has been also described in Portuguese and Italian studies (29, 30); nonetheless, the studies were based only on *SAG2* PCR-RFLP and five microsatellite loci (*TUB2, TgM-A, W35, B17*, and *B18*) or on *B1*-PCR-HRM (high resolution melting) genotyping assays, respectively. Similarly, a recent Serbian study reported that 22.2% (2/9) of detected strains correspond to the genotype III according to PCR-RFLP of *GRA6, alt. SAG2, PK-1, BTUB, C22-8, CS3* and *Apico* markers (31); nevertheless, one of the isolates had a recombinant type II allele in the *C22-8* marker. A Polish survey detected high prevalence of type III alleles in *T. gondii* DNA extracted from retail raw pork meat products (32). Similar results

were obtained in the myocardial tissues of wild boars ranging in the southwestern locations in Spain (33) close to the sampling areas covered in the present study. Surprisingly, a high frequency of type I alleles was described in European literature (27, 34, 35) although type I were not detected in the present study except *Apico* marker identified within the Pruginaud (PRU) variant of the clonal genotype II. Remarkably, ToxoDB #3 genotype (type II PRU-variant) had not been described previously in studies using similar methodologies in infected European pigs (28, 31, 36). Different typing methodologies and sampling efforts used in the available references of the literature complicate the conclusions; however, it appears that overlapping strains from the three clonal genotypes are infecting European pig livestock, and recombination events within feline population are possible (28, 35, 36). This fact may be favored by the production systems involving pigs raised in semi-free ranging conditions that are exposed to potential sources of *T. gondii*, e.g., oocyst-contaminated environment or animal carcasses (37).

Currently, only two studies (29, 31) reported isolation of *T. gondii* from domestic swine in Europe; a third study (38) reported the seroconversion and DNA detection in the brain of mice inoculated with the homogenates of meat from 16 seropositive pigs reared in the indoor systems in the Aragon region of Spain. In wild swine, viable *T. gondii* were isolated from 21 wild boar heart tissue samples in France (23); all samples were identified as genotype II by PCR-RFLP (*SAG1, SAG2,* and *GRA7*) and microsatellite loci analysis (*TUB2, TgM-A, W35, B17, B18, and M33*). No additional data were reported thus increasing the interest in the present investigation along with the fact that no previous assays aimed to determine the virulence of European isolates from pigs.

The *CS3* locus has been described previously as a highly predictive marker of mortality in mice inoculated with *T. gondii* isolates (16). Our *CS3* typing results disagree with previous Brazilian and Chinese studies reporting high mortality rates associated with the type I or II alleles of the *CS3* gene and low or null rates associated with the type III alleles (16, 39, 40). However, opposite results have been reported in studies from Brazil (41, 42) and Spain (14). Clearly, additional investigations are required to determine the role of this locus and its interaction with related well-known virulence factors, such as ROP5 and ROP18 (43), in *Toxoplasma* virulence in mice and other hosts.

In this study, an apparent correlation between the genotype and mortality and morbidity rates in mice is described; nonetheless, further studies are needed. *Toxoplasma gondii* clonal lineages I, II and III have been traditionally classified according to their virulence in outbred laboratory mice into highly virulent (type I), intermediate virulent (type II) and nonvirulent (type III) (44–46). Nevertheless, current population structure of *T. gondii* is not limited to the three clonal lineages and is considerably more complex with at least 16 haplogroups known worldwide (47, 48); moreover, pre-established virulence classification is apparently also disputed. In the present study, an isolate was classified as clonal type III according to 11 RFLP-markers (TgPigSp1) and therefore was expected to be nonvirulent; however, the isolate had 87.5% cumulative mortality in the standardized mouse model, whereas a type II isolate (genotype #3, TgPigSp4) was completely nonvirulent while it has been described as moderately virulent previously. In the case of the TgPigSp1 isolate, already starting from the dose of 10^2 tachyzoites/mouse, the mortality rates were 80–100%. High mortality rate detected in the present study is in agreement with a previous report from Japan, where oral doses of 10^2-cyst of a type III cat isolate were found to cause 100% mortality in mice; nevertheless, no clinical signs of infection were seen when micro minipigs were infected in a similar manner (49). Although a relative correspondence between the virulence of *T. gondii* strains in mice and their virulence in humans is traditionally assumed (50), there are many studies that point out different behavior of the parasite in different hosts (49, 51, 52). Thus, the mouse virulence model should be interpreted as a relative and not an absolute characterization method; therefore, the mortality values described here will be valid for a mouse model but not for assessing the virulence degree of the TgPigSp1 and TgPigSp4 isolates in a swine model. Increasing evidence of a host-dependent virulence and a broken linkage with genotype, emphasize the need to investigate still unknown *T. gondii* strains virulence factors.

The parasite load is associated with the histological lesions detected in the tested mouse tissues, indicating a higher degree of virulence of the TgPigSp1 isolate compared to that of the TgPigSp4 isolate thus supporting the results of the cumulative mortality and morbidity rates. A high incidence of the lesions in the tissues of mice infected by TgPigSp4 at 30 dpi, especially in the brain and lung, corresponds to the development of a chronic infection similar to mice that were infected with low virulence type II isolates (53); however, high cumulative mortality of TgPigSp1 does not permit any additional comparison.

In addition to the cumulative morbidity and mortality rates, the virulence degree can be evaluated by nonlethal infection parameters; a pioneering study identified the intra-genotype variations in the weight gain and both anti-*T. gondii* IgG antibodies and haptoglobin levels in the serum of mice infected with a panel of type II isolates (54). Interestingly, variations in these parameters were observed in strains of the same genotype isolated from different hosts. Recent evaluation of the virulence degree in Caribbean, Brazilian and European *T. gondii* strains of six different genotypes showed a wide variability in the mortality rate and in parasite burden in the mouse tissues at 8 dpi (55). Additional studies aiming to combine genotypic and phenotypic characterization of the European isolates will be of major interest to determine the possible presence of hidden intragenotype variations within the clonal *T. gondii* strains, which were formerly classified as moderately virulent (type II) and nonvirulent (type III).

It should be noted that the isolates included in the present experiments on phenotypical characterization have been obtained recently (low number of cell culture passages), fact that enables to conserve their *in vivo* biological behavior avoiding the adaptation to the cell culture conditions. This feature is important since various factors, such as life stage of the parasite or the number of passages in mice or in cell culture, have been repeatedly demonstrated in the

literature to notably influence the evaluation of the virulence parameters (17, 49, 52).

The present study provides new information on the *T. gondii* strains circulating in swine in Europe and opens interesting avenues toward the epidemiological importance of trending organic farming and semi-free systems with regard to food safety, especially when domestic pigs are raised in sympatry with wildlife; in this sense, studies on the circulation of *T. gondii* from a One Health approach are of major interest. Considerable sampling and isolation efforts along with genotyping improvements were made to change the paradigm of the genetic structure of *T. gondii* population (56); similarly, implementation of virulence/phenotypical characterization of a large number of *T. gondii* strains by accurate models, including mortality and evaluation of nonlethal infection parameters, may let us to solve the raised controversy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare Committee of the Community of Madrid (PROEX 274/16).

AUTHOR CONTRIBUTIONS

MF-E, RC-B, EC-F, and LO-M conceived and designed the laboratory tests. MF-E, RC-B, JR-C, RV, and JB performed experiments. MF-E, RC-B, RV, JR-C, and EC-F analyzed the data. LO-M, EC-F, and JB contributed reagents, materials, analysis tools. MF-E, RC-B, JR-C, JB, EC-F, and LO-M drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER VI

GENERAL DISCUSSION

CAPÍTULO VI

DISCUSIÓN GENERAL

Toxoplasmosis, caused by the apicomplexan parasite *Toxoplasma gondii*, is a relevant public and animal health issue worldwide. *Toxoplasma gondii* is known as one of the major causes of reproductive failure in small ruminants, resulting in significant economic losses to the European livestock industry (Innes et al., 2009; Katzer et al., 2011). Apart from that, toxoplasmosis is considered an important food safety concern in the EU, being subject to surveillance and monitoring by the EFSA (EFSA and ECDC, 2021). As farm animals represent simultaneously a major source of infection for humans and relevant reservoirs of *T. gondii* for wildlife species, there is an interest in the characterization of *T. gondii* strains circulating in livestock.

An extensive investigation over the last decades, led to the initial description of a widely clonal *T. gondii* genetic population in North America and Europe, a concept that was also extended to Africa and Asia, in contrast to an extremely diverse South American population (Su et al., 2012; Lorenzi et al., 2016; Bertranpetit et al., 2017). However, Europe is one of the areas with more limited data, with a majority of the studies implementing questionable and non-consensus typing methodologies including a low number of molecular markers, and with serious sampling disparities between regions with vast areas that remain unexplored. In the particular case of Spain, there are only seven studies reporting data on *T. gondii* genotyping and involving 121 samples. Fuentes et al. (2001) typed human clinical samples collected from 33 immunocompromised patients and congenital infection cases, whereas Montoya et al. (2008) analysed 47 brains from cats of diverse origins. In both cases PCR-RFLP method based only on *SAG2* (both 5'- and 3'-ends) marker was implemented, therefore currently supposing inconclusive data. Apart from that, a few wildlife (wild boars, rodents, wild ungulates and foxes) studies are available, but despite the use of at least five PCR-RFLP markers, the amplification success was really low (Calero-Bernal et al., 2013, 2015; Fernández-Escobar et al., 2020). Finally, prior to this Doctoral Thesis, there were only two completely genetically characterized Spanish isolates; the first was obtained from an ovine abortion case (TgShSp1) and the second from a chronically infected yellow-legged gull (*Larus michahellis*) (TgA 21067), and both presented a type II profile by PCR-RFLP (11 markers) or MS (15 loci) typing, respectively (Sánchez-Sánchez et al., 2019b; Gamble et al., 2019). All above studies corroborated a predominance of type II strains but also showed a significant prevalence of other clonal and recombinant types; however, it is important to interpret with caution these quite limited results. In addition to the scarce information on circulating genotypes in Spain, the knowledge about the phenotypic characteristics of these strains is virtually nil. Until date, the only data available were those obtained from the TgShSp1 isolate, which demonstrated a low degree of virulence in an *in vivo* mouse model (0% mortality after IP infection with up to 10^5 tachyzoites) along with limited growth and enhanced tachyzoite-to-bradyzoite conversion *in vitro.* On the other hand, the TgShSp1 isolate was efficient in vertical transmition in pregnant mice, and compared to pregnant sheep, it triggered lower offspring mortality and morbidity rates after infection with sporulated oocysts (Sánchez-Sánchez et al., 2019b).

From this point, the main objective of the present Doctoral Thesis was to obtain a representative panel of isolates from Spanish livestock, concretely from sheep and Iberian pigs, that might allow us to explore the genetic and phenotypic diversity of circulating strains in these food animal species, implementing molecular methodologies, and *in vivo* and *in vitro* models. To achieve the initial sub-objectives (Sub-objective 1.1 and 2.1), isolation procedures were firstly carried out on ovine abortion-derived tissues occurred all over the Spanish territory and submitted for *T. gondii* diagnosis, as well as on myocardial tissues from chronically infected adult sheep slaughtered for human consumption. The important role of *T. gondii* as a cause of reproductive failure in sheep, the access to abortion-derived tissues thanks to our collaboration with the ULE, and the high

seroprevalences previously described in European and Spanish sheep flocks, perfectly justified these first two strategies. Sampling covered a notable proportion of the Spanish sheep farming area, since samples were collected in 15 different locations belonging to seven regions that together represented approximately three-quarters of the sheep population census (MAPA, 2019a). To begin with, a total of 11 toxoplasmosis-related ovine abortion outbreaks occurring during the 2015, 2016 and 2017 lambing seasons were confirmed and considered for parasite isolation. Moreover, serological screening revealed 62.3% of seropositive slaughtered adult sheep, in agreement with high seroprevalence figures found in previous surveys from southern Spain and other Mediterranean countries (García-Bocanegra et al., 2013; Almería et al., 2018; Stelzer et al., 2019).

The notable sampling effort for isolation made in this investigation is only comparable in Europe to two previous French studies (Dumètre et al., 2006; Halos et al., 2010), and makes it the first survey representative of *T. gondii* population genetic diversity circulating through Spanish sheep flocks. In addition, unlike in French studies in which only chronically infected adult sheep were tested, here we included abortion cases, increasing the extend and probable diversity reached. However, as mentioned previously, this is not the first report of obtaining isolates from sheep in Spain; Sánchez-Sánchez et al. (2019b) obtained the isolate TgShSp1 (ToxoDB #3) from an ovine abortion case occurred in Palencia province (North Spain). It should be noted that this isolate was included in present investigations for further in-depth genetic (and phenotypic) analysis, aiming to enrich the results. Furthermore, in the European context several previous studies achieved the isolation of *Toxoplasma* from sheep in France (Dumètre et al., 2006; Halos et al., 2010), Italy (Vismarra et al., 2017b), Serbia (Marković et al., 2014) and UK (Owen and Trees, 1999). Nonetheless, most of these isolation procedures were conducted to genotype the strains detected in bioassayed mice tissues; only Halos et al. (2010) reported the subsequent propagation in cell culture after bioassay in mice and the preservation of the isolates, calling into question the availability of the isolates included in the rest of studies for further investigations, such us phenotypic characterization. In present research, *T. gondii* isolation was successful from five ovine abortion outbreaks that occurred in different farms in Central, North and East Spain during subsequent lambing seasons, as well as from 20 chronically infected adult sheep raised in different location of Central and West Spain and slaughtered for human consumption. A total of 30 isolates (TgShSp2-31) were obtained that, along with the above-mentioned TgShSp1 isolate, represented a significant cross-section of the *T. gondii* Spanish population infecting sheep, covering a wide part of the country.

Complementarily, it was attempted to expand the panel of Spanish isolates by implementing isolation procedures on heart tissues from Iberian pigs raised under semi-free conditions and destined for human consumption. Sera of Iberian pigs from extensive-raised herds originating from different locations in the Southwest of the country were tested for the presence of anti-*Toxoplasma* antibodies, covering the three leading regions in Iberian pig production in Spain: Extremadura, Andalucía, and Castilla y León (MAPA, 2019b). An average seroprevalence of 21.9% (79/361) was found, in accordance with previous data (9.5–58.2%) obtained from the same host and breeding systems in Spain (García-Bocanegra et al., 2010b; Hernández et al., 2014; Pablos-Tanarro et al., 2018; Castillo-Cuenca et al., 2019). The Iberian pig breed is traditionally linked to the Dehesa ecosystem, where animals are raised in semi-freedom conditions sharing space and natural resources (acorn, grass, etc.) with other sympatric domestic and wild species up to the end of the fattening period (usually more than 14 months of age). Thus, since age and outdoor access are among the main risk factors for *Toxoplasma* infection (Pablos-Tanarro et al., 2018; Stelzer et al., 2019), free-ranging fattening Iberian pigs are an excellent model for *T. gondii*

isolation. Some previous studies carried out in Europe also achieved *Toxoplasma* isolation from domestic swine (Djokić et al., 2016; Kuruca et al., 2019; Paştiu et al., 2019), and even from Iberian pigs (De Sousa et al., 2006). Again, among the European porcine isolates only De Sousa et al. (2006) described the subsequent propagation of the isolates in cell culture and their preservation. In the present research, a total of five isolates (TgPigSp1-5) were obtained, which represent the first isolates available from such host in Spain.

