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Licenciada em Biologia área científica de Biologia

Contribution for the knowledge of toxoplasmosis in Portugal

Dissertação para obtenção do Grau de Doutor em Biologia

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Setembro 2019

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É o tempo da travessia:

E, se não ousarmos fazé – la,

Teremos ficado, para sempre, à margem de nós mesmos.

Fernando Pessoa

Agradecimentos

Agradeço sinceramente reconhecida,

Ao meu orientador Doutor João Paulo Gomes, Responsável da Unidade Investigação do Departamento de Doenças Infecciosas do Instituto Nacional de Saúde Doutor Ricardo Jorge, pelo incentivo e motivação e pelo rigor científico, sapiência e experiência com que me orientou e enriqueceu este trabalho. Muito obrigada pela tua amizade. Admiro te muito pelo investigador que és!

À minha coorientadora Professora Isabel Sá Nogueira, Professora Associada com Agregação e Coordenadora do Programa Doutoral em Biologia da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, por ter reconhecido valor cientifico na minha tese e a ter aceite no Programa Doutoral que coordena e pelo seu profissionalismo e cordialidade.

Ao Doutor Jorge Machado, Coordenador do Departamento de Doenças Infecciosas do Instituto Nacional de Saúde Doutor Ricardo Jorge, pela iniciativa e reconhecimento da minha capacidade para levar a cabo esta tarefa, pelo encorajamento, pela liderança e pela amizade.

Aos membros da Comissão de Acompanhamento de Tese, Doutora Gabriela Rodrigues, Professora Auxiliar da Faculdade de Ciências da Universidade de Lisboa e Doutor Baltazar Nunes, Investigador do Departamento de Epidemiologia do Instituto Nacional de Saúde Doutor Ricardo Jorge, pela revisão do manuscrito e sugestões concedidas e pelo incentivo e afabilidade.

Ao Doutor Vitor Borges Investigador do Núcleo de Bioinformática do Departamento de Doenças Infeciosas do Instituto Nacional de Saúde Doutor Ricardo Jorge, uma alma antiga numa cabeça de vanguarda, pela sabedoria, amabilidade e maneira única de ser.

À Doutora Helena Ângelo pela mão de quem vim parar a esta ciência e de quem sou sucessora, espero ter estado e continuar a estar à sua altura.

Às minhas amigas e colegas do Laboratório Nacional de Referencia de Infeções Parasitárias e Fúngicas do Departamento de Doenças Infecciosas do Instituto Nacional de Saúde Doutor Ricardo Jorge, que trabalharam, me acompanharam, encorajaram e animaram em todo o período da tese bem como em todos os dias dos últimos 16 anos; Idalina Ferreira, com quem dei os primeiros passos no mundo da parasitologia, com quem fiz as primeiras incursões no biotério *com os nossos ratinhos* e de quem ouvi e oiço tantos conselhos sábios, obrigada de todo o coração por tudo isto e pela sua amizade, é insubstituível; Anabela Vilares uma das minhas grandes lutas, pela forma entusiasta, sabedoria e rigor com que trabalhas, pelo ânimo e paixão pelos parasitas, pela

disponibilidade e amizade que nunca me falharam, Susana Martins pela entrega com que trabalhas e pela juventude que trazes a esta equipa, Tania Reis pelo profissionalismo, pela serenidade e pela disponibilidade sempre pronta, Cristina Verissimo, Helena Simões e Raquel Sabino pelas palavras de apoio e motivação que sempre me disseram e por fim mas não no fim, Assunção António pelos *Bons dias* mais afáveis do INSA e pelo optimismo que sempre me transmite.

Aos meus amigos Raquel Guiomar e Nuno Verdasca, companheiros, confidentes e conselheiros há quase 25 anos, muito obrigada pela ajuda sempre voluntária, pelo apoio incondicional, pela vossa solidariedade e amizade sincera. Raquel a tua opinião é muito importante para mim, Nuno não podia viver sem as tuas formatações e ajustes informáticos.

Aos meus familiares e amigos que me acompanham sempre.

À memória dos meus avós queridos.

Aos meus pais que gostam de mim mais do que eu própria e me mimam como se eu ainda tivesse cinco anos, adoro viver com a graça, o humor, as histórias, a cultura e o mimo de um; a sensatez, a retaguarda, a sabedoria, os auspícios e o mimo do outro. São o meu pilar.

Aos meus três filhos únicos Joana Maria, António Maria e Francisco Maria, gigantes amores da minha vida, a vossa existência, as vossas conversas, lógicas, ideias, piadas, gargalhadas e até o vosso mau feitio, atestam-me a alma e inspiram me todos os dias.

Ao cúmplice de uma vida, o meu marido, Miguel, que me conhece como só a minha mãe, que testemunha, acompanha e engrandece a minha vida desde há muitos anos.

Resumo

O parasita *Toxoplasma gondii* infecta mais de um terço da humanidade sendo a toxoplasmose uma ameaça permanente, uma vez que os seres humanos permanecem infectados a vida toda. Esta tese de doutoramento teve como objetivo enriquecer o conhecimento sobre a toxoplasmose em Portugal e implementar abordagens optimizadas para a propagação de *T. gondii* no laboratório de referência.

Começámos por avaliar a seroprevalência da população portuguesa comparando três estudos transversais ao longo de três décadas (1979/80, 2001-2002, 2013), com especial foco nas mulheres em idade fértil. Observámos uma tendência decrescente da seroprevalência ao longo do tempo (de 47 % em 1979/80 para 22 % em 2013), aumentando esta com a idade. O cenário observado para as mulheres em idade fértil indica que mais de 80 % são suscetíveis à infecção primária encontrando-se assim em risco de contrair toxoplasmose.