In order to attain the sub-objectives 1.2 and 2.2 (Genetic characterization of *T. gondii* isolates), the implementation of PCR-RFLP method based on *SAG1*, *SAG2* (5'- 3' *SAG2*, and *alt. SAG2*), *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1* and *Apico* markers led to the classification of most isolates (86.1%; 31/36) as the genotype II PRU variant (ToxoDB #3), coexisting with much more infrequent clonal genotypes III (ToxoDB #2) (11.1%; 4/36) and II (ToxoDB #1) (2.8%; 1/36). These figures are in concordance with available European genotyping data on sheep, domestic pigs, and other livestock animals (Gutierrez et al., 2012; Verma et al., 2015; Kuruca et al., 2019). Within type II, the specific predominance of the PRU variant (type II alleles for all loci except *Apico*), should be highlighted. This variant has already been previously described in domestic and wild animals in Europe but is predictably underestimated due to the limited use of the *Apico* marker (Prestrud et al., 2008; Berger-Schoch et al., 2011; Herrmann et al., 2012; Verma et al., 2015; Sroka et al., 2020). Consistent with literature, no specific dominance of any RFLP genotype was related to abortion cases or chronic infections; probably, the high susceptibility of sheep to *T. gondii* infection makes the time point of the infection (during pregnancy or not) the only factor involved in the different outcome. Notable frequency of type III alleles has been also described in *T. gondii* strains infecting pigs from Italy (Papini et al., 2017; Vergara et al., 2018; Gazzonis et al., 2018), Portugal (De Sousa et al., 2006) or Serbia (Kuruca et al., 2019), sheep from Ireland (Gutiérrez et al., 2012); chicken from Portugal (Verma et al., 2015); or cattle from Switzerland (Berger-Schoch et al., 2011), among others. Similarly, a recent Serbian study reported that 22.2% (2/9) of detected strains infecting domestic pigs corresponded to the genotype III according to PCR-RFLP of *GRA6*, alt. *SAG2, PK-1, BTUB, C22-8, CS3* and *Apico* markers (Kuruca et al., 2019); nevertheless, one of the isolates had a recombinant type II allele in the *C22-8* marker. Similar results were also found in isolates obtained from Iberian pigs in Portugal, with 26.7% (4/15) of them presenting a type III genotype based on PCR-RFLP of the *SAG2* marker, confirmed by MS typing (De Sousa et al., 2006; Bertranpetit et al., 2017). These findings are quite valuable given the geographic proximity and the fact that they are from the same breed of pigs. Some authors claimed that type III alleles are more frequently detected in southern Europe compared to other parts of the continent (Kuruca et al., 2019), but the reality is that France, Italy and Portugal are the countries that have published the most *T. gondii* genotyping studies, with a low contribution from northern countries, what generates large areas without information.

As the use of a limited number of molecular markers, genotyping of isolates obtained by bioassay discarding the original clinical samples could underestimate the intraspecific diversity, promoting the selection of more proliferative strains or those present in higher loads in the original tissues at the expense of others. Given the high *Toxoplasma* seroprevalence found in European sheep flocks, along with the significant increase with age, multiple exposures to the parasite during the lifetime of the animal are suggested (Stelzer et al., 2019; Dubey et al., 2020d). Genetic characterization by PCR-RFLP of ovine original clinical samples in present investigation led to the description of co-infection events in an abortion outbreak occurred in 2017 in Palencia province (North Spain), with not only type II but also type I alleles at the *SAG3* marker detected in different foetal brain tissues. Mixed infections have been described previously in diverse

European genotyping studies focused on livestock (Aspinall et al., 2002; Berger-Schoch et al., 2011; Sroka et al., 2019) and wildlife species (Herrmann et al., 2012; Calero-Bernal et al., 2015). Aspinall et al. (2002) found RFLP mixed patterns corresponding with alleles I and II at the *SAG2* locus when analysing commercial lamb meat products in UK, whereas Berger-Schoch et al. (2011) detected different alleles at alt. *SAG2* and *SAG3* loci in sheep diaphragm samples collected at Swiss slaughterhouses. Here, type I alleles were also detected alone at the *SAG3* locus in a different foetal brain tissue collected in the above-mentioned outbreak in Palencia and in the myocardium of a slaughtered adult animal from Southwest Spain. These results may be indicative of additional mixed infection or the presence of recombinant strains but, in any case, calling attention to the extension of this type I alleles through livestock, as reported in other European studies (Aspinall et al., 2002; Turčeková et al., 2013; Verma et al., 2015; Battisti et al., 2018; Vergara et al., 2018). Most of the aforementioned articles only involved tissue samples direct genotyping, with consequent low amplification success. Nevertheless, Verma et al. (2015) and Moskwa et al. (2017) showed a complete clonal type I profile in two isolates obtained from an aborted bovine foetus in Portugal (firstly reported by Canada et al., 2002) and from an aborted foetus of European bison (*Bison bonasus bonasus L.*) in Poland, respectively. Finally, considering that the RFLP profile of the TgShSp4 and TgShSp5 isolates did not show the type I alleles at the *SAG3* locus found in the foetal brains that were their source of origin, the selection of strains during the bioassay was proved.

Within the large number of ovine samples collected, PCR-DNA sequencing-based genotyping considering three polymorphic genes, *SAG3*, *GRA6* and *GRA7*, revealed a low sequence diversity with only one relevant and widespread SNP (single nucleotide polymorphism) (G1691T) in the *SAG3* partial sequence analysed. Cited SNP splits the clonal type II (ToxoDB #1) and type II PRU variant (ToxoDB #3) ovine isolates and clinical samples into two well-defined groups, with 100% sequence homology with the Me49 reference strain or including the G1691T SNP, respectively. Such dichotomy was also present in ovine isolates from France whose sequences had been previously deposited in GenBank by Bertranpetit et al. (2017) in an attempt to describe the origin and historical expansion of the *T. gondii* population at a global level. This fact suggests common evolutionary forces over Spanish and French *T. gondii* populations or most likely common origins in livestock from both countries due to a historical and intense trade exchange of sheep between Spain and France (Bertranpetit et al., 2017). In addition, two sequences belonging to Ethiopian goat isolates also presented such dichotomy, and a further out-of-Europe extension of the mutation can be hypothesized.

In present studies, the genotypes ToxoDB #3, #2 and #1 have been described as the most prevalent circulating in two major Spanish livestock species (sheep and pig), whose individual prevalence is in line with European figures. In addition, it was demonstrated how the increase in the number of RFLP markers applied, a noteworthy sampling effort in number of samples, geographic coverage and types of samples, together with the use of PCR-sequencing methodologies, can lead to a significant increase in the definition of the intraspecific diversity of *T. gondii* population. The current bias towards certain more scientifically prolific countries, combined with the heterogenicity of methodologies implemented and different sampling efforts in the available references in the literature hamper extremely to draw general conclusions. However, if we combine our results with those available in the literature, it appears that overlapping strains from the three clonal genotypes are overall infecting European livestock, and recombination events within feline population are possible.

Then, and regarding the sub-objective 1.3 and 2.3 (Phenotypic characterization of *T. gondii* isolates), a group of 12 isolates from the recently obtained panel were selected to characterize their virulence degree. Isolates were selected on the basis of host (sheep or pig), PCR-RFLP genotype, geographic origin, and original clinical sample (abortion-derived or myocardial tissues), trying to maximize the diversity coverage. Thus, strains belonging to the three genotypes most prevalent in Spanish livestock species (ToxoDB #3, #2 or #1), originally from diverse and distant provinces, and obtained from ovine abortion cases or chronically infected adult sheep and fattened Iberian pigs, were included.

The virulence degree of *T. gondii* strains has been conventionally determined by cumulative mortality rate calculation in outbred laboratory mice as the standard method recently reviewed by Saraf et al. (2017). Although the mouse is not a host of clinical or economic interest, rodents are considered good oocyst-soil contamination indicators and relevant sources of infection for felids (Fernández-Escobar et al., 2020). Mouse models are the most frequently used due to the ease of handling, low cost, availability of specific reagents as well as outbred and inbreed lineages, among other factors. Traditionally, type I isolates have been classified as 100% lethal to mice regardless the dose, whereas types II and III have been considered intermediate (99-30% lethality) and non-virulent (<30% lethality), respectively and in a dose-dependent manner (Sibley and Boothroyd, 1992; Su et al., 2002). However, it is currently known that the population structure of *T. gondii* is much more complex, comprising at least 16 different haplogroups that likewise cluster a great diversity of strains (Lorenzi et al., 2016), and even the conventionally established virulence classification of clonal types is under debate. This simplistic criterion ignores important variations in relevant aspects of the development of the infection. There are some studies that, reflecting this superficiality of the mortality in mice calculation, combine the evaluation of other interesting non-lethal parameters in their *in vivo* experiments. Along with cumulative mortality calculation, in Hamilton et al. (2019) the virulence degree of some Caribbean, Brazilian and European isolates was evaluated by parasite burdens detected in brain, lungs and eyes, as well as by pathological lesion scoring in brain and lungs at 8 dpi, in a valuable comparative study. Evaluation of morbidity is quite infrequent, probably due to the inherent subjectivity. In Salman et al. (2021), in addition to assess cumulative mortality, parasite and cysts loads in the brain, and weight loss, they also implemented a detailed ethogram for clinical scoring during infection, revealing important differences between isolates evaluated. Interestingly, Bezerra et al. (2019) employed different behavioural tests for evaluation of learning and memory, locomotor activity and aversion to feline odour of mice after *Toxoplasma* infection, demonstrating important differences between Me49 (type II) and VEG (type III) reference strains. In the same line, Jungersen et al. (2002) assessed weight loss, anti-*T. gondii* IgG antibodies and haptoglobin levels in serum for describing variations between the outcome of the infection with different isolates classified as type II. Furthermore, it is widely known how long-term maintenance under laboratory conditions involves strong phenotypic changes in *Toxoplasma* strains, as occurs in many of the strains considered as reference, transferred successively between laboratories and maintained in cell culture or mice passages for an indeterminate time period. Drastic changes in virulence related to laboratory adaptation has been reported for type I, type II, and type III strains (Frenkel et al., 1976; Lindsay et al., 1991; Khan et al., 2009; Sánchez-Sánchez et al., 2019b; Colos-Arango et al., unpublished data).

In view of what has been exposed, and based on the method proposed by Saraf et al. (2017), we designed a comprehensive virulence assessment procedure in Swiss mice, including lethal (cumulative mortality) and non-lethal parameters (*i.e.*, cumulative morbidity, as well as tropism,

parasite burden and histopathological lesions in different tissues), and further implemented it on a panel of 12 recently obtained Spanish *T. gondii* isolates with a low number of cell culture passages, avoiding adaptation to *in vitro* laboratory conditions. Of the 12 isolates evaluated, nine had been classified as type II PRU variants according to 11 RFLP markers, while examples of clonal type II (TgShSp2) and clonal type III (TgShSp24, TgPigSp1) were also included. To begin with, all isolates were classified as non-virulent (cumulative mortality < 30%) according to the traditional criteria established by Su et al. (2002) except for the TgPigSp1 isolate (ToxoDB #2), with a surprising 87.5% cumulative mortality rate that defines it as a moderately/highly virulent isolate. Most of the type II isolates (clonal and PRU variants) showed cumulative mortality rates of 0 or close to 0, and low clinical scores with ruffled coat and ascites as main manifestations. There was an exception in TgShSp16 (ToxoDB #3) isolate, which stood out with a ratio of 21% lethality and presented several cases of severe weight loss and nervous signs in mice, consequently involving humane euthanasia, as well as spontaneous deaths. TgShSp16 isolate also produced the highest mean parasite burden in the brain at 30 dpi, which was between 10 and 100 times higher than that of the rest of the type II isolates, and it was the only case in which brain cysts were found during histopathological analysis of this tissue. Comparable results were found in the case of the type III (ToxoDB #2) TgShSp24 isolate, which presented an almost 20% cumulative mortality rate, several cases of sudden death in mice infected and similar mean parasite loads in the brain at 30 dpi. Both isolates reached similar total parasite burden values at the chronic stage of the infection, ranging between 6 and 55 times higher than those of the rest of the isolates. Concretely, the brain was the organ that most contributed to these differences, although TgShSp16 or TgShSp24 always stood out in all the organs evaluated. Finally, histopathological results supported parasite quantification findings outlining the enhanced ability to disseminate of both TgShSp16 and TgShSp24 isolates, especially that of TgShSp24. At the other end of the scale, in addition to its notable lethality, TgPigSp1 isolate exhibited 100% of cumulative morbidity, causing severe clinical signs in virtually all mice and resulting in sudden death in 75% of animals infected. None of the mice infected by TgPigSp1 isolate and scheduled for sacrifice at 30 dpi survived until that point, making impossible to assess parasite loads or histopathological lesions during the chronic phase of infection. Nevertheless, already in the early phase of infection, the isolate showed average parasite loads remarkably higher than those of the rest of the isolates (including TgShSp16 or TgShSp24) in all organs of early tropism (lung, liver and kidney) and even in cardiac tissue (data not shown), being probably the cause of the acute death of the animals. Moreover, although these were very low values, the TgPigSp1 isolate was the only one that was detected in the brain of infected mice (2/5) at 7 dpi. Parasite presence in the brain at such early time may seem surprising but in Hamilton et al. (2019), parasite DNA was detected at 8 dpi in some of the isolates evaluated.

Concerning type II isolates findings, Hamilton et al. (2019) in a similar experimental design obtained comparable results with a non-European ToxoDB #1 isolate (TgCkStk12) showing 0% mortality and negligible parasite burdens in mouse brain, lungs, and eyes, while the Moredun M4 isolate (Scotland, ovine origin, ToxoDB #3) presented 20% mortality and intermediate parasite burden values in mouse tissues evaluated. Important differences were also found between Danish type II isolates in Jungersen et al. (2002) in terms of mortality, weight loss and both haptoglobin and anti-*T. gondii* antibodies serum levels after mice infections; similarly, most of the isolates were obtained from slaughtered adult sheep or ovine abortion cases (isolates obtained from slaughtered pigs, foxes or cats were also evaluated). Although the majority of type II *T. gondii* strains confirmed their low virulence in mice in mortality assays (only SVS O15 isolate presented 67% intermediate lethality), notable differences in infection dynamics were described. In Bezerra

et al. (2019), apart from evaluating the behavioural changes triggered after mice infection with type II (Me49) and III (VEG) strains, mortality, weight loss, anti-*T. gondii* IgG production and parasite burden in brain were also assessed. As a result, mice group infected with VEG strain presented higher weight loss, anti-*T. gondii* IgG production and parasite load in brain in the later course of the chronic infection. Nonetheless, it should be noted that the mortality rates in Hamilton et al. (2019) and Jungersen et al. (2002) publications were based on a unique dose of tachyzoites IP inoculated, whereas in Bezerra et al. (2019) mice were orally inoculated with 10 cysts of Me49 or VEG strains. Apart from that, in the Danish study the classification of isolates was carried out by serotyping, a much less precise method noncomparable to common genotyping techniques (Jensen et al., 1998; Jungersen et al., 2002). In addition to the lack of consensus on the genotyping techniques, it is necessary to draw attention to the enormous heterogeneity in mortality rate in mice assessments. Different routes of inoculation, mouse strains, dosages and stages of the parasite used make comparisons and drawing solid conclusions impossible; virtually no studies implemented the standardized method proposed by Saraf et al. (2017), as done here and by Uzelac et al. (2020).

In the present research, TgShSp24 isolate (ToxoDB #2) produced a significantly higher parasite burden in mouse brains at 30 dpi than all the rest of isolates (type II) except for TgShSp16. In addition, the high parasite load detected at 7 dpi in the case of mice infected with the TgPigSp1 isolate (also belonging to ToxoDB #2 genotype) suggests that its capacity to replicate in mouse tissues is even much higher than that of the isolate TgShSp24. Despite being type III isolates, conventionally considered non-virulent, and taking into account the data on mortality, morbidity, parasite burden and tissue lesions, the TgShSp24 and TgPigSp1 isolates demonstrated a virulence degree in mice that was generally higher than that of the type II isolates. This fact is especially notable in the case of the TgPigSp1 isolate, when already starting from the dose of 10^2 tachyzoites/mouse, the mortality rates were 80–100%. Such high mortality rate agrees with a previous report from Japan (Taniguchi et al., 2018), where oral doses of 10^2-cyst of a type III cat isolate were found to cause 100% mortality in mice; nevertheless, no clinical signs of infection were seen when micro minipigs were challenged in a similar manner.

In vitro models represent an excellent alternative to *in vivo* experiments for the study of intracellular organisms such as *T. gondii* making possible to investigate some aspects of the lytic cycle of the parasite and reducing the use of laboratory animals (Regidor-Cerrillo et al., 2011; Müller and Hemphill, 2013; Dellarupe et al., 2014; Frey et al., 2016; Jiménez-Pelayo et al., 2017; García-Sánchez et al., 2019). Within the subobjective 1.3. and as a valuable complement for the *in vivo* virulence degree evaluation, an ovine trophoblast cell line (AH-1) was chosen for characterization of *in vitro* invasion rates and proliferation kinetics of a selection of six isolates of ovine origin included in previous *in vivo* experiments. AH-1 cells originated from immortalized primary cultures of ovine placenta (Haldorson et al., 2006). Therefore, they represent a perfect model for proposed characterization assays since they share the ovine origin of the isolates and trophoblasts are target cells during congenital infection, with a proven immunomodulatory role (Wheelhouse et al., 2009). This cell line was previously used to demonstrate the role of trophoblasts in the initiation and propagation of placental inflammation during ovine enzootic abortion (*Chlamydia abortus*) (Wheelhouse et al., 2009). Invasion and proliferation kinetics assays showed significant differences between the clonal type III isolate (TgShSp24) and the other five type II isolates (clonal and PRU variant) included. TgShSp24 presented the highest invasion rate and reached a tachyzoite production at 72 hpi (TY_{72h}) nine to three times higher than that of the rest of the isolates. Nevertheless, important variations were also found between type II

isolates. The TgShSp1 isolate (ToxoDB #3) had the lowest rates in both parameters measured, closely followed by TgShSp2 (ToxoDB #1) and TgShSp16 (ToxoDB #3), whereas TgShSp3 and TgShSp11 were in an intermediate situation. Quantification of tachyzoite produced during the time-course matched perfectly with microscopic monitoring of the infection. TgShSp24 isolate developed notably larger parasitophorous vacuoles and more frequent infection events than the rest of the isolates and the same differences observed in terms of proliferation between type II isolates were evident also by microscopy.

There is abundant literature regarding the *in vitro* behaviour of *T. gondii* strains, but most of them have the purpose of evaluating the antiparasitic efficacy of some drugs or to demonstrate the role of some host or parasite effectors during some phase of the lytic cycle (Contreras-Ochoa et al., 2012; Witola et al., 2014; Barbosa et al., 2015; Da Silva et al., 2017). In such investigations, laboratory-strains of reference (mostly RH and Me49) are usually employed to minimize variables. Thus, there are few examples of *in vitro* phenotypic characterization of recently obtained *T. gondii* isolates. An example could be found in the research carried out by Brenier-Pinchart et al. (2010); authors evaluated the invasion, multiplication, and cyst formation rates in HFF cells of a set of four type II strains isolated from human congenital infections. Hence, although there were no relevant differences in terms of multiplication rates, invasion and cyst formation rates varied between isolates included. Previous investigations in our group studied the growth rate in Vero cells as well as the plaque formation and tachyzoite-bradyzoite conversion capacities in HFF cells of the recently obtained TgShSp1 isolate (ToxoDB #3) and the reference strain Me49 (ToxoDB #1) (Sánchez-Sánchez et al., 2019b). Findings indicated an enhanced virulence for the laboratory-adapted Me49 strain compared to the TgShSp1 isolate, with a history of a quite low number of cell-culture passages. Concretely, TgShSp1 proliferated at a slower rate and had delayed lysis plaque formation compared to Me49 strain, but it formed more cyst-like structures *in vitro* (Sánchez-Sánchez et al., 2019b). If we compare with our results, the TgShSp1 isolate seems to present a tachyzoite production at 48 hpi quite similar in both cell types (around 50 parasites / ng DNA; data not shown), but it must be taken into account that the initial MOI was higher in HFF cells experiments and therefore data are not directly comparable. In an interesting research, Meneceur et al. (2008) described differences in *in vitro* growth rates in a human acute monocytic leukaemia THP-1 cell line between a large panel of *T. gondii* isolates obtained from human patients with congenital or acquired toxoplasmosis including type I, II, III, recombinants and atypical strains. They provided evidence of significantly lower proliferation rates in type II than in type I or III strains. Again, the differences in experimental designs as well as in the type of cells used must be considered. The present study was pioneering in the use of ovine trophoblast cells for virulence evaluation of recently obtained *T. gondii* isolates from natural sheep infections.

In consideration of both *in vivo* and *in vitro* models findings, most type II isolates (ToxoDB #1 and #3) possessed non-virulent characteristics, except for the TgShSp16 isolate (#3), which showed a 21% cumulative mortality rate and an especially relevant enhanced ability to disseminate *in vivo* to organs such as the brain, despite low-intermediate *in vitro* invasion and proliferation rates. The type III (#2) isolates presented the most virulent profile among the strains evaluated. On the one hand, the TgShSp24 isolate achieved *in vivo* characteristics quite similar to TgShSp16, also combined with a marked *in vitro* replication capacity in ovine trophoblast cultures, much higher than that of the rest of ovine isolates; on the other hand, the high lethality of the isolate TgPigSp1 is undeniable. These findings directly contradict former classifications that considered type III strains as the least virulent of the three *T. gondii* clonal types (Sibley and Boothroyd, 1992; Su et al., 2002). These results also revealed the apparently broken linkage

between virulence and genotype as well as the enormous intragenotype phenotypic variability that exists in the *T. gondii* Spanish population. Limitations of RFLP-genotyping were demonstrated, drawing attention to the need to implement genetic tools that may allow us to obtain much more detailed, precise, and complete genetic information (such as whole-genome sequencing methods) which in turn may explain the biological variability found.

The *CS3* marker was used by Khan et al. (2005) along with a wide set of molecular markers for developing a high-resolution genetic linkage map for *T. gondii*, leading to define the chromosomic structure of its genome as well as other relevant genome features. Later, the *CS3* has been described as a marker highly predictive of *T. gondii* strains lethality in mice, with several studies in which Brazilian and Chinese isolates exhibiting high mortality rates (normally above 80%) also presented type I or II alleles for the *CS3* locus, while non-virulent isolates (0–9.3% mortality) showed type III alleles (Pena et al., 2008; Yai et al., 2009; Wang et al., 2013; Silva et al., 2014; Rocha et al., 2018). Therefore, our *CS3* typing results completely disagree with those above-mentioned investigations due to the presence of alleles II among the type II isolates assessed (0–20.8% lethality) and alleles III in the case of type III isolates (18.2–87.5%). Nevertheless, contradictory results had been also reported in the literature with non-virulent Brazilian isolates presenting type I (Langoni et al., 2012) or type II (Rêgo et al., 2017) alleles at the *CS3* locus. Hence, the fact that all strains included in the abovementioned studies are polymorphic, none of them with a European or North American origin (considered "clonal" regions), suggests the need for further investigations to unravel the definitive role of the locus in *Toxoplasma* virulence and clarify possible differences among biogeographical regions.

As an intracellular pathogen, for the establishment of the infection and for tackling the host immune response, *T. gondii* carries out the sequential secretion of multiple effectors from highly specialized secretory organelles, including the well-known micronemes, rhoptries and dense granules. A wide list of MICs, ROPs and GRAs effectors have been described but, quantitative trait locus (QTL) mapping analyses of the virulence of F1 progeny derived from sexual recombination experiments with representative strains of the three *T. gondii* archetypal genotypes resulted in the identification of *ROP18* and *ROP5* as key virulence factors in acute virulence in mice (Saeij et al., 2006; Behnke et al., 2012). *ROP18* is an extremely polymorphic rhoptry kinase, that is highly expressed in types I and II strains but an insertion in the promoter of the gene prevents its expression in type III strains (Niedelman et al., 2012). *ROP18* is known to prevent the immunity-related GTPases (IRGs) from disrupting the PV, among other functions related to counteracting the host immune response. On the other hand, the *ROP5* locus, which consists of a family of 4-10 tandem duplicates of highly polymorphic genes encoding for rhoptry pseudokinases, is another demonstrated important virulence factor in mice. It is currently known that *ROP18* requires of *ROP5* to prevent IRGs accumulation in the PV membrane and that these two secreted proteins determine the majority of *T. gondii* strain differences in IRGs evasion (Behnke et al., 2012; Niedelman et al., 2012). The sequences of these two genes have been studied in detail for the three archetypal strains, so that it has been established the existence of a virulent allele (that determines a high virulence degree in mice in the isolates that present it) and a non-virulent allele (that determine a low virulence degree) for each locus. It is known that type I strains possess virulent alleles for both loci, while type II strains present the virulent version of *ROP18* but the non-virulent form of *ROP5*. Finally, type III strains exhibit the non-virulent allele of *ROP18* but the virulent form of *ROP5*. In-depth research on these sequences has led to conclude that the allelic combination of *ROP18*/*ROP5* could be used to predict the virulence degree in mice of globally distributed *T. gondii* isolates (Dubey et al., 2014; Shwab et al., 2016). Here, we

determined that all type II isolates (ToxoDB #1 and #3) included in virulence assays presented the *ROP18*/*ROP5* allelic combination of 2/2, regardless of the mortality rate reached (TgPigSp4, unpublished data). The allelic combination of 2/2 has been associated with 0% lethality in mice, with the exception of laboratory strains Me49 and ARI strains (40 and 60%, respectively), reflecting the influence of long-term laboratory conditions on parasite behaviour (Dubey et al., 2014; Shwab et al., 2016). On the other hand, type III isolates TgShSp24 and TgPigSp1 exhibited a *ROP18*/*ROP5* allelic combination 3/3, the most unspecific profile due to its association with levels of mortality strongly varying from 100 to 0% (Shwab et al., 2016; Hamilton et al., 2019; Uzelac et al., 2020) (TgPigSp1, unpublished data). While there appears to be a correlation between the *ROP18*/*ROP5* allelic combination observed in the isolates evaluated and their virulence degree to some extent, additional genetic factors might also be involved.

In the present Doctoral Thesis, efforts have been made to genetically and phenotypically characterize a wide panel of Spanish *T. gondii* isolates recently obtained, by means of molecular techniques and *in vivo* and *in vitro* models. Recent investigations also accomplished *in vivo* and *in vitro* virulence assays of non-laboratory *Toxoplasma* strains, along with allelic profile characterization of *ROP18* and *ROP5* among other relevant loci (Fukumoto et al., 2020; Uzelac et al., 2020; Salman et al., 2021). Fukumoto et al. (2020) carried out lethality evaluations in CD-1 mice, as well as *in vitro* invasion and cyst formation assays in HFF cells, of different partly genotyped Japanese strains. Another Japanese group studied the mortality and morbidity rates in BALB/c mice, along with the growth rate and spontaneous cyst formation ability in HFF cells and primary mouse peritoneal macrophages, of the recently obtained TgCatJpObi1 isolate (Salman et al., 2021). Finally, Uzelac et al. (2020) assessed the mortality in Swiss-Webster mice, as well as *in vitro* growth and plaque formation in Vero cells, of four recombinant strains originated from different host in Serbia. Findings achieved here through our *in vitro* and *in vivo* models are not directly comparable with cited studies due to the clonal genetic character of our selected strains and once more the different methodologies implemented; however, it could be said that the Japanese isolates TgCatJpObi1 (ToxoDB #4) (Salman et al., 2021) and TgCatJpOk3 (haplogroup 2) (Fukumoto et al., 2020) are phenotypically similar to most of the Spanish type II isolates, with non-virulent *in vivo* and *in vitro* features and presenting the same *ROP18*/*ROP5* allelic combination of 2/2. In our studies, we showed some new examples of the inter- and intragenotype phenotypic variation in *in vivo* and *in vitro* virulence features between recently obtained isolates. Thus, we were able to demonstrate that current widely used genetic characterization methods are not sufficient to sort *Toxoplasma* population virulence.

In summary, in the different investigations that cluster this Doctoral Thesis, it has been possible to obtain a representative panel of *T. gondii* circulating isolates from sheep and Iberian pigs reared in Spain, which has served to describe at some extent the genetic diversity that exists in the parasite population as well as to study the variability in its pathogenesis, using *in vivo* and *in vitro* models. It is well known how parasite genetic diversity influences its epidemiology and pathogenicity, with consequent implications in therapeutic and vaccination strategies as well as disease control. The fact of having a representative panel of isolates in-depth characterized may constitute the basis for further investigations focused on virulence factors description, evaluation of treatments and vaccines efficacy, validation of diagnostic techniques, or even the development of new molecular genotyping methods based on next-generation sequencing technologies.

CHAPTER VII

CONCLUSIONS

CAPÍTULO VII

CONCLUSIONES

<u>Specific objective 1</u>: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish sheep livestock and its genetic and phenotypic characterization

First. A representative panel of 30 *T. gondii* isolates has been obtained from abortion cases and chronic infections in adult animals coming from selected sheep farming areas in Spain. Their cryopreservation with a low number of passages in cell culture guarantees reliable further characterization studies in which their original characteristics are preserved.

Second. The success of *T. gondii* isolation from brains of aborted foetuses and hearts from adult animals evidences the relevance of sheep derived tissues as a source of infection for scavengers in general and felids in particular, and the risk to public health posed by the consumption of undercooked ovine meat, respectively.

Third. The genetic characterization of the *T. gondii* isolates panel by the well-established PCR-RFLP methodology reveals a low genetic diversity, with a strong predominance of the genotype II PRU variant (ToxoDB #3) coexisting with much less frequent clonal genotypes III (ToxoDB #2) and II (ToxoDB #1).

Fourth. Comparison between PCR-RFLP results obtained from isolates and from DNA-positive clinical samples reveals a more complex genetic diversity in the later, suggesting the existence of a selective bottle-neck during the isolation procedure.

Fifth. PCR-sequencing of a fragment of the *SAG3* polymorphic gene lead to the detection of a widespread single-nucleotide polymorphism across the Spanish sheep population, thus increasing the resolution of genetic diversity characterization.