Focámo-nos também no estudo do parasita e caracterizámos geneticamente 48 estirpes isoladas a partir de amostras biológicas de pacientes diagnosticados no Instituto Nacional de Saúde (INSA), para os quais efetuámos uma avaliação retrospectiva que estimou 1,6% de novos casos de toxoplasmose congénita nos últimos 10 anos. Este estudo revelou variações genéticas nas estirpes de *T. gondii* causadoras de infecção, mais especificamente, a existência de uma proporção considerável (21 %) de estirpes recombinantes, que se acredita estarem associadas a fenótipos específicos.

Finalmente, avaliámos diversas abordagens laboratoriais com o objectivo de reduzir a utilização do número de ratinhos sacrificados na actividade laboratorial de referência. Concluímos que a propagação de uma forma alternada do parasita numa uma linhagem celular e em ratinhos constitui um procedimento laboratorial promissor, pois, para além de reduzir o número de animais sacrificados em mais de 80 %, permite também que *T. gondii* não perca a sua virulência, mantendo potencialmente o seu genoma inalterado.

Globalmente, esta tese de doutoramento não só contribuiu para o conhecimento da toxoplasmose em Portugal, ao nível do individuo, elucidando a tendência cronológica do estado imunitário da população portuguesa, bem como ao nível do parasita, identificando o perfil genético das estirpes de *T. gondii* circulantes causadoras de infecção humana e a sua virulência no ratinho. Finalmente, esta dissertação de doutoramento estabeleceu procedimentos que modificaram o *modus operandi* do laboratório de referência que visam uma redução significativa do número de ratinhos sacrificados.

Palavras-chave *Toxoplasma gondii*; Genotipagem; Estirpes recombinantes; Toxoplasmose congénita; Seroprevalencia; Vigilância laboratorial.

Abstract

Toxoplasma gondii infects over a third of the world's humanity and toxoplasmosis constitutes a life-long threat since humans remain infected throughout life. This PhD dissertation aimed to enrich the knowledge on toxoplasmosis in Portugal and to implement updated approaches regarding *T. gondii* propagation in the reference laboratory.

We started by evaluating the seroprevalence in Portugal by comparing three cross-sectional studies spanning three decades (1979/80, 2001-2002, 2013), with focus on childbearing women. Seroprevalence showed a decreasing trend over time (from 47 % in 1979/80 to 22 % in 2013) and increased with age. The scenario observed for childbearing women indicates that more than 80 % of these are susceptible to primary infection and thus to congenital toxoplasmosis.

We also focused on the parasite and genetically characterized 48 strains isolated from biological samples from patients attending to the NIH, for which a retrospective evaluation estimated 1.6 % of new cases of congenital toxoplasmosis in the last 10 years. This study revealed genetic variations in *T. gondii* and more specifically the existence of a considerable proportion (21 %) of recombinant strains, which are believed to be associated with specific phenotypes.

Finally, we evaluated laboratory approaches towards the reduction of sacrificed mice in toxoplasmosis reference laboratories. We observed that the alternate passaging of the parasite in a cell line and in mice constitutes a promising laboratory procedure as, besides the reduction of sacrificed mice in more than 80 %, it enabled *T. gondii* to retain the virulence potential while keeping a putative stable genome.

Globally, this PhD dissertation not only increased the knowledge on toxoplasmosis in Portugal by elucidating the chronological trend of the immune status of the population and the general genetic profile of the *T. gondii* strains causing human infection, but it also modified the *modus operandi* of the reference laboratory towards the significant reduction of scarified mice.

Keywords *Toxoplasma gondii*; Genotyping; Recombinant strains; congenital toxoplasmosis; Seroprevelenve; Laboratory surveillance.

Table of Contents

Agradecimentos VII
Resumo IX
Abstract XI
Table of contents XIII
Figure index XV
Table index XVI
List of abbreviations XVII
Notes of the author: thesis organization, format and outline XIX

1. Chapter I: General Introduction 1

1.1. *Toxoplasma gondii,* the parasite 3

1.1.1. Historical background and taxonomy 3

1.1.2. Biology 5

1.1.3. Life Cycle 6

1.1.4. Mechanisms of cell invasion 8

1.1.5. The Genome 10

1.1.6. Genetic variation and disease severity 12

1.1.7. Assessment of strain virulence 15

1.2. Toxoplasmosis, the human disease 16

1.2.1. Pathophysiology 16

1.2.2. Transmission 16

1.2.3. Clinical Features 17

1.2.3.1. The immunocompetent patient 17

1.2.3.2. The immunosuppressed patient 18

1.2.3.3. Ocular disease 18

1.2.3.4. Congenital toxoplasmosis 19

1.2.4. Diagnosis 20

1.2.4.1. The immunocompetent patient 20

1.2.4.2. The immunosuppressed patient 21

1.2.4.3 Ocular disease 21

1.2.4.4. Congenital Toxoplasmosis 21

1.2.5. Prevention and Treatment 22

1.2.5.1. The immonocompetent patient 23

1.2.5.2. The immunosuppressed patient 23

1.2.5.3. Ocular disease 23

1.2.5.4. Congenital toxoplasmosis 23

1.2.6. Epidemiology and Burden of disease 23
1.3. State of the Art 25
1.4. Aims of the thesis 26

2. Chapter II: ***Toxoplasma gondii*** **seroprevalence in the Portuguese population: comparison of three cross-sectional studies spanning three decades** 27
2.1. Abstract 29
2.2. Introduction 29
2.3. Material and Methods 31
2.3.1. Study design and sampling 31
2.3.2. Serological analyses 32
2.3.3. Statistical analysis 33
2.4. Results 33
2.5. Discussion 37

3. Chapter III: Genetic and virulence characterization of ***Toxoplasma gondii*** **isolates causing human infection in Portugal** 43
3.1. Abstract 43
3.2. Introduction 44
3.3. Material and Methods 46
3.4. Results 48
3.5. Discussion 52

4. Chapter IV: Parallel propagation of *Toxoplasma gondii* ***in vivo*****,** ***in vitro*** **and alternate models: towards less dependence on the mice model**. 55
4.1. Abstract 57
4.2. Introduction 58
4.3. Material and Methods 60
4.4. Results 65
4.5. Discussion 69

5. Chapter V: Final overview, concluding remarks and future perspectives 73

References 81

Supplementary Material 97

Figure índex

Figure 1.1 Graphic representation of a tachyzoite. 6

Figure 1.2 A) Bradyzoites inside a cyst of a laboratory mouse brain (100 x, 40 x e 10 x objective), B) Free stained tachyzoites on laboratory mouse ascites, C) Sporolated and unsporulated Oocysts. 6

Figure 1.3 Life cycle of *Toxoplasma gondii*. 8

Figure 1.4 Mechanism of cell invasion. 10

Figure 1.5 Genetic linkage maps for the 14 chromosomes of *Toxoplasma gondii*. 12

Figure 1.6 Clinical signs. A) Cerebral calcifications, B) Retina lesions due to ocular toxoplasmosis C) Newborn with hydrocephalus. 19

Figure 2.1 (A and B) (A) shows the evolution of *Toxoplasma gondii* seroprevalence in Portugal over the past three decades; (B) shows the sample sizes for the three National Serological Surveys, the precise estimated seroprevalences and respective 95% confidence interval (CI). 34

Figure 2.2 Comparison of *Toxoplasma gondii* seroprevalence trends, according to the data from three National Serological Surveys, by age groups. 36

Figure 3.1 Annual distribution of acquired and congenital *Toxoplasma gondii* infection, 2009-2018. Number of cases by year of diagnosis. 49

Figure 3.2 Annual distribution of *Toxoplasma gondii* infection acquired by gender, 2009-2018. Number of cases by year of diagnosis. 49

Figure 4.1 Workflow of RH strain inoculations. 61

Figure 4.2 Experiment A: Continuous RH strain mice propagation. 65

Figure 4.3 Phenotypic evaluation on the course of experiment A. 65

Figure 4.4 Phenotypic evaluation on the course of experiment B. 66

Figure 4.5 Experiment B: Continuous RH strain cell line propagation. 67

Figure 4.6 Experiment C: intercalate passages of the RH strain in the cell line and in mice. 68

Figure 4.7 Comparison of tachyzoites yield between propagation experiments. 68

Table índex

Table 1.1 Geographical distribution of *Toxoplasma gondii* genotypes and potencial relationships with human disease. 15

Table 2.1 Trends of *Toxoplasma gondii* seroprevalence in Portugal by region calculated with an interval confidence of 95 %. 34

Table 2.2 Multivariate analysis adjusted for gender, age group and region. 35

Table 2.3 *Toxoplasma gondii* seroprevalence by gender and year of analysis calculated with an interval confidence of 95 %. 36

Table 2.4 *Toxoplasma gondii* seroprevalence in childbearing women by age group calculated with an interval confidence of 95 %. 37

Table 3.1 Annual distribution of acquired and congenital *Toxoplasma gondii* infection by age group, 2009-2018. Number of cases by year of diagnosis. 50

Table 3.2 Virulence and genotyping analysis of *Toxoplasma gondii* strains and laboratorial and clinical features of the biological samples/cases. 51

Table 4.1 Loci, amplicon length and primers used for PCR. 64

Supplementary Table S 4.1 BioProject PRJEB34235 - European Nucleotide Archive (ENA) accession numbers. 99

List of Abbreviations

Throuout this PhD dissertation acronyms are extended upon first usage.

AIDS	Acquired Immunodeficiency Syndrome
AF	Amniotic fluid
AH	Aqueous humor
ATCC	American Type Culture Collection
BAL	bronchoalveolar lavage fluid
BB	Baby blood
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CB	Cerebral biopsy
CI	Confidence intervals
CSF	Cerebrospinal fluid
CG	Cytosine-guanine
cM	Centimorgan
DALY's	Disability-adjusted life- years
DAT	Direct Agglutination test
DMEM	Dulbecco's Modified Eagle Medium
DNA	Desoxyribonucleic acid
DGS	Direção Geral de Saúde
ELFA	Enzyme-Linked Fluorescent Assay
ELISA	Enzyme Linked Immunosorbent assay
Gra	Dense granule protein
HCL	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HFF	Human Foreskin Fibroblast
IB	Imunoblot
ISAGA	Immunoglobulin Immunosorbent Agglutination Assay
Mb	Mega base pair
MLST	Multilocus Sequence Typing
Ms	Microsatellites
NB	Newborn
NBB	Newborn blood
NGS	New Generation Sequencing

NIH	National Institute of Health
NV	Non virulent
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
p.i.	Post-inoculation
PL	Placenta
QALYs	Quality-adjusted life-years
RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphisms
Rop	Roptry
Sag	Surface antigen
SNP	Single Nucleotide Polymorphism
TgMA	Myosine A
TUB2	Beta Tubulina
UCB	Umbilical cord blood
T. gondii	*Toxoplasma gondii*
WHO	World Health Organization
WGS	Whole Genome Sequence
VH	Vitreous humor
USA	United States of America

Notes of the author: thesis organization, format and outline

The work performed in this PhD thesis was carried out at the National Reference Laboratory of Parasitic and Fungal Infections, Department of Infectious Diseases, National Institute of Health Doctor Ricardo Jorge, Lisbon, Portugal.