Sixth. A comprehensive and normalized procedure for virulence in mice assessment, including lethal (cumulative mortality) and non-lethal parameters (such as cumulative morbidity, or tropism, parasite burden and histopathological lesions in different tissues) is proposed. Characterization of several *in vitro* virulence traits (invasion rates and proliferation kinetics) using host target cells are suggested in order to achieve a more in-depth virulence evaluation.

Seventh. The phenotypic characterization of selected isolates by *in vivo* and *in vitro* approaches shows that, most type II isolates possess non-virulent traits, although there are significant intra-genotype differences. The only type III isolate included presents the most virulent profile among the isolates evaluated, which contradicts former classifications of type III *T. gondii* isolates as the least virulent strains among the three major linages.

Eighth. The *CS3*/*ROP18*/*ROP5* allelic combination of selected isolates shows no relation with their virulence and even disagrees with pre-established associations, providing evidence that other genetic factors might be involved in the virulence of *T. gondii* in mice.

Specific objective 2: Obtaining of a representative panel of *Toxoplasma gondii* isolates from Spanish Iberian pigs and its genetic and phenotypic characterization

Nineth. A group of five isolates was obtained from Iberian pigs raised in different locations of southwestern Spain. This group represents the first isolates available from such host in our country. Free-ranging fattening Iberian pigs constitute an appropriate model for *T. gondii* isolation, highlighting the risk to public health posed when such highly valued meat is consumed undercooked.

Tenth. The genetic characterization of the isolates obtained from Iberian pigs confirms the predominance of the genotype II PRU variant (ToxoDB #3) in Spanish livestock, although the clonal genotype III (ToxoDB #2) is also detected, in agreement with available data from domestic pigs and other domestic animals in Europe.

Eleventh. The evaluation of the virulence in mice of isolates obtained from Iberian pigs shows a type II PRU variant isolate with non-virulent characteristics, contrasting with a virulent type III isolate, that exhibits remarkably high cumulative mortality and morbidity rates in mice. These findings support the observations found with isolates obtained from sheep, refuting traditional virulence degree classifications of *T. gondii* strains and highlighting the intragenotype phenotypic variability also among type III strains, which is not explained by their *CS3*/*ROP18*/*ROP5* allelic combination.

<u>Objetivo específico 1</u>: Obtención de un panel representativo de aislados de *Toxoplasma gondii* de ganado ovino español y su caracterización genética y fenotípica

Primera. Se ha obtenido un panel representativo de 30 aislados de *T. gondii* a partir de casos de aborto e infecciones crónicas en animales adultos procedentes de zonas seleccionadas de producción ovina en España. Su criopreservación tras un número reducido de pases en cultivo celular garantiza la conservación de sus características originales para su utilización en futuros estudios de caracterización.

Segunda. El éxito del aislamiento de *T. gondii* a partir de cerebros de fetos abortados y de corazones de animales adultos evidencia la relevancia de los tejidos ovinos como fuente de infección para carroñeros en general y felinos en particular, junto con el riesgo para la salud pública que representa el consumo de carne de ovino poco cocinada.

Tercera. La caracterización genética del panel de aislados de *T. gondii* mediante la consolidada metodología de PCR-RFLP revela una baja diversidad genética, con un fuerte predominio de la variante PRU del genotipo II (ToxoDB #3) coexistiendo con genotipos clonales III (ToxoDB #2) y II (ToxoDB #1) mucho menos frecuentes.

Cuarta. La comparación entre los resultados de PCR-RFLP obtenidos de los aislados y de las muestras clínicas positivas a la detección de ADN del parásito revela una diversidad genética más compleja en estas últimas, lo que sugiere la existencia de un cuello de botella selectivo durante el procedimiento de aislamiento.

Quinta. La secuenciación de un fragmento del gen polimórfico *SAG3* permite detectar un polimorfismo de un solo nucleótido extendido en la población ovina española, aumentando así la resolución de la caracterización de la diversidad genética.

Sexta. Se propone un procedimiento normalizado para la evaluación de la virulencia de aislados del parásito por medio del estudio de su virulencia en ratón. Este procedimiento incluye parámetros letales (mortalidad acumulada) y no letales (como la morbilidad acumulada, o el tropismo, las cargas parasitarias y las lesiones histopatológicas en diferentes tejidos). Adicionalmente, se propone la caracterización de varios parámetros de virulencia *in vitro* (tasas de invasión y cinética de proliferación) utilizando células diana del hospedador del que proceden para lograr una evaluación de la virulencia de los aislados más exhaustiva y completa.

Séptima. La caracterización fenotípica de una selección de aislados en modelos *in vivo* e *in vitro* demuestra que la mayoría de los aislados de tipo II poseen características no virulentas, aunque existen significativas diferencias intragenotipo. El único aislado de tipo III incluido entre los aislados evaluados presenta el perfil más virulento, lo que contradice las clasificaciones convencionales que establecen a los aislados de *T. gondii* de tipo III como los menos virulentos entre los tres linajes principales.

Octava. La combinación alélica *CS3*/*ROP18*/*ROP5* de los aislados seleccionados no muestra relación con su virulencia, estando en contradicción con otros estudios previos y sugiriendo que otros factores genéticos podrían estar involucrados en la virulencia de *T. gondii* en ratón.

<u>Objetivo específico 2</u>: Obtención de un panel representativo de aislados de *Toxoplasma gondii* de cerdos ibéricos españoles y su caracterización genética y fenotípica

Novena. Se obtuvo un panel de cinco aislados del parásito procedentes de cerdos ibéricos criados en diferentes localizaciones del suroeste de España. Este panel incluye los primeros aislados obtenidos de dicho hospedador en nuestro país. El éxito en el aislamiento a partir de cerdos ibéricos de engorde criados en libertad pone de relieve el riesgo para la salud pública que supone el consumo de una carne tan valorada cuando está poco cocinada.

Décima. La caracterización genética de los aislados obtenidos de cerdo ibérico confirma el predominio de la variante PRU del genotipo II (ToxoDB #3) en la ganadería española, aunque también se detecta el genotipo clonal III (ToxoDB #2), coincidiendo con los datos disponibles de cerdo y otros animales domésticos en Europa.

Undécima. La evaluación de la virulencia en ratón de aislados obtenidos de cerdos ibéricos muestra un aislado de genotipo variante PRU del tipo clonal II con características no virulentas, contrastando con un aislado de genotipo clonal III virulento para los ratones, presentando tasas de mortalidad y morbilidad acumuladas notablemente elevadas. Estos hallazgos apoyan los resultados obtenidos en los aislados procedentes de oveja, refutando las clasificaciones tradicionales del grado de virulencia de las cepas de *T. gondii* y destacando la variabilidad fenotípica intragenotipo también entre las cepas de tipo III, que no se explica por su combinación alélica *CS3*/*ROP18*/*ROP5*.

CHAPTER VIII

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CAPÍTULO VIII

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APPENDIXES

ANEXOS

APPENDIX 1. Review article "*Toxoplasma gondii* genotyping: a closer look into Europe"

Toxoplasma gondii genotyping: a closer look into Europe

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Keywords

Toxoplasma gondii, genotypes, Europe, restriction fragment length polymorphism, microsatellite typing, sequence typing

Abstract

Toxoplasma gondii is a major zoonotic agent which may cause harmful effects in pregnant and immunocompromised hosts. Despite many efforts on its genetic characterization, an entirely clear picture of the population structure in Europe has not been achieved yet. The present study aimed to summarize the available genotyping information and provide a map of the circulating strains distribution. There is consensus on type II *T. gondii* genotypes prevailing in Europe, but the absence of harmonization in the use of typing methods limits detailed knowledge. Standardized, high-end typing tools and integrative strategies are needed to fill the gaps and complete an accurate image of the *T. gondii* genetic population in Europe.

Toxoplasma gondii paradigm of the One Health concept in Europe

Toxoplasma gondii, the etiologic agent of toxoplasmosis, is an apicomplexan obligate intracellular protist of major medical and veterinary relevance. The complex life cycle of *T. gondii* is defined as facultative heteroxenous, with virtually all warm-blooded animals as intermediate hosts (including humans, domestic and wild mammals and birds), and members of the Felidae family acting as definitive hosts (Dubey, 2010) (Figure 1). Toxoplasmosis is a zoonosis of global distribution (Dubey, 2010; Robert-Gangneux & Dardé, 2012) and represents an excellent example of the **One Health** concept, since *T. gondii* is present and circulates through all **compartments** defined in this paradigm (Aguirre et al., 2019; Djurković-Djaković et al., 2019). Due to its wide host range the parasite is of importance not only in public health, but also in livestock industry and wildlife management programmes. A FAO/WHO report considered *T. gondii* as the fourth world most relevant parasite (FAO/WHO, 2014). In addition, globalization and trade could contribute to the inter-regional and intercontinental spread of new parasite strains (Bertranpetit et al., 2017; Galal et al., 2019).

In humans, this parasite infects up to a third of the total global population (Bigna et al., 2020; Rostami et al., 2020). The infection can be acquired by ingesting oocysts (in food, water, or particles of soil contaminated with faeces of shedding felids; environmental pathway) or tissue cysts (in meat of infected animals that are slaughtered or hunted for human consumption; meat-borne pathway) (Cook et al., 2000; Belluco et al., 2018) (Figure 1). The infection is normally asymptomatic but results in chronicity; however, a primary infection in pregnant women could cause congenital transmission and imply serious damage to the foetus (Jones et al., 2001). In immunocompromised individuals, severe neurologic and pulmonary clinical signs are frequently observed consequences of a re-activated or new infection (Robert-Gangneux et al., 2018; Wang et al., 2017). Finally, ocular toxoplasmosis is also an increasingly recognised clinical issue in some parts of the world (Maenz et al., 2014).

In livestock, *T. gondii* infection is associated with significant economic losses linked to reproductive failure in several domestic species such as sheep and goats (Dubey et al., 2020, Dubey et al., 2020; Stelzer et al., 2019). Infection by *T. gondii* in livestock is also a risk to public health when animals destined to human consumption are involved (Opsteegh et al., 2016). Moreover, the parasite is a cause of concern in wildlife and zoo animals since *T. gondii* may cause lethal infection in particular species (*e.g.*, New World monkeys and Australian marsupials) presumably due to an evolutionary history practically developed in the absence of felids (Thompson, 2013).

Although important oocyst-associated human toxoplasmosis outbreaks have been documented in the past few years (Pinto-Ferreira et al., 2019), the relevance of the environmental route remains poorly investigated. *Toxoplasma gondii* oocysts has been detected in all type of **matrices** worldwide (Shapiro et al., 2019). Consumption of raw vegetables seems be an important risk factor for *T. gondii* infection and there are several reports on produce contaminated by *T. gondii* oocysts (summarized in Almeria & Dubey, 2021). Bivalves, such as mussels and oysters, can accumulate *T. gondii* oocysts by water filtration (Coupe et al., 2018; Marquis et al., 2019). *Toxoplasma gondii* oocysts have also been reported in water (Minuzzi et al., 2020) and soil (Cong et al., 2020).

Strategies to reduce the disease burden of toxoplasmosis should be based on close collaboration between both practitioners and veterinarians under the One Health umbrella. The relative contributions of the different transmissible stages, sources and transmission pathways remain partly unknown. This lack of information on the attribution to specific infection sources has hampered the development of effective intervention strategies. That fact could be partly due to the absence of a systematic surveillance system for this zoonotic foodborne pathogen (van der Giessen et al., 2021). In addition, there are major geographical differences in the epidemiology of the infection as well as in consumption habits around the world, which affect the importance of different transmission routes and specific food products for the occurrence of the infection (Galal et al., 2019).

In Europe, *T. gondii* is considered as an important foodborne parasite that ranked high according to the multicriteria decision analyses (Bouwknegt et al., 2018) and disease-burden estimations for toxoplasmosis (Havelaar et al., 2015). Congenital toxoplasmosis is notifiable in 29 of 35 surveyed European countries and pregnant women are screened in some countries such as Austria, Belgium, and France; nevertheless, underreporting is a major problem in most countries. In animals, risk-based surveillance system of EU livestock needs to be improved to reduce human meat-borne infections (van der Giessen et al., 2021). At present, *T. gondii* surveillance is strongly affected by several important limitations: (i) small numbers of tested animals; (ii) the use of different indirect and direct detection methods, which, in most cases have not been validated by an independent body; (iii) unknown age of tested animals; and (iv) no information on type of breeding (EU One Health Zoonoses Report 2019).

Concerning the genetic diversity of *T. gondii* circulating in Europe, type II strains and, to a lesser extent, type III strains, are the dominating populations, both in domestic and wild environments (Khan et al., 2007; Lorenzi et al., 2016). However, the current globalization of trade seems to be causing risk situations that pose new research and public health challenges (Galal et al., 2019). For instance, cases of severe human toxoplasmosis have been reported in France due to the consumption of imported South and North American horsemeat contaminated with highly pathogenic strains of the parasite (Elbez-Rubinstein et al., 2009; Pomares et al., 2011).

Because of the importance of genetic characterization of *T. gondii* strains for epidemiological and clinical studies, this work is aimed to summarize present knowledge on the genetic population structure of *Toxoplasma gondii* in Europe and the distribution of genotypes within the different compartments comprised in the One Health concept/approach (*i.e.*, human, domestic and wild animals, and environment).

Toxoplasma gondii, **a complex organism with complex genetics**

The Toxoplasma gondii life cycle, an avenue for a rich genetic diversity

The global distribution of the parasite and a complex life cycle including a broad host range and several transmission routes, along with the capacity for **genetic recombination** during the sexual phase have led to a wide genetic and phenotypic diversity within *Toxoplasma gondii* populations circulating worldwide (Figure 1).

Almost all life cycle stages of *T. gondii* are haploid, with the exception of a diploid stage during the short phase of **zygote formation** in the small intestine of felines (Martorelli et al., 2019),

after which haploid sporozoites are the result of a **postzygotic meiosis** in the environment (Dubey, 2010) (Figure 1). Thus, most stages of the life cycle are characterised by allelic homozygosity, facilitating genetic recombination and supporting direct measurements of population-level heterozygosity. Unlike for many apicomplexan parasites, in the case of the genera *Toxoplasma* and *Neospora* the sexual phase is not mandatory and zoites can propagate by asexual replication indefinitely (Beck et al., 2009) (Figure 1).

During 1990s, restriction fragment length polymorphism (RFLP) and other methods (*e.g.*, isoenzyme typing) allowed researchers to establish the existence of three clonal **lineages** linked to mouse virulence. Type I isolates were 100% lethal to mice, irrespectively of the dose, and types II and III moderately or non-virulent in a dose-dependent manner (Dardé et al., 1992; Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Howe et al., 1996) (Box 1). Since then, global population structure and genetic variability of *T. gondii* has been extensively investigated. The rapid development of multilocus-sequencing methods, and the description of a wide panel of new **PCR-RFLP** and **microsatellite (MS)** markers led to the consolidation of the predominance of the three initial clonal/archetypal types or lineages in Europe and North America, but new concepts of "**recombinant**" and "**atypical/noncanonical**" strains appeared on the scene (Khan et al., 2005; Su et al., 2006; Khan et al 2007; Ajzenberg et al., 2002, 2005) (Box 1).

Toxoplasma genome-wide sequencing approaches to understand diversification

The total haploid genome of *T. gondii* contains 13 chromosomes, with a total genome size of about 65 million of base pairs (Mbp) and more than 8300 protein coding genes identified (Lorenzi et al., 2016; Xia et al., 2021). Comparative genomic studies carried out with *T. gondii* and several members of the Apicomplexa subphylum have demonstrated how *T. gondii* is demarcated from its closest relatives. The key are tandem amplification and diversification of certain groups of genes involved in host-parasite interactions (*e.g.*, secretory proteins and surface adhesins involved in host cell attachment and immune evasion), that determine also key differences among the 16 major **haplogroups** defined currently for the species (see following section) (Lorenzi et al., 2016).

The genome-wide polymorphism rate between the three archetypal clonal lineages, which seem to dominate the *Toxoplasma gondii* population in Europe and North America, has been estimated to be approximately 1%, characterised by an extensive bi-allelism falling into type I, II and III single nucleotide polymorphisms (**SNP**) (Sibley and Ajioka, 2008; Grigg et al., 2001; Khan et al., 2005; Boyle et al., 2006). The origin of this clonality, explained as low genetic diversity within each lineage and low divergence between them has been suggested to be due to a recent emergence from a common ancestor only 10,000 years ago (Su et al., 2003) during the domestication process of cats and various livestock species. In addition, an extensive bypassing of the sexual cycle may have led to a continuous asexual propagation, resulting in rare possibilities for meiotic crosses between the highly similar parental strains (Sibley and Ajioka, 2008) only observed occasionally in naturally infected cats (Herrmann et al., 2012). Nevertheless, this hypothesis is not applicable to the South American model, where a notably higher prevalence of the infection, a larger spectrum of susceptible intermediate host species along with an increased diversity of wild felid species might have promoted more frequent recombination events resulting in the contrasting extreme diverse, largely non-clonal population there (Shwab et al., 2014; Bertranpetit et al., 2017).

Global Toxoplasma gondii *population genetic structure*

Until date, there have been several comprehensive attempts to unravel the population structure of the parasite aided by great advances in molecular typing techniques. In an extensive and in-deep study based on phylogenetic analysis of above 950 typed isolates from all over the world 16 well-defined haplogroups were identified (Su et al., 2012) and assorted into 6 major clades A-F based on whole genome sequencing (Lorenzi et al., 2016).

In North America and Europe the population structure of *T. gondii* appears to be dominated by three clonal lineages (I, II and III; corresponding to haplogroups (HG) 1, 2 and 3). A fourth clonal lineage (HG12) is largely confined to North America, where it is more common in wild animals. In contrast, much greater genetic diversity is observed in South America, where the population fits an epidemic structure, with a few major clonal complexes and abundant less related isolates (Pena et al., 2008; Khan et al., 2007, 2011).

It has been suggested that African and Asian situations could be a mixture between both (above) scenarios, with abundance of isolates belonging to type I, II, III clonal lineages, coexisting with other clonal groups that emerged from the strong expansion of recombinant or atypical isolates, but exhibiting a less divergent character than in South America; however, both continents remain poorly explored, especially in tropical regions (Galal et al., 2018; Chaichan et al., 2017). Phylogenetic and geostatistical approaches led Bertranpetit et al. (2017) to hypothesize with a South American origin of *T. gondii* and its initial spread through North America, Asia, Europe and finally Africa, through different migration routes, linked to the co-evolution of Felidae family members and humans. During this expansion, a growing tendency to transmission between a huge variety of domestic intermediate hosts and the decrease in the diversity of felids could create a genetic bottleneck responsible for the clonality present in the population outside South America.

Toxoplasma gondii **genotyping tools in Europe: is there a consensus?**

Available genotyping methodologies (see Box 1) have been unregularly applied in different areas, over different **matrices** and in a different manner by distinct research groups. The present section aims to examine the use of common methodologies within the European context. After a comprehensive screening of studies on European *T. gondii* strains genotyping deposited in PubMed database, 101 and 43 studies including PCR-RFLP/PCR-Sequencing or MS typing, respectively, were selected. Typing results on both, isolated viable parasites and DNA positive specimens/clinical samples have been considered. Furthermore, data from overseas territories and zoo-kept animals were not included. Despite the large number of studies aiming at a genetic characterization of European *T. gondii* strains, the data are limited due to several factors. After analysis of the extracted data, it seems to be evident that there is a notable divergence in the identity and number of markers used among the studies (Figure 2A, B, C). The selected studies comprised the use of different 15 PCR-RFLP (Figure 2A), 15 PCR-Seq or 15 MS (Figure 2B) markers. The use of an insufficient number of molecular markers is a problematic issue, especially in the case of PCR-RFLP and PCR-sequencing typing studies, because a large part of diversity might be missed or genotypically different parasites not efficiently distinguished. Unfortunately, an important proportion (40%, 40/101) of these studies implemented a single-locus typing method, (therefore) being completely outdated and possessing major limitations for reliable strain classification (Figure 2C). The most frequently used marker was *SAG2* (5’ and

3' ends of the gene) probably because it was among the first PCR-RFLP markers described, supposing a milestone on *Toxoplasma gondii* genetic studies (Figure 2A) (Howe and Sibley, 1995; Sibley and Boothroyd, 1992). On the other hand, assays are not very informative when based on genes infrequently used such as *ROP1* (Haque et al., 1999; Turčeková et al., 2013), or on markers, like *B1* gene, mostly applied in a certain type of environmental specimens (*i.e.*, water, soil, air, vegetables, or fruit) (Figure 2A) (Burg et al., 1989; Sroka et al., 2008, 2009, 2010). Regarding microsatellites typing procedures, the number of markers has not been observed as a problematic issue since the use of five "genotyping" markers or the complete panel of 15 "genotyping" plus "**fingerprinting**" MS markers is quite widely used (Figure 2B).
Overall, however, independent of the typing techniques used, it is observed that the collected information is highly unbalanced between countries (information from 21 different countries was found), with large regions of the continent from which there is no data, what is hardly representative of the entire European population (Figure 2D).
Aiming to describe the reliable information available about genotypes circulating in Europe and taking into account the proportion of studies implementing different numbers of PCR-RFLP/PCR-Seq or MS genotyping markers (Figure 2C), a minimum of four and five regions analysed was established as a "cut-off", respectively. These cut-offs on minimum numbers of markers typed were applied, because the resolution of genetic data is dependent of the number of markers analysed (yielding PCR amplification) (Ajzenberg et al., 2010; Su et al., 2010). To this end, 51 (n=804) and 42 studies (n=831) including PCR-RFLP/PCR-Sequencing or MS typing, respectively, were considered to represent a robust pan-European overview (Table 1).

Global picture of the genetic population in Europe

MS and PCR-RFLP typing are the most widely used methods, but except for predominant lineages and some unique strains, equivalence between assigned genotypes by each technique remains at some extent confusing; thus, remarks will be given separately. The classification of an isolate into archetypal, recombinant or atypical, or even distinguish it from a mixed infection is a sensitive and non-definitely settled issue. In most cases this requires the availability of viable parasites in a sample that could be separated into different clonal populations. The unambiguous identification of mixed or recombinant infections is almost impossible in only DNA positive materials and largely depends on the number and the discriminating power of markers used for genotyping. Therefore, from a critical viewpoint, recombinant, atypical and mixed infections should be treated as a whole (MRA category), differentiating them from the widely prevalent archetypal clonal strains. Based on the One Health concept, we sorted genotypic information according to samples or isolates origin into four "compartments", namely humans, domestic and wild animals, and environment (Table 1, Figure 3).

Toxoplasma gondii *genetic diversity based on PCR-RFLP or PCR-sequencing methodologies*

Applying the cut-off criterion and concerning strain types detected in humans, only three countries are represented (Germany, Poland and Serbia) in five studies with a total of 33 samples typed (Djurkovic-Djakovic et al., 2006; Marković et al., 2014; Stajner et al., 2013; Nowakowska et al., 2006; Herrmann et al., 2014). Among them, almost 90% (29/33) corresponded with type II strains, only one type III was detected, and MRA infections were described in three cases. The

presumed predominance of type II in Europe is evident but not conclusive since data could be representative only of central Europe.

Most European (geno)typed samples have been collected from infected domestic (pets and livestock) and free-living animals. Regarding domestic animals, the range of countries represented is wider but not enough, with molecular studies from Austria, Czech Republic, Denmark, France, Germany, Ireland, Italy, Poland, Portugal, Serbia, Spain, Switzerland and The Netherlands (22 studies with a total of 501 samples) (Table 1). Likewise, studies could be sorted according to the host, including data from sheep, goat, cattle, pig, horse, chicken, dog, and cat, standing out chicken and pig species in terms of sampling effort, with 102 and 76, samples typed, respectively. Type II strains were detected in 86% (431/501) of samples, together with a 6.2% (31/501) of type III, 2% of type I (10/501) and approximately 6% (29/501) of MRA infections (Table 1). Concerning wild animals, European studies include data from Croatia, Czech Republic, Denmark, Germany, Italy, Norway, Poland, Scotland, Serbia, Spain, and the UK, with a total of 261 samples collected in 25 different studies. It involves data from a wide variety of hosts such as rodents, marine mammals, wild cats, wild swine, mesocarnivores, wild ungulates, and wild avian species. Within the group of wild animals, mesocarnivores were those with the highest number of studies (eight) and samples analysed (144). For strains circulating in sylvatic cycles approximately 66% were reported to be type II (172/261), 20.7% MRA (54/261), 12.6% type III (33/261), and 0.8% type I (2/261) (Table 1).
Regarding genotypes present in environmental samples, the situation is even more restricted, with only two studies having met the requirements accounting for a total of nine samples. Type II strains were detected in seven samples of vegetables in the Czech Republic (Slany et al., 2019) whereas type I alleles were observed in DNA extracted from two ticks (*Dermacentor reticulatus*) collected in field areas of Poland (Table 1) (Wojcik-Fatla et al., 2015).

As a whole, literature data on PCR-RFLP typing or PCR-sequencing suggest a clear predominance of type II strains circulating in Europe, that comprises of 79.5% (639/804) of the total samples collected in 51 different studies included (Table 1). Type I strains are truly scarce, representing 1.7% (14/804) of samples, whereas type III strains imply 8.1% of total samples (65/804). Finally, MRA infections were reported for 10.7% (86/804) of the records. Despite the limitation on the data, it could be pointed out the enhanced burden of type III strains, as well as MRA infections, in the case of wildlife animal species in comparison with the rest of European matrices considered. In Figure 2D, geographic distribution of genotyping studies across Europe is represented. Germany, Italy and Serbia are the countries with the highest number of PCR-RFLP/Seq based genotyping investigations in their territories.

Toxoplasma gondii genetic diversity based on MS methodologies

Under the view of the available literature, the number of samples typed by less than 5 MS loci is negligible compared to the 831 samples typed in 42 different studies by using more than five MS markers (Table 1). Apart from type I, II, III or MRA infections, by MS typing was also possible to identify specific genotypes such as *Africa1*, *Caribbean2, Caribbean3* even characterizing only five loci (*B18*, *TUB*, *Tg-MA*, *W35* and *B17*).
Unlike the previously mentioned methods, the MS-based methodology has been widely used in the genetic characterization of human samples, involving a total of 428 samples in 20 different studies. Despite the participation of a greater number of European countries, France clustered

77.3% of the human samples analysed (Ajzenberg et al., 2015; Ajzenberg et al., 2009), followed by far by Portugal (11.7%) (Ajzenberg et al., 2009; Vilares et al., 2017), Denmark (4.7%) (Jokelainen et al., 2018), and Belgium (4.4%) (Gisbert-Algaba et al., 2020); most of the other countries contributed with up to three single isolates (Austria, England, Germany, Romania, Serbia, and The Netherlands). Concerning strain types detected in human population, 86.4% corresponded with type II strains, the types I and III were found in low proportions (2.6 and 3% respectively), and those of MRA infections corresponded to 6.3% of cases. In addition, six cases of human infection with *Africa1* strains and one case with *Caribbean2* were detected in France, Denmark and Belgium (Ajzenberg et al., 2010; Jokelainen et al., 2018; Su et al. 2012; Fekkar et al., 2011) (Table 1). The predominance of type II in Europe is again clear but once more it should be borne in mind that extensive areas of the continent are still not represented.

The second most studied compartment was that of domestic animals, involving a total of 238 samples in 15 different investigations. Once again, France (36.9%) and Portugal (20.6%), together with Austria (27.3%), stood out in the number of genotyped samples. Data from Finland, Germany, Italy, Romania, Serbia, and The Netherlands are also available. In respect of the different hosts studied, most of the samples were collected from chicken (93) and sheep (91) (Verma et al., 2015; Shwab et al., 2018; Bertrantpetit et al., 2017). In pets and livestock type II strains were detected in 91.2% (217/238) of samples, along with a 6.7% (16/238) of type III and 1.7% of type I (4/238). Apart from that, only one sample presented a MRA profile (0.4%, 1/238). Concerning wildlife, European studies included data from Belgium, Czech Republic, England, Finland, France, Italy, Norway, Portugal, Serbia, and Spain, with a total of 160 samples collected in 15 different publications; a wide variety of hosts were included in such surveys, highlighting the red foxes (*Vulpes vulpes*) (n=54) (De Craeye et al., 2011; Aubert et al., 2010) and wild boars (*Sus scrofa ferus*) (n=44) (Richomme et al., 2009; Gisbert-Algaba et al. 2020). Among strains circulating in wild animals, 88.8% corresponded to type II (142/160), 6.2% (10/160) to MRA infections and 3.8% (6/160) to type III. Only one case of type I and another of *Caribbean3* were detected (0.6% each, 1/160) in a pigeon from Portugal and a wild boar from Italy, respectively (Vilares et al., 2014; Sgroi et al., 2020).

As occurred in previous section, typing reports on environmental samples are quite rare. Only Santoro et al. (2020) reported genotyping results from Mediterranean mussels (*Mytilus galloprovincialis*) collected in southern Italy, with four samples surprisingly belonging to type I and one sample typed as a recombinant or mixed profile. As this is the only study, including such a small sample size, general conclusions should be drawn after further complementary surveys.

On balance, the prevalence figures obtained from reviewing the available data on *T. gondii* strains genotyped by MS in Europe are quite similar to those obtained by PCR-RFLP and PCR-sequencing methods. The predominance of type II strains in Europe is again evident, involving 87.7% (729/831) of the total samples analysed in 42 studies that meet the criteria of at least 5 markers characterized (Table 1). As seen in previous section, type I strains remain infrequent, representing 2.4% (20/831) of samples. On the other hand, the prevalence of type III and non-assorted, recombinant strains or mixed infections were slightly lower compared to PCR-RFLP and PCR-sequencing methods with almost 4.2% (35/831) and 4.7% (39/831) of total records, respectively. Finally, MS-typing was able to resolve other non-canonical haplogroups, *i.e.*, *Caribbean1*, *Caribbean3* or *Africa1*, allowing to identify *T. gondii* strains possibly imported to Europe (1%, 8/831), either by human migration or trade. Overall, France, Portugal, Austria, and Belgium are the countries with the highest number of MS genotyping results in their territories;

on the contrary, there are large areas of the continent from which there is no information, especially northern European countries (Figure 2B).
An eBURST analysis (Feil et al., 2004) of all *Toxoplasma gondii* DNA samples typed by 15 MS regions (n=487) clearly separated type I, type II, type III and MRA genotyping results but also showed vast diversity among the dominating type II typing results. There seems to be no clear genetic divergence between type II samples from different parts of Europe (*e.g.*, the northern part, Denmark, Norway and the eastern part, Czech Republic, Romania), as shown in Figure 4. This is only partially in accord with results reported in France for *T. gondii* strains involved in human toxoplasmosis where in rural regions *T. gondii* associated with cases of congenital toxoplasmosis were genetically different between the eastern and western part of the country based on MS typing results (Ajzenberg et al., 2015).