The main body of this Ph.D. dissertation is based on three manuscripts (listed below) that are presented as individual chapters (II to IV). Two of them have already been published (the remaining one is submitted for publication at the time this thesis was completed) in peer reviewed international journal, being presented in this thesis essentially as a reproduction of the content that was published. These three manuscripts reflect the objectives delineated for this Ph.D. thesis and consequently the experimental studies performed throughout the author Ph.D. work, also the order of presentation of these chapters reflet the chronological order of the publication of the respective manuscripts. Besides, these manuscript-based chapters, each one including extensive and specific introduction, material and methods, results and discussion sections. The present doctoral dissertation still encompasses a general introduction (chapter I) and a conclusive overview of the main findings and conclusions, with regards to future perspectives and lines of work (chapter V). In brief, each chapter includes the following contents:

Chapter I

This chapter consists of a general introduction that intents to provide the reader an global overview of the major *Toxoplasma gondii* features such as, historical background, taxonomy, biology, life cycle, mechanisms of cell invasion, genome and genetic variation; and also the pathophysiology, transmission, clinical features, diagnosis, treatment and epidemiology of Toxoplasmosis, the infectious disease caused by *Toxoplasma gondii*. The state of the art that express the reasons why the author selected the objectives of this Ph.D. project and the main objectives of this doctoral dissertation are listed at the end of this chapter.

Chapter II

Toxoplasma gondii seroprevalence in the Portuguese population: comparison of three cross-sectional studies spanning three decades. This chapter corresponds to a manuscript with the following reference: BMJ Open. 2016; 6(10): e011648. "*Toxoplasma gondii* seroprevalence in the Portuguese population: comparison of three cross-sectional studies spanning three decades." Maria João Gargaté, Idalina Ferreira, Anabela Vilares, Susana Martins, Carlos Cardoso, Susana Silva, Baltazar Nunes, João Paulo Gomes. Published online 2016 Oct 5. doi: 10.1136/bmjopen-2016-011648.

Chapter III

Genetic and virulence characterization of *Toxoplasma gondii* isolates causing human infection in Portugal. Part of this chapter corresponds to a manuscript with the following reference, so some sections constitute a faithful reproduction of what was published: Parasitol Res (2017) 116:979–985. "Molecular and virulence characterization of *Toxoplasma gondii* strains isolated from humans in Portugal." Anabela Vilares, Maria João Gargaté, Idalina Ferreira, Susana Martins, João Paulo Gomes. doi: 10.1007/s00436-017-5374-5.

Chapter IV

Contains the following study that was recently submitted for publication in a *peer reviewed* international journal (Parasitology Research):"Parallel propagation of *Toxoplasma gondii in vivo*, *in vitro* and alternate models: towards less dependence on the mice model". Maria João Gargaté, Anabela Vilares, Idalina Ferreira, Tania Reis, Susana Martins, Vitor Borges, João Paulo Gomes. 2019.

Chapter V

This chapter provides a global overview of the subjects addressed throughout the chapters, where the main findings and conclusions achieved in this Ph.D. thesis are highlighting. Of note, in order to avoid redundancies, this section solely summarizes and discusses the major findings of this work because a detailed discussion of specific results was already provided in each chapter. Future perspectives and follow-up of these investigations are also presented. Considering the different layouts required by the different journals where the manuscripts were published, including tables, figures and references, all chapters were formatted in a unique style, with all references being cited by the name of the first author according alphabetical order and year of the publication and listed in a single section denominated "References". The supplemental material is presented at the final of this Ph.D. thesis, enumerated accordingly with the chapter they concern to in a section referred as "Supplementary material".

Chapter I

General Introduction

1. General Introduction

1.1. *Toxoplasma gondii*, the parasite

1.1.1. Historical background and taxonomy

The *Toxoplasma gondii* (*T. gondii*) is an apicomplexan protozoan parasite and one of the most successful parasites worldwide due to its ability to infect all warm blooded animals including humans. One third of the world's human population is assumed to be infected with *T. gondii* (Louis M. Weissa; Weiss and Dubey, 2009; Innes, 2010). This ubiquitous obligate intracellular organism was first discovered in 1908 by Charles Nicolle and Louis Manceaux at the Pasteur Institute in Tunis, who found the parasite in the liver and spleen of a North African rodent, named *gundi* (*Ctenodactylus gundi*) and initially presumed it was a species of *Leishmania*. In the same year, Alfonso Splendore, a Brazilian scientist discovered the parasite in a rabbit (*Oryctolagus cuniculus*) and again mistakenly identified it as *Leishmania* (Ferguson D, 2009). However in 1909 following experimental infection and microscopic analysis, the parasite was renamed to *Toxoplasma gondii* as described by Nicolle and Manceaux (Nicolle and Manceaux, 1909; Ferguson D, 2009) due to the bow shaped morphology of the extracellular stage of the parasite - tachyzoite; *Toxo* is derived from Greek for bow, *plasma* meaning life and *gondii* because its original host was *C. gundi*. In 1914 Castellani was probably the first to describe a *T. gondii*–like parasite in smears of the blood and spleen from a 14-year-old boy from Ceylon who died from a disease characterized by severe anemia, fever and spleenomegaly. (Louis M. Weissa; Cheng *et al.*, 2015).

In 1923, Janků observed parasitic cysts in the retina of an eleven month old child who was suffering from hydrocephalus (Janků, 1928). In the same year the first identified case of congenital toxoplasmosis was reported by Wolf and Cowen from a 3 day old child who had developed seizures (Wolf *et al.*, 1939). The baby only survived for one month and following post mortem cerebral calcification, retinochoroiditis, and hydrocephalus were observed. In this same year Sabin isolated *T. gondii* from two children from Cincinnati, named R.H. (initials of the patients name), aged 6 years old and the other named W.B.D. aged 8 years, with encephalitis (Sabin, 1941). This strain, designated "RH" became the laboratory prototypical Type I strain and since 1938 it has been passed in mice in many laboratories worldwide. In 1941, Pinkerton and Henderson reported atypical pneumonia on two adults who died and in whom they demonstrated *T. gondii* as the etiological agent. These were the first reports of acute toxoplasmosis in adults without neurological signs. In the 1950's *T. gondii* parasites were discovered in enucleated eyes (Wilder, 1952), and this type of ocular toxoplasmosis was presumed to be a consequence of congenital transmission of the parasite. However, more recent studies have also described a

greater number of cases than expected of ocular toxoplasmosis due to postnatal acquired infection (Montoya and Remington, 1996; Burnett *et al.*, 1998; Gilbert *et al.*, 2008).

The development of a serological test, the *dye test*, in 1948 by Albert Sabin and Harry Feldman was a major advance in the study of toxoplasmosis (Sabin and Feldman, 1948). The ability to identify *T. gondii* infections based on this serological method allowed epidemiological studies on the incidence of infection, demonstrating the widespread world-wide prevalence of this infection in humans. It also allowed the identification of clinical signs compatible with the diagnosis of congenital toxoplasmosis (Sabin and Feldman, 1948; Feldman and Miller, 1956). As such, Sabin determined that the simultaneous occurrence of clinical signs of hydrocephalus or microcephalus, intracerebral calcification and chorioretinitis, could be used to identify cases of congenital toxoplasmosis, what is nowadays known as the classical triad of symptoms of congenital toxoplasmosis (Sabin, 1941, 1942).

In 1965 Desmonts and colleagues confirmed the transmission by the carnivorous route, which had been previously presupposed by Weinman and Chandler (Weinman and Chandler, 1954) and by Jacobs et al. (1960). In 1972, Wallace and his colleagues began epidemiological surveys in regions where habitants ate raw or undercooked meat and observed a high frequency of infection in humans, demonstrating the transmission route through carnivorism. Anteriorly in 1969, Kean et al. described the first outbreak of toxoplasmosis in Cornell University medical students after eating insufficiently cooked hamburgers. However, in 1959 Rawal in a study in vegetarians observed a frequency equivalent to that found in carnivores, and the diffusion of the parasitizes in this group was not clarified. In 1970 the cycle of this parasite is definitively clarified, with the discovery by Dubey et al. of the sexual development of *T. gondii* in the intestine of cats (Frenkel *et al.*, 1970), thus felids are still presumed to be the only definitive host. An important step in the history of *T. gondii* occurred in the 1980's when AIDS patients were found to develop clinical symptoms of the parasite (Luft and Remington, 1992). *T. gondii* was identified as a major opportunistic infection for these immunocompromised patients, where either newly acquired infection or recrudescence of latent infection would frequently cause encephalitis (Luft and Remington, 1992).

The most recent developments for *T. gondii* are its possible effect on behavior changes in both animals (Berdoy *et al.*, 2000; Ingram *et al.*, 2013), and humans. Although the link to *T. gondii* infection and behavioral problems in humans is not completely clear, several reports have associated infection to schizophrenia (Torrey *et al.*, 2012), increased risk taking and road traffic accidents (Flegr *et al.*, 2009) and an increased risk of suicide (Lester, 2012). Also, the emergence of genetically different strains of the parasite have been linked to several fatal cases of acquired

infection in immuno-competent individuals (Carme *et al.*, 2002, 2009; Ajzenberg *et al.*, 2016), further highlighting the potential public health risk of the parasite.

According to the current taxonomic classification, *T. gondii* belongs to the *Kingdom Protista*, *sub - Kingdom Protozoa* (Goldfuss, 1918), *Phylum Apicomplexa* (Levine, 1970), *Class Sporozoasida* (Leukart, 1879), *Subclass Coccidiasina* (Leukart, 1879), *Order Eimeriorina* (Leger, 1911), *Family Toxoplasmatidae* (Biocca, 1956) (Hill *et al.*, 2005; Dubey, 2010) and g*enus Toxoplasma* (Nicolle and Manceaux, 1909). There is only one species, *T. gondii*; one of the most successful parasitic organisms.

1.1.2. Biology

There are three infective stages of *T. gondii*: a rapidly dividing invasive tachyzoite (Figure 1.1), a slowly dividing bradyzoite in tissue cysts, and an environmental stage, the sporozoite, protected inside an oocyst (Figure 1.2). Tachyzoites are crescent-shaped cells, approximately 5 µm long and 2 µm wide, with a pointed apical end and a round posterior end. They are limited by a complex membrane, named the pellicle, closely connected with a cytoskeleton involved in the structural integrity and motility of the cell. They possess a nucleus, a mitochondria, a Golgi complex, ribosomes, an endoplasmic reticulum, and a multiple-membrane-bound plastid-like organelle called the apicoplast (Roos *et al.*, 1999). As other members of the phylum Apicomplexa, they concentrate in their apical area a specialized cytoskeletal structure (the conoid, implicated in cell invasion) and numerous secretory organelles (rhoptries [ROPs], dense granules, and micronemes). Tachyzoites are the dissemination form and they are able to invade cells of all vertebrate, where they multiply in a parasitophorous vacuole. Bradyzoites result from the conversion of tachyzoites into a slow-dividing stage and form tissue cysts. These cysts are spheroid in brain cells or elongated in muscular cells, vary in size and can contain only two bradyzoites or thousands with a latent metabolism. Cysts remain intracellular throughout their lifetime and the death of the host cell may produce the disruption of the cyst wall and the consequent liberation of bradyzoites. The resistance of bradyzoites to the acid pepsin (1- to 2-h survival into pepsin-HCl) allows their transmission through ingestion. Oocysts are ovoid structures with two sporocysts (after sporulation) and with an exceptionally resistant double wall that enables the parasite to survive for long periods in adverse conditions. (Robert-Gangneux and Dardé, 2012).