Integrative analysis: evidences from a pan-European perspective

The extensive knowledge of the biology and epidemiology of *T. gondii* means that the biggest problem we face is not the detection of routes or sources of transmission as in other emerging zoonotic diseases, but rather the enormous variety of susceptible hosts that makes it an underestimated and silent concern, only visible in specifically vulnerable groups of populations (immunosuppressed or pregnant hosts). This review examines the distribution of various *T. gondii* genotypes through the European continent taking in account the different One Health compartments. As a whole, the predominance of clonal type II strains is evident, but exhaustive published data collection suggests the existence of a significant percentage of divergent strains (MRA), detected by the different techniques taken into account, and concentrated in the wildlife compartment. Hence, the dichotomy “domestic versus wild” so manifest in the American continent is probably present also in Europe (Mercier et al., 2011; Jiang et al., 2018; Galal et al., 2019). Nonetheless, the possible genetic diversity of *T. gondii* found infecting the wildlife has been less studied than in domestic animals, with fewer samples available, with less effort/success on parasite isolation and consequently limited PCR amplification and a limited resolution of typing assays (Herrmann et al., 2012b; Verin et al., 2013; Bacci et al., 2015; Uzelac et al., 2019). Furthermore, in many of the studies in which the isolation of the parasite is achieved (mainly in mice or cell culture), and a high number of RFLP or MS genotyping markers are applied, the genotypes described are mainly clonal type II. On the other hand, the selection of certain strains at expense of others during isolation procedures has been demonstrated in literature (Verma et al., 2017; Fernández-Escobar et al., 2020), and therefore, data obtained directly from clinical samples should not be ignored but need verification. In short, findings should be always interpreted cautiously, as well as with interest, since strains that circulate in sylvatic cycles are a source of infection for domestic animals and humans and have been associated with greater pathogenicity (Dubey et al., 2014).
Clonal type III-related strains were also highlighted, mainly detected in animal hosts. Some authors claimed that type III alleles are more frequently detected in southern Europe compared to other parts of the continent (Kuruca et al., 2019), but the reality is that France, Italy and Portugal are the countries that have published the most *T. gondii* genotyping studies, with a lower contribution from northern countries (Figure 2B), implying large areas without information. Type I alleles are particularly underrepresented in Europe. Most articles describing type I alleles during genotyping (Turčeková et al., 2013; Papini et al., 2015; Sroka et al., 2020;

Mancianti et al., 2015; Battisti et al., 2018; Santoro et al., 2020) only involved direct genotyping from tissue samples DNA, with an often lower success in the amplification of typing markers. Nevertheless, Verma et al. (2015) and Moskwa et al. (2017) showed a complete clonal type I profile in two isolates obtained from an aborted bovine foetus in Portugal (firstly reported by Canada et al., 2002) and from an aborted foetus of European bison (*Bison bonasus bonasus* L.) in Poland, respectively. This could be further proof of the enhanced pathogenicity of type I strains, leading to reproductive failure in species known for their greater resistance to infection (Bovidae family). Clonal type I isolates fully typed by 15 MS markers have been also described infecting humans (Ajzenberg et al., 2010).

Standardization of typing methods is definitively necessary for the integration of genetic data. The BRC biobank (Biological Resource Center for Toxoplasma) was one of the approaches that comes closest to this objective, storing around 1500 strains from different hosts (humans or animals) and from different countries around the world and genotyped by the widely applied 15 MS markers (Rocaboy et al., 2020; Ajzenberg et al., 2010). There are important limitations of traditional methodologies used for *T. gondii* typing, because only quite specific and restricted sites within a comparatively large *T. gondii* genome are assessed. Whole-genome sequencing (WGS) data analysis has emerged as the most suitable approach for a thorough analysis of the genetic diversity in *Toxoplasma gondii*, its evolutionary history, and population structure. Despite WGS is difficult to apply as a routine technique for strain typing, the number of studies using this technology is growing rapidly, mainly due to its enormous potential and the continuously reducing costs. WGS data are publicly available only for a few isolates from Europe (namely PRU, MAS, FOU, BOF, TgH26044, TgH21016, TgH20005, Cz-H3, among others). Of these, only two isolates (PRU, Cz-H3) belong to the dominant clonal type II; all others have been isolated in Europe (*i.e.*, France, Belgium) but at least partially represent strains likely originating from other continents, like FOU and BOF (Africa) or MAS (South America) (Lorenzi et al, 2016). The only European Type II isolate PRU (Pruginaud) was assorted to Clade D, a clade which was established based on WGS data and comprised in addition to other type II strains also of North American HG12 strains or atypical North or South American strains. The generation of WGS data on further strains including European type II strains could help to better understand the real genetic diversity within the dominant European strains, to explore the possible exchange of sequence blocks between clonal lineages in Europe and probably to link genetic differences not covered by the traditional widely used typing methods with phenotypic differences (*e.g.*, virulence in mice) evidenced in literature between European isolates (Fernández-Escobar et al., 2020, 2021).

Concluding remarks

Despite many important efforts on *T. gondii* genotyping in Europe, the situation is still blur and need an extra and closer look. Still many questions remain unsolved (Outstanding Questions) and will constitute medium term challenges for researchers. Some important facts, like the lack of consensus over the methodologies and markers applied, the huge differences in sample quality, the sampling disparities existing among regions and the fact that vast areas remain unexplored, as well as the scarcity of data from human cases and environment are the main limitations to have a comprehensive picture. In this sense, epidemiological surveillance systems must be strengthened at many levels, in humans and in livestock industry (for example on farms,

slaughterhouses, and during home veterinary inspection of hunted and home slaughtered animals). Therefore, close collaboration between the medical and veterinary sectors is crucial. Although several archetypal and atypical genotypes have been described, there is consensus on type II *T. gondii* prevailing in Europe, but standardized, high-end typing tools and integrative strategies within the One Health approach are needed to fill the existing gaps and provide a clear picture of the *T. gondii* population in Europe.

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Glossary

Archetypal or canonical strains: original and ideal genetic types (type I, II and III) from which the rest of the genetic types derive and which have served as a reference for their classification.
Atypical strains: those with unique polymorphisms at some loci (not detected in the three major lineages).
Clade: a group consisting of a given ancestor and all its descendants.
Clonal population: consisting of individuals of the same genotype derived from a single individual after successive cycles of asexual division.
Fingerprinting MS markers: those whose characterization allows a higher level of discrimination for differentiating closely related strains belonging to the same haplogroup or lineage. This high-resolution analysis is required for establishing a common source of infection in outbreaks,

possibly discriminating geographical origin of the organism, or even identifying laboratory contaminations issues during diagnosis.

Haplogroup: a classification comprising many different genotypes thought to be related.

Microsatellite (MS): tandem repeats of short (1 to 6 bp) DNA motifs that are ubiquitous in eukaryotic genomes and undergo length changes due to insertion or deletion of one or multiple repeat units. The most commonly proposed mutation mechanism for MS sequences is strand slippage, occurring predominantly during DNA replication. Therefore, for the same locus there may be different alleles, with a different length depending on the number of repetitions of the motif.

Multilocus sequence typing (MLST): typing analysis based on DNA sequence polymorphisms (*e.g.*, SNPs, insertion, or deletion events) detected in genomic regions of interest, including introns and coding regions.

One Health: an integrative approach to designing and implementing programmes, policies, regulations and research in which the interconnected **domains** that comprises humans, animals (domestic and wildlife) and environment health are taken into consideration.

Population structure: understood as its genetic diversity degree and distribution, which can be expressed in terms of allelic and genotypic frequencies.

Postzygotic meiosis: Meiosis is a cell division process in which a diploid (2n) cell undergoes two successive divisions, with the ability to generate four haploid (n) cells, involving genetic recombination and therefore increased genetic diversity. In diploid organisms this meiotic phase occurs during the formation of haploid gametes, while in haploid organisms (such us *Toxoplasma gondii*) this genetic recombination occurs after the formation of the zygote to recover the haploid genetic background.

Recombinant strains: Strains that possess types I, II or III alleles, identical to those found in the three major clonal lineages, but these have segregated differently among the loci analysed. These strains are considered to be the result of genetic crosses between strains of the main lineages.

Restriction fragment length polymorphism (RFLP): such analysis is based on the ability of restriction endonucleases to recognize SNPs present in PCR products and subsequently display distinct DNA banding patterns on agarose gels electrophoresis.

Single nucleotide polymorphisms (SNP): represent a difference in a single nucleotide within a genetic sequence.

Outstanding Questions

- To what extent are the different anthropogenic factors involved in shaping *T. gondii* population structure in Europe?
- Do globalization and human migrations have any real impact on the genetic population of *T. gondii*?
- Is there an unidentified risk of transmission and expansion of the strains circulating in the sylvatic cycle?
- Is there an unexplored biodiversity hotspot in the wild in Europe?
- What is the future of traditional typing techniques (PCR-RFLP or MS typing)? Are they going to e replaced by Next-Generation Sequencing techniques?

Text boxes

Box 1: Usefulness of genotyping, aims and brief historical background)

Differences in virulence for laboratory mice belong to the early observations in *T. gondii* research [1]. Against this background it was exciting that monoclonal antibodies were able to separate differentially mouse virulent *T. gondii* into two or three groups, suggesting differences in particular genes [2, 3, 4, 5]. Isoenzyme data had revealed a basically clonal population structure and separated sets of isolates into different zymodemes [6, 7, 8, 9]. PCR-based restriction fragment length polymorphism (PCR-RFLP) analyses applying independent single-copy gene loci, suggested - at this time - that the *T. gondii* population in both animals and humans mainly consisted of only three clonal lineages designated archetypal types I, II and III [10]. In addition to PCR-RFLP, Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR, [11]), pyrosequencing [12, 13], and high-resolution melting [14] were used as methods to discriminate mouse virulent and non-virulent isolates as well as the canonical types I, II and III. However, all these analyses were biased by the predominance of *T. gondii* isolates sampled in the northern hemisphere, especially in Europe and North America. Later, pioneering long term activities in sampling *T. gondii* isolates world-wide (e.g. [15, 16]) or the establishment of a Toxoplasma Biological Resource Centre located in France [17] provided an excellent and continuing foundation for further population genetic analyses which clearly revealed that the population structure of *T. gondii* was much more diverse, especially in South America but also in other parts of the world (e.g. in Africa). As yet, at least 16 haplogroups have been identified worldwide based on PCR-RFLP [18], microsatellite [19, 20] and sequenced-based markers covering more than 30 loci distributed across all chromosomes and the apicoplast of *T. gondii* [21]. It needs to be noted, that many of the strains assorted to a single haplogroup showed unique genotypes pointing to a much larger diversity in *T. gondii* as available data suggest [22, 23]. It is matter of debate whether genotyping *T. gondii* helps to understand virulence of particular strains in humans [24]. However, there is growing evidence that nonarchetypal strains (recombinant genotypes, atypical or exotic strains) which seem to be poorly adapted to humans and livestock are reason for a higher disease burden in South America [25] and probably in other parts of the world. Global trade may cause the introduction of such strains to other regions far away from their region of origin [26, 27] and genotyping was able to identify such imported strains [27].

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Tables

Table 1. Prevalence of the *Toxoplasma gondii* genetic types observed in isolates and DNA positive specimens/clinical samples in Europe according to the four compartments within the One Health concept (human, domestic animals, wildlife, and environment) and based on PCR-RFLP, PCR-sequencing or MS data. Percentages are given in brackets.

	Humans		**Domestic animals (1)**		**Wildlife(2)**		**Environment(3)**		**TOTAL**	
	RFLP/ Seq (%)	**MS (%)**	**RFLP/ Seq (%)**	**MS (%)**	**RFLP/ Seq (%)**	**MS (%)**	**RFLP/ Seq (%)**	**MS (%)**	**RFLP/ Seq (%)**	**MS (%)**
Type I	0 (0)	11 (2.6)	10 (2)	4 (1.7)	2 (0.8)	1 (0.6)	2 (22.2)	4 (80)	**14 (1.7)**	**20 (2.4)**
Type II(4)	29 (87.9)	370 (86.4)	431 (86)	217 (91.2)	172 (65.9)	142 (88.8)	7 (77.8)	0 (0)	**639 (79.5)**	**729 (87.7)**
Type III	1 (3)	13 (3)	31 (6.2)	16 (6.7)	33 (12.6)	6 (3.8)	0 (0)	0 (0)	**65 (8.1)**	**35 (4.2)**
MRA	3 (9.1)	27 (6.3)	29 (5.8)	1 (0.4)	54 (20.7)	10 (6.2)	0 (0)	1 (20)	**86 (10.7)**	**39 (4.7)**
Likely importation/ migration related genotypes	-	7 (1.6)	-	0 (0)	-	1 (0.6)	-	0 (0)	-	**8 (1)**
TOTAL	33	428	501	238	261	160	9	5	**804**	**831**

MRA: Mixed infections and recombinant or atypical genotypes; Likely importation/migration related genotypes (*Africa 1*, *Caribbean 2*, *Caribbean 3*); - : PCR-RFLP method is not valid for intra-genotype differentiation. Domains: (1) livestock (poultry, cattle, small ruminants, equines, pigs) and pets (carnivores); (2) rodents, marine mammals, wild ungulates (Cervidae, Bovidae, swine), carnivores, wild cats, and birds; (3) water, soil, air, fresh produce, ticks, and bivalves; (4) PCR-RFLP profiles suggesting a type II PRU variant (type II alleles combined with type I allele at Apico marker) were included within Type II category.