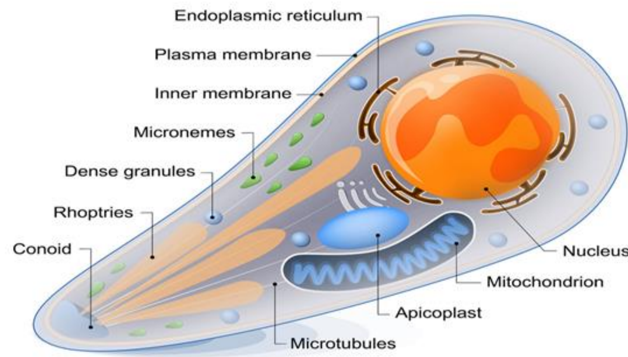


Figure 1.1 Graphic representation of a tachyzoite.
Image source: VectorStock.com/11563382.

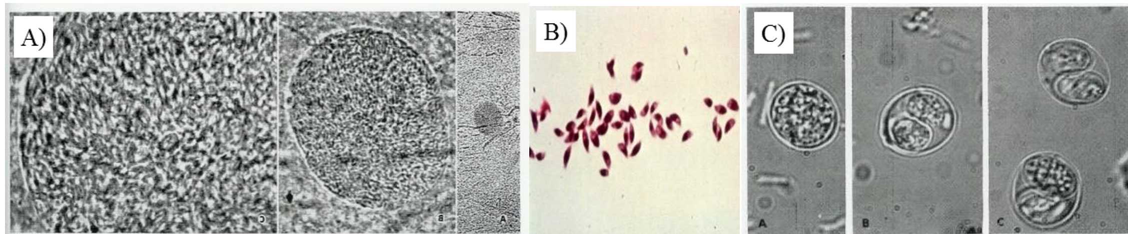


Figure 1.2 A) Bradyzoites inside a cyst of a laboratory mouse brain (100x, 40x e 10x objective), B) Free stained tachyzoites on laboratory mouse ascites, C) Sporolated and unsporulated Oocysts.
Image source: National Reference Laboratory of Parasitic and Fungal Infections.

1.1.3. Life cycle

The life cycle of the parasite was fully understood with the discovery of *T. gondii* oocysts in cat faeces (Hutchison *et al.*, 1969, 1971) it evidenced the central role of the cat as the only definitive host harboring the sexual developmental stages within the small intestine and spreading millions of oocysts through feces to the environment. The life cycle (Figure 1.3) consists of asexual reproduction in the intermediate hosts (including humans) and sexual reproduction in the intestinal mucosa of the definitive host. A unique feature that characterizes *T. gondii* life cycle is that it can be transmitted not only between intermediate and definitive hosts (sexual cycle) but also between intermediate hosts via carnivorism (asexual cycle) or even between definitive hosts. Oocysts take 3-7 days to sporulate in the environment and become infective by meiosis process leading to the formation of a sporulated oocyst with two sporocysts, each containing four haploid sporozoites (Holliman *et al.*, 2003). Intermediate hosts in nature (including birds and rodents) become infected after ingesting soil, water or plant material contaminated with these oocysts. After oocyst ingestion, sporozoites are released and penetrate the intestinal epithelium. Then, they transform into tachyzoites and become surrounded by a parasitophorous vacuole that protects

them from host defense mechanisms. The tachyzoite multiplies asexually by endodygeny and they spread first to mesenteric lymph nodes and then to the several organs by invasion of the bloodstream (Hill *et al.*, 2005; Jones and Dubey, 2010). After several multiplication cycles, tachyzoites give rise to bradyzoites, and tissue cysts arise as early as 7 to 10 days post infection and may remain throughout lifetime in the hosts, predominantly in the brain or muscles (Jones and Dubey, 2010; Dubey, 2010; Robert-Gangneux and Dardé, 2012). After the ingestion by a cat of cysts present in tissues of an intermediate host, gastric enzymes destroy the cyst wall. Bradyzoites settle within enterocytes, where they undergo a number of asexual multiplications, with the development of merozoites within schizonts. This process is followed by the formation of male and female gametes (gametogony) (Ferguson, 2002). After fertilization, oocysts formed within enterocytes are released by the disruption of the cell and are excreted as unsporulated forms in cat feces. On the other hand, after the ingestion of the tissue cysts by an intermediate host through raw or undercooked meat, cysts are ruptured as they pass through the digestive tract, causing the release of bradyzoites. The bradyzoites will infect the intestinal epithelium of the new host and differentiate back into the rapidly dividing tachyzoite stage for dissemination throughout the body. In addition, if the acute phase occurs during pregnancy, the parasite can cross the placenta and infect the fetus (congenital transmission) (Robert-Gangneux and Dardé, 2012).

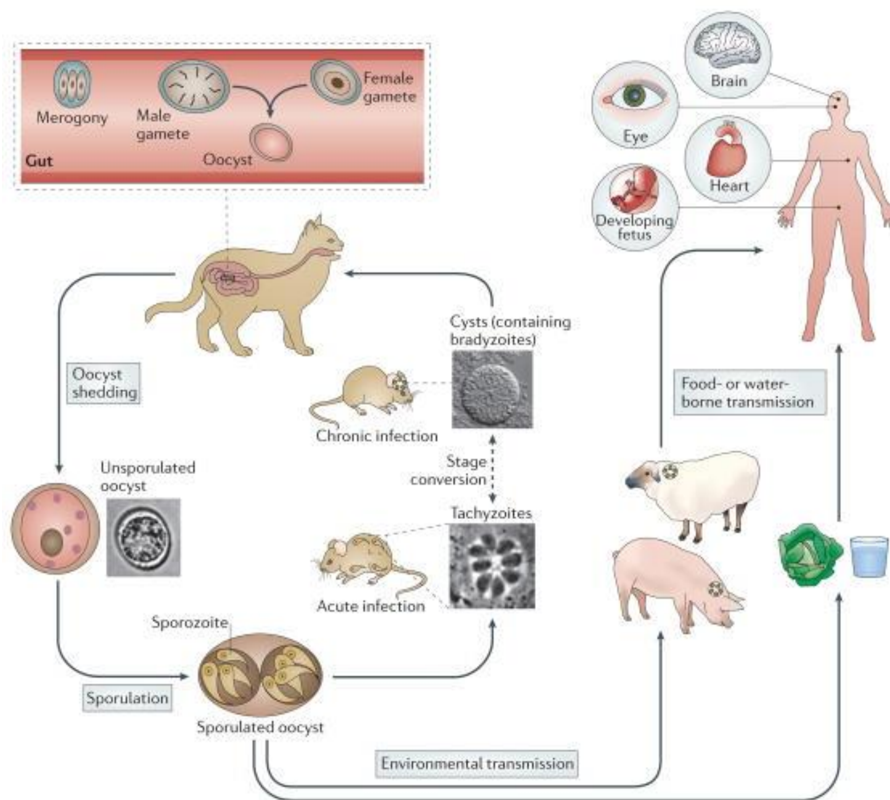


Figure 1.3 Life cycle of *Toxoplasma gondii*. Cats are the definitive host where sexual replication takes place. Following replication within enterocytes of the gut (merogony), male and female gametes are formed within the host cell. Fusion of gametes leads to the formation of diploid oocysts that are shed in cat faeces and undergo meiosis in the environment to yield eight haploid progeny. Oocysts contaminate food and water, providing a route of infection for intermediate hosts. In the intermediate host (birds and rodents) asexual replication occurs. Acute infection is characterized by fast replicating tachyzoites that disseminate throughout the body. Differentiation to slow-growing bradyzoites within tissue cysts leads to long-term chronic infection. Ingestion of tissue cysts via omnivorous or carnivorous feeding can lead to transmission to other intermediate hosts or to cats, which re-initiates the sexual phase of the life cycle. Many animals serve as intermediate hosts, including farm animals. Humans become infected by eating undercooked meat containing tissue cysts or by the ingestion of oocysts in contaminated water (de Moura *et al.*, 2006; Jones and Dubey, 2012). *T. gondii* can infect the brain and other organs as well as the foetus following a congenital infection.
Image adapted from Hunter and Sibley. Nature Reviews Microbiology 2012 Nov; 10:766-778 (Hunter and Sibley, 2012).

1.1.4. Mechanism of cell Invasion

T. gondii is a successful parasite because it can spread across many biomes and species, and has developed specialized processes to invade and replicate efficiently within cells. It is an obligate intracellular parasite, implying that it cannot survive outside a cell, which provides the parasite a safe, secure home full of nutrients and a refuge from the immune system of the host (Tosh *et al.*, 2016). Invasion is an active process based on parasite motility and the sequential secretion of proteins from specialized secretory organelles, the micronemes, the rhoptries, and the

dense granules. The micronemes and rhoptries are localized at the apical end of the parasite, where they function to form the anchor and tight junction to host cell (Huynh *et al.*, 2006; Dlugonska, 2008). Secreted dense granule synthesizes proteins to facilitate the remodeling of host processes to help replication of the parasite (Bougdour *et al.*, 2014). A fourth organelle, the apicoplast that contains essential metabolic pathways is also envolved in the cell invasion process (Arisue and Hashimoto, 2015). *T. gondii* invasion starts with parasite attachment to the host cell plasma membrane. First, the parasite contacts its apical end to the plasmatic membrane of a cell and then secretes proteins that will promote parasite adhesion. It requires the calcium-dependent secretion of adhesins from micronemes, such as the microneme protein MIC2, which recognize host cell receptors and promote parasite reorientation and attachment. Cell invasion relies on a complex interaction between the host cell surface and the parasite, a process called *gliding motility,* a complex motor system promoted by actin-myosin interactions and dynamic rearrangements of the parasite cytoskeleton (Carruthers and Boothroyd, 2007).

Entry is a rapid process (15 to 30 s), apical membrane antigen (AMA1) secreted from micronemes and the secretion of rhoptry (ROP) neck proteins (RONs) inserted into the host cell membrane (Dubremetz, 2007) plays part in the anchoring process and creation of a tight junction between the parasite and the host cell plasma membranes (Blader *et al.*, 2015), called the moving junction. The moving junction complex (AMA1-RON2) allows for host cell membrane invagination and movement of the parasite into the cell (Dobrowolski and Sibley, 1996; Håkansson *et al.*, 2001). When the parasite fully enters the host cell, it is covered by a membrane made of a conglomerate of host lipid bilayer and secreted parasite lipids and proteins, resulting in a specialized vacuole called the parasitophorous vacuole (PV) (Suss-Toby *et al.*, 1996). Host transmembrane proteins and proteins found in lipid rafts are excluded during the formation of the PV (Blader *et al.*, 2015). The PV will be the focal point of interaction with the host cell, through which the parasite will import nutrients and export secreted proteins to create an environment ready for replication (Blader and Koshy, 2014). Soon after invasion, the PV localizes in the perinuclear region and associates with several host organelles, including the endoplasmic reticulum, Golgi complex, and mitochondria (de Melo *et al.*, 1992; Sinai *et al.*, 1997; Walker *et al.*, 2008). The formation of the nascent parasitophorous vacuole membrane (PVM) requires the secretion of proteins from the ROPs. ROP18 one of these proteins is associated with the cytosolic face of the PVM and exerts protein kinase activity, which has a profound effect on parasite growth and virulence (El Hajj *et al.*, 2007), and ROP16 is able to manipulate host gene expression, affecting interleukin secretion (Laliberté and Carruthers, 2008). Within the PV, tachyzoites divide during a 6 to 9 h cycle, by endodyogeny and they exit the cell usually after 64 to 128 parasites have accumulated in the PV (Black and Boothroyd, 2000). The egress of *T. gondii* tachyzoites from the host cell is performed through the rupture of the PV and the host cell plasma membrane,