Figures

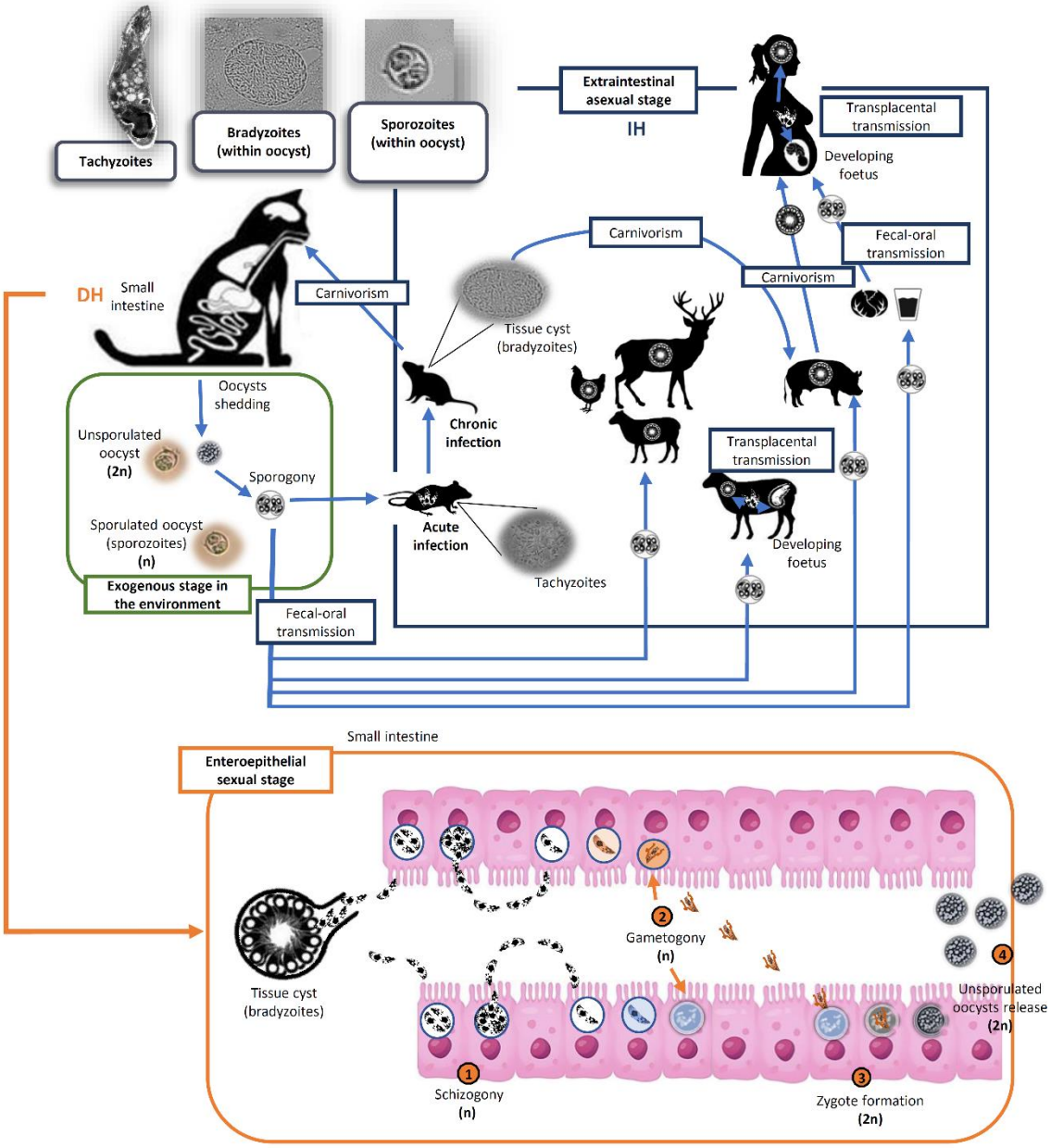

Figure 1. *Toxoplasma gondii* life cycle and transmission routes.

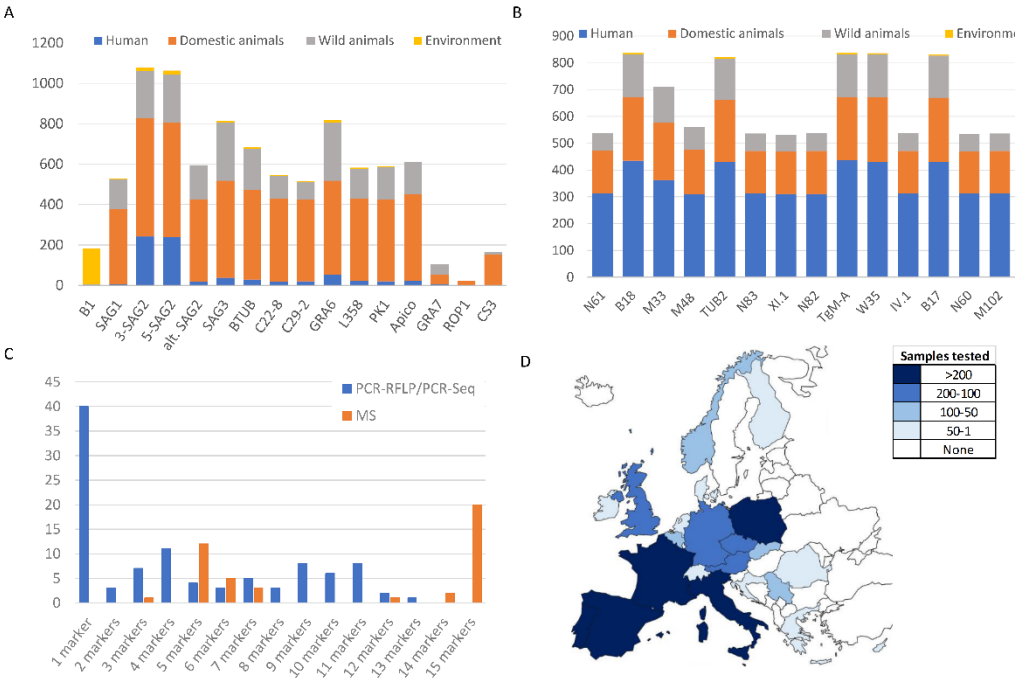

Figure 2. Analyses of the methodologies used within European *Toxoplasma gondii* strains genotyping studies. **A.** Number of samples typed by each PCR-RFLP marker in genotyping European studies. **B.** Number of samples typed by each MS marker in genotyping European studies. **C.** Proportion of studies implementing different number of PCR-RFLP or MS genotyping markers. **D.** Geographic distribution of genotyping studies across Europe. Only studies meeting the cut off criterion (at least 4 PCR-RFLP/PCR-Seq or 5 MS markers applied) have been considered.

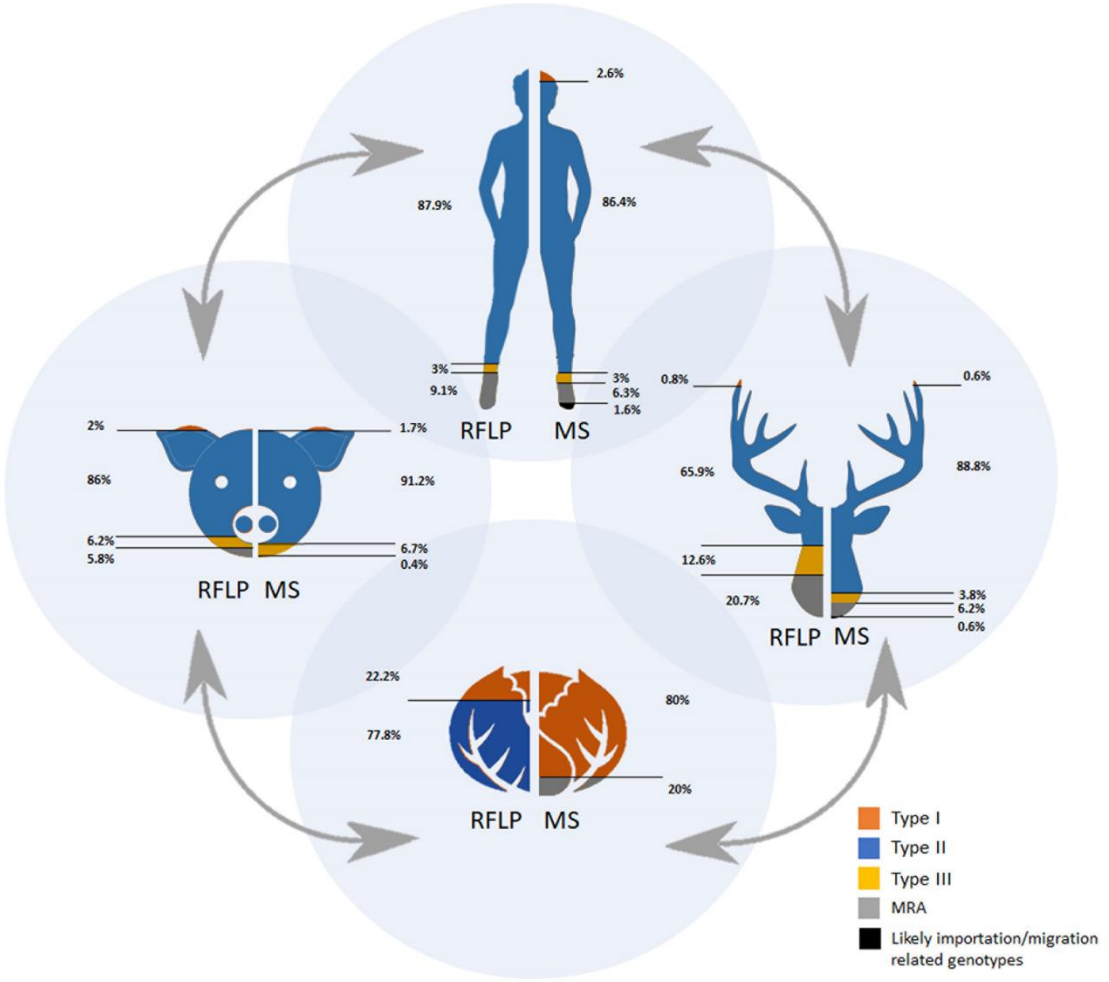

Figure 3. Occurrence of *Toxoplasma gondii* genetic types by each of the One-Health compartments.

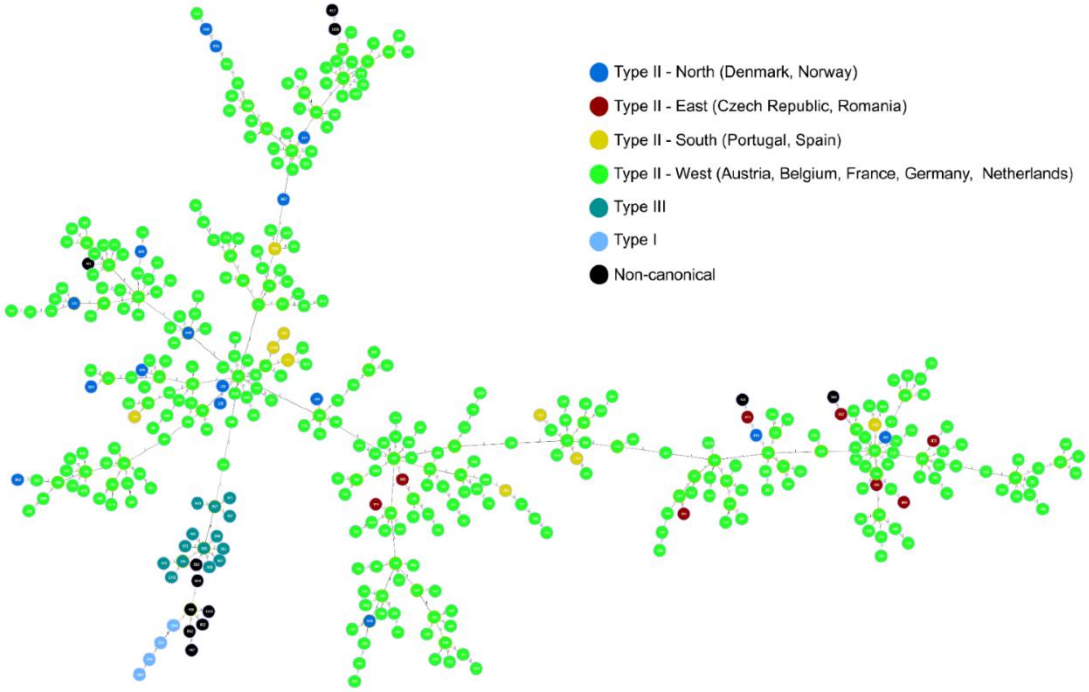

Figure 4. eBURST analysis using the Full-MST option of European *Toxoplasma gondii* samples typed by 15 MS regions. In total n=487 isolates, microsatellite typed at all 15 microsatellite markers were included. Of isolates with identical typing patterns only a single sample was included and the remaining random-based excluded. Type I, type III and non-canonical *T. gondii* are clearly separated from type II while no clear regional patterns can be observed in type II *T. gondii*.

APPENDIX 2. Summarized phenotypic data from relevant *Toxoplasma gondii* isolates.

Isolate ID	ToxoDB # or available genetic information	Mouse mortality (%)	Lethality*	Country	Reference
Europe					
DEG	#3	0	Non-virulent	France	Shwab et al. (2016)
TgShSp1	#3	0	Non-virulent	Spain	Sánchez-Sánchez et al. (2019)
SVS P14	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS F17	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O14	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS Fox2	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O12	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O16	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O17	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O18	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O10	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O11	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O13	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O14	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O20	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
SVS O21	Not determined. Serotype II	0	Non-virulent	Denmark	Jungersen et al. (2002)
EQ39	#54	6.8	Non-virulent	Serbia	Uzelac et al. (2020)
BOF	#6	8.3	Non-virulent	Belgium	Shwab et al. (2016)
G13	Not determined. Recombinant	10.7	Non-virulent	Serbia	Uzelac et al. (2020)
SSI 119	Not determined. Serotype II	11	Non-virulent	Denmark	Jungersen et al. (2002)
M4	#3	20	Non-virulent	UK	Hamilton et al. (2019)
K1	Not determined. Recombinant	38.8	Intermediate	Serbia	Uzelac et al. (2020)
SVS O15	Not determined. Serotype II	67	Intermediate	Denmark	Jungersen et al. (2002)
EQ40	Not determined. Recombinant	69.4	Intermediate	Serbia	Uzelac et al. (2020)
MAS	#17	100	Highly virulent	France	Shwab et al. (2016)
FOU	#6	100	Highly virulent	France	Dubey et al. (2014)
GPHT	#6	100	Highly virulent	France	Dubey et al. (2014)
ENT	Haplogroup 1	100	Highly virulent	France	Khan et al. (2009b); Khan et al. (2007)
MOR	Haplogroup 1	100	Highly virulent	France	Khan et al. (2009b)
North America					
TgShUS32	#131	0	Non-virulent	USA	Shwab et al. (2016)
TgWtdUS8	#74	0	Non-virulent	USA	Shwab et al. (2016)
TgWtdUS10	#54	0	Non-virulent	USA	Shwab et al. (2016)
CTG	#2	0	Non-virulent	USA	Shwab et al. (2016)
M7741	#133	11	Non-virulent	USA	Shwab et al. (2016)
VEG	#2	13	Non-virulent	USA	Dubey et al. (2014)
PTG (Me49)	#1	40	Intermediate	USA	Shwab et al. (2016)
TgBBeCa1	#90	50	Intermediate	Canada	Shwab et al. (2016)
ARI	#5	60	Intermediate	USA	Dubey et al. (2014)
B41	#4	71.4	Intermediate	.	Shwab et al. (2016)
TgRaw3	#32	75	Intermediate	USA	Shwab et al. (2016)
P89	#8	76	Intermediate	USA	Shwab et al. (2016)
TgShUs55	#32	80	Intermediate	USA	Dubey et al. (2014)
TgWtdPa4	#216	83	Intermediate	USA	Dubey et al. (2014)
TgCgCa1	#66	90	Intermediate	Canada	Shwab et al. (2016)
ROD	#72	90	Intermediate	USA	Shwab et al. (2016)
TgFoxPa7	#216	91	Intermediate	USA	Dubey et al. (2014)
TgShUS28	#73	100	Highly virulent	USA	Shwab et al. (2016)
GT1	#10	100	Highly virulent	USA	Shwab et al. (2016)
CAST	#28	100	Highly virulent	USA	Shwab et al. (2016)
TgBbUS1	#147	100	Highly virulent	USA	Dubey et al. (2014)
TgFoxPa9	#141	100	Highly virulent	USA	Dubey et al. (2014)
TgFoxPa8	#216	100	Highly virulent	USA	Dubey et al. (2014)
TgSwanUs3	#216	100	Highly virulent	USA	Dubey et al. (2014)
TgWtdPa5	#216	100	Highly virulent	USA	Dubey et al. (2014)

Isolate ID	ToxoDB # or available genetic information	Mouse mortality (%)	Lethality*	Country	Reference
South America					
TgCkBr149	#186	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr150	#186	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr157	#186	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr65	#186	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr66	#186	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkNi27	#140	0	Non-virulent	Nicaragua	Shwab et al. (2016)
TgCkBr168	#129	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCtCo8	#128	0	Non-virulent	Colombia	Shwab et al. (2016)
TgCatPr9	#115	0	Non-virulent	Puerto Rico	Shwab et al. (2016)
TgCkBr166	#114	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr156	#87	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr50	#86	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr173	#81	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr155	#76	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr159	#76	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkGy18	#68	0	Non-virulent	Guyana	Shwab et al. (2016)
TgCtCo3	#62	0	Non-virulent	Colombia	Shwab et al. (2016)
TgCkNi32	#52	0	Non-virulent	Nicaragua	Shwab et al. (2016)
TgRsCr1	#52	0	Non-virulent	Costa Rica	Shwab et al. (2016)
TgCkNi45	#50	0	Non-virulent	Nicaragua	Shwab et al. (2016)
TgDgCo14	#46	0	Non-virulent	Colombia	Shwab et al. (2016)
TgDgCo16	#46	0	Non-virulent	Colombia	Shwab et al. (2016)
TgCkGy1	#31	0	Non-virulent	Guyana	Shwab et al. (2016)
TgCkGy7	#30	0	Non-virulent	Guyana	Shwab et al. (2016)
TgCkBr114	#29	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkGy8	#25	0	Non-virulent	Guyana	Shwab et al. (2016)
TgCkCr2	#24	0	Non-virulent	Costa Rica	Shwab et al. (2016)
TgCatBr28	#21	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCtCo13	#18	0	Non-virulent	Colombia	Shwab et al. (2016)
TgCatBr61	#11	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr116	#9	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr3	#8	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr4	#8	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr58	#8	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr59	#8	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr60	#8	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr73	#8	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCatBr74	#8	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr161	#2	0	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr152	#186	25	Non-virulent	Brazil	Shwab et al. (2016)
TgCkNi35	#102	25	Non-virulent	Nicaragua	Shwab et al. (2016)
TgCkGy22	#48	25	Non-virulent	Guyana	Shwab et al. (2016)
TgCkBr142	#28	25	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr110	#25	25	Non-virulent	Brazil	Shwab et al. (2016)
TgCkBr160	#17	25	Non-virulent	Brazil	Shwab et al. (2016)
TgDgCo20	#29	30	Intermediate	Colombia	Shwab et al. (2016)
TgCatBr38	#136	40	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr67	#121	40	Intermediate	Brazil	Shwab et al. (2016)
TgCtCo12	#18	43	Intermediate	Colombia	Shwab et al. (2016)
P_{BR}	#84	43.1	Intermediate	Brazil	Shwab et al. (2016)
TgCkGy34	#123	50	Intermediate	Guyana	Shwab et al. (2016)
TgDgCo13	#79	50	Intermediate	Colombia	Shwab et al. (2016)
TgCkCr9	#43	50	Intermediate	Costa Rica	Shwab et al. (2016)
TgCkBr163	#17	50	Intermediate	Brazil	Shwab et al. (2016)
TgCkNi1	#16	50	Intermediate	Nicaragua	Shwab et al. (2016)
TgCkGy2	#12	50	Intermediate	Guyana	Shwab et al. (2016)
TgCatBr39	#11	50	Intermediate	Brazil	Shwab et al. (2016)
TgCkBr112	#7	50	Intermediate	Brazil	Shwab et al. (2016)
TgCkCr7	#43	60	Intermediate	Costa Rica	Shwab et al. (2016)
TgCatBr44	#34	60	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr77	#11	60	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr81	#124	67	Intermediate	Brazil	Shwab et al. (2016)
TgCkCr1	#91	67	Intermediate	Costa Rica	Shwab et al. (2016)

Isolate ID	ToxoDB # or available genetic information	Mouse mortality (%)	Lethality*	Country	Reference
South America					
TgCatBr32	#21	67	Intermediate	Brazil	Shwab et al. (2016)
TgCkBr162	#17	67	Intermediate	Brazil	Shwab et al. (2016)
TgDgCo9	#14	67	Intermediate	Colombia	Shwab et al. (2016)
TgDgBr18	#106	75	Intermediate	Brazil	Shwab et al. (2016)
TgCkBr143	#105	75	Intermediate	Brazil	Shwab et al. (2016)
TgCkBr107	#70	75	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr69	#34	75	Intermediate	Brazil	Shwab et al. (2016)
TgCkBr113	#30	75	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr68	#11	75	Intermediate	Brazil	Shwab et al. (2016)
TgCtCo2	#10	75	Intermediate	Colombia	Shwab et al. (2016)
TgCkBr111	#7	75	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr54	#6	75	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr26	#80	80	Intermediate	Brazil	Shwab et al. (2016)
TgCkCr10	#43	80	Intermediate	Costa Rica	Shwab et al. (2016)
TgCkBr136	#41	80	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr70	#34	80	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr23	#21	80	Intermediate	Brazil	Shwab et al. (2016)
TgCatBr51	#11	80	Intermediate	Brazil	Shwab et al. (2016)
TgCtCo5	#61	89	Intermediate	Colombia	Shwab et al. (2016)
TgCatPr6	#112	92	Intermediate	Puerto Rico	Shwab et al. (2016)
TgCPBr25	#175	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCPBr27	#165	100	Highly virulent	Brazil	Shwab et al. (2016)
TgMr	#163	100	Highly virulent	Argentina	Bernstein et al. (2020)
TgCPBr1	#162	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCPBr26	#148	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr45	#135	100	Highly virulent	Brazil	Shwab et al. (2016)
TgDgCo7	#122	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCatBr20	#120	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatPr8	#118	100	Highly virulent	Puerto Rico	Shwab et al. (2016)
TgCatBr41	#117	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr64	#111	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr177	#109	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr57	#108	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr37	#107	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr34	#104	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCtCo15	#101	100	Highly virulent	Colombia	Shwab et al. (2016)
GUY-DOS	#97	100	Highly virulent	French Guayana	Shwab et al. (2016)
TgCkBr109	#96	100	Highly virulent	Brazil	Shwab et al. (2016)
RUB	#96	100	Highly virulent	French Guayana	Shwab et al. (2016)
GUY-MAT	#95	100	Highly virulent	French Guayana	Shwab et al. (2016)
TgCkBr16	#94	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr61	#93	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr40	#92	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr186	#88	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr72	#85	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr54	#82	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr169	#78	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr141	#77	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr48	#75	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr108	#70	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr76	#67	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr89	#65	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr19	#64	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr13	#63	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCtCo6	#61	100	Highly virulent	Colombia	Shwab et al. (2016)
GUY-KOE	#60	100	Highly virulent	French Guayana	Shwab et al. (2016)
VAND	#60	100	Highly virulent	French Guayana	Shwab et al. (2016)
TgCkBr40	#59	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr45	#56	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr46	#56	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr80	#55	100	Highly virulent	Brazil	Shwab et al. (2016)

Isolate ID	ToxoDB # or available genetic information	Mouse mortality (%)	Lethality*	Country	Reference
South America					
TgDgBr15	#53	100	Highly virulent	Brazil	Shwab et al. (2016)
TgDgBr6	#51	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatPr5	#49	100	Highly virulent	Puerto Rico	Shwab et al. (2016)
TgCatBr25	#47	100	Highly virulent	Brazil	Shwab et al. (2016)
TgDgCo8	#46	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCkBr126	#45	100	Highly virulent	Brazil	Shwab et al. (2016)
TgDgCo11	#44	100	Highly virulent	Colombia	Shwab et al. (2016)
TgDgCo5	#44	100	Highly virulent	Colombia	Shwab et al. (2016)
TgDgCo6	#44	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCatBr19	#42	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr9	#42	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr75	#40	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCtCo10	#38	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCtCo11	#38	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCtCo4	#38	100	Highly virulent	Colombia	Shwab et al. (2016)
TgDgCo17	#38	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCkBr59	#36	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkCr3	#35	100	Highly virulent	Costa Rica	Shwab et al. (2016)
TgCkCr4	#35	100	Highly virulent	Costa Rica	Shwab et al. (2016)
TgCkCr5	#35	100	Highly virulent	Costa Rica	Shwab et al. (2016)
TgCatBr48	#34	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCtCo1	#28	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCkNi9	#27	100	Highly virulent	Nicaragua	Shwab et al. (2016)
TgCkNi4	#23	100	Highly virulent	Nicaragua	Shwab et al. (2016)
TgCkBr38	#22	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr10	#21	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr22	#21	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr31	#21	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr37	#21	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr11	#19	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr16	#19	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr5	#19	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr147	#17	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr148	#17	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr151	#17	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr154	#17	100	Highly virulent	Brazil	Shwab et al. (2016)
CASTELLS	#15	100	Highly virulent	Uruguay	Shwab et al. (2016)
TgCatBr15	#14	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCtCo14	#14	100	Highly virulent	Colombia	Shwab et al. (2016)
TgDgCo12	#14	100	Highly virulent	Colombia	Shwab et al. (2016)
TgDgCo15	#14	100	Highly virulent	Colombia	Shwab et al. (2016)
TgDgCo18	#14	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCkBr153	#14	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr1	#11	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr7	#11	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr52	#11	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr56	#11	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr78	#11	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCtCo7	#10	100	Highly virulent	Colombia	Shwab et al. (2016)
TgCatBr12	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr17	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr2	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr21	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr30	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCkBr144	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr42	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr47	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr53	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr55	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr62	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr71	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgCatBr75	#6	100	Highly virulent	Brazil	Shwab et al. (2016)
TgSb	#14	100	Highly virulent	Argentina	Bernstein et al. (2020)

Isolate ID	ToxoDB # or available genetic information	Mouse mortality (%)	Lethality*	Country	Reference
Africa					
TgCkGh2	#132	0	Non-virulent	Ghana	Shwab et al. (2016)
TgCkGh1	#137	0	Non-virulent	Ghana	Shwab et al. (2016)
TgCkNg1	#15	0	Non-virulent	Nigeria	Shwab et al. (2016)
GAB3-2007-GAL-DOM14	*Africa 1*	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB2-2007-GAL-DOM6	Recombinant	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG6	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM13	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM16	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM17	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM9	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-OVI-ARI3	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-OVI-ARI6	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG1	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG3	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG9	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM11	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-OVI-ARI4	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM5	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG2	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG7	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG8	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM1	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM14	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM15	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM18	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM2	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM6	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM7	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM8	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-OVI-ARI1	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-OVI-ARI2	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-OVI-ARI5	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM4	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM3	Type III	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB4-2007-GAL-DOM1	Type III-like	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG10	Type III-like	0	Non-virulent	Gabon	Mercier et al. (2010)
GAB5-2007-GAL-DOM3	*Africa 3*	33	Intermediate	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM13	Type III	33	Intermediate	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG5	Type III	33	Intermediate	Gabon	Mercier et al. (2010)
GAB1-2007-FEL-CAT1	Type III	50	Intermediate	Gabon	Mercier et al. (2010)
GAB1-2007-OVI-ARI7	Type III	50	Intermediate	Gabon	Mercier et al. (2010)
GAB1-2007-CAP-AEG4	Type III-like	50	Intermediate	Gabon	Mercier et al. (2010)
GAB5-2007-GAL-DOM1	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB5-2007-GAL-DOM2	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM15	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB6-2007-GAL-DOM19	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM1	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM2	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM3	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM4	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM11	*Africa 1*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB2-2007-GAL-DOM4	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB2-2007-GAL-DOM5	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM10	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM16	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM7	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM9	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM6	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM8	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB5-2007-GAL-DOM4	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB5-2007-GAL-DOM5	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB5-2007-GAL-DOM6	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB1-2007-GAL-DOM10	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)

Isolate ID	ToxoDB # or available genetic information	Mouse mortality (%)	Lethality*	Country	Reference
Africa					
GAB8-2007-GAL-DOM12	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB3-2007-GAL-DOM12	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB2-2007-GAL-DOM1	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB2-2007-GAL-DOM3	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB2-2007-GAL-DOM2	*Africa 3*	100	Highly virulent	Gabon	Mercier et al. (2010)
GAB7-2007-GAL-DOM7	Type III	100	Highly virulent	Gabon	Mercier et al. (2010)
Asia					
TgDgSl1	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl15	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl17	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl19	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl2	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl20	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl24	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl3	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl7	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl8	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl9	#4	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl6	#2	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl12	#20	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl13	#20	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl14	#20	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl16	#20	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl22	#20	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgDgSl23	#20	0	Non-virulent	Sri Lanka	Shwab et al. (2016)
TgCtPRC10	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC11	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC12	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC16	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC2	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC4	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC6	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC7	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC8	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC9	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC13	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC15	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC17	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC5	#9	0	Non-virulent	China	Shwab et al. (2016)
TgCtPRC1	#18	0	Non-virulent	China	Shwab et al. (2016)
TgCatJpObi1	#4	0	Non-virulent	Japan	Salman et al. (2021)
TgCatJpOk3	Haplogroup 2	0	Non-virulent	Japan	Fukumoto et al. (2020)
TgCatCHn4	#9	0	Non-virulent	China	Yang et al. (2017b)
TgCtwh6	#9	0	Non-virulent	China	Wang et al. (2013b)
TgCtsd3	#9	0	Non-virulent	China	Wang et al. (2013b)
TgCtsd2	#9	10	Non-virulent	China	Wang et al. (2013b)
TgCatJpOk2	Not determined	20	Non-virulent	Japan	Fukumoto et al. (2020)
TgSpHn2	#9	70	Intermediate	China	Yang et al. (2017a)
TgCatJpOk1	Not determined	80	Intermediate	Japan	Fukumoto et al. (2020)
TgSpHn1	#9	87	Intermediate	China	Yang et al. (2017a)
TgCatJpOk4	Haplogroup 2	100	Highly virulent	Japan	Fukumoto et al. (2020)
TgCtgy5	#9	100	Highly virulent	China	Wang et al. (2013a)
TgCatJpGi1/TaJ	Type III	100	Highly virulent	Japan	Taniguchi et al. (2018)
TgCatJpTy1/k-3	Type II	100	Highly virulent	Japan	Taniguchi et al. (2018)
TgCtwh12	#9	100	Highly virulent	China	Wang et al. (2013b)
TgCtwh14	#9	100	Highly virulent	China	Wang et al. (2013b)
TgCtxz1	#10	100	Highly virulent	China	Wang et al. (2013b)
TgCtxz5	#205	100	Highly virulent	China	Wang et al. (2013b)
TgCtxz8	#205	100	Highly virulent	China	Wang et al. (2013b)

* *Toxoplasma gondii* strains were classified according to mortality in mice into “Highly virulent” (100% mortality); “Intermediate” (99–30%), and “Non-virulent” (Non, <30%) (Su et al., 2002).