releasing free parasites in the medium an active process that is dependent upon a rise in the calcium concentration (Sibley, 2010). This process can be triggered by parasite produced abscisic acid or other vacuole acidification and/or by the NTPases segregation. Egress occurs within minutes and once outside the infected host cell, the parasites use gliding motility to move and invade a new cell (Håkansson *et al.*, 1999; Heintzelman, 2015; Periz *et al.*, 2017) (Figure 1.4).

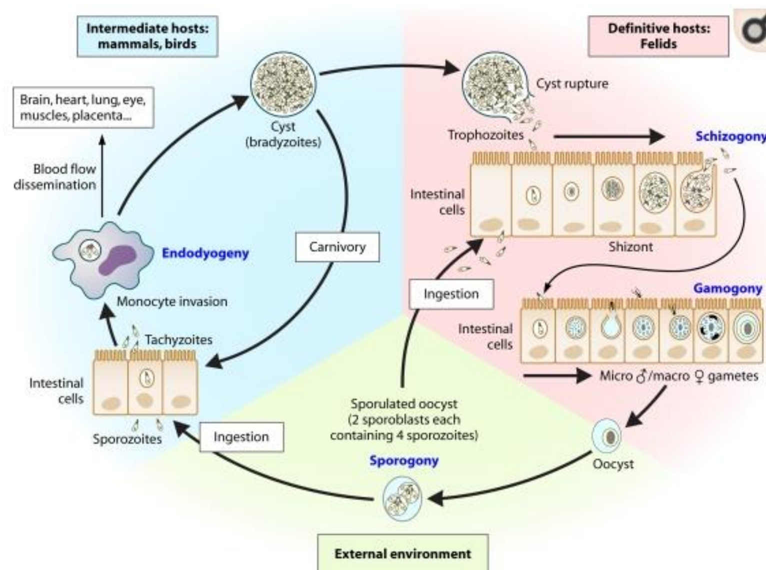


Figure 1.4: Mechanism of cell invasion.
Image source: Clinical Microbiology Reviews (Robert-Gangneux and Dardé, 2012).

1.1.5. The Genome

T. gondii has a ~65 Mb genome, comprising of 14 chromosomes which range from approximately 2 Mb – 7.5 Mb as shown in Figure 1.5 (Khan *et al.*, 2005). The genome is closely related to another apicomplexan protozoan parasite, *Neospora caninum (N. Caninum)*, and it is thought that around 28 million years ago the two parasites diverged from a common ancestor, due to the speciation of the definitive hosts (cats - *T. gondii*, dogs - *N. Caninum*) (Reid *et al.*, 2012). In comparison to other apiocomplexan parasites, such as *Cyptosporidium parvum* (*C. Parvum*) and *Theileria parva* (*T. parva*), the genome of *T. gondii* is significantly larger, it contains more introns, more predicted genes and has a lower gene density (Delbac *et al.*, 2001). One possible reason for the difference in size compared to other apicomplexans can be due to the large number of secondary hosts which this parasite is able to establish within (Roos, 2005). In 2005, a composite genome map was derived from genetic crosses and linkage analysis of the three main archetypal *T. gondii* lineages (I, II, III) (Khan *et al.*, 2005). The genetic linkage map that was generated identified 250 species specific markers, of which 12 are most commonly used for strain

genotyping by PCR-RFLP. The Figure 1.5 details each of these individual markers and also highlights markers which are strain specific and those which are present on all three archetypal strains. A fourth clonal lineage, designated haplotype 12, is largely confined to North America and more common in wild animals (Khan *et al.*, 2011) was designated. Su and colleagues classified *T. gondii* strains into 15 different haplotypes, defining six major clades (Su *et al.*, 2012). However, Minot and colleagues disagree with this theory (Minot *et al.*, 2012). The online genome database ToxoDB (http://toxodb.org/toxo/) provides further detailed information about the genome and the functional genomics of *T. gondii*. It also provides genome sequence information (including the facility to BLAST sequences), gene expression and proteomics data (Gajria *et al.*, 2008), which helps support research on the parasite. The most recent study published in 2016 compared four tissue-cyst forming coccidian parasites and showed that three of these organisms *N. caninum, Hammondia hammondi and T. gondii* have a similar total genome size of 62–65 Mb while the *Sarcocystis neurona* genome is larger due to expanded repeats and much larger introns. All four genomes have identical GC compositions and are predicted to encode from 7,000 to more than 8,000 genes located on 14 chromosomes, as with *T. gondii*. This work reveals that tandem amplification and diversification of secretory pathogenesis determinants is the primary characteristic that distinguishes the closely related genomes of these parasites and also disclosed that the unusual population structure of this parasite is characterized by clade-specific inheritance of large conserved haploblocks that are significantly enriched in tandemly clustered secretory pathogenesis determinants. The shared heritage of these conserved haploblocks, which show a different ancestry than the genome as a whole, may thus influence transmission, host range and pathogenicity (Lorenzi *et al.*, 2016).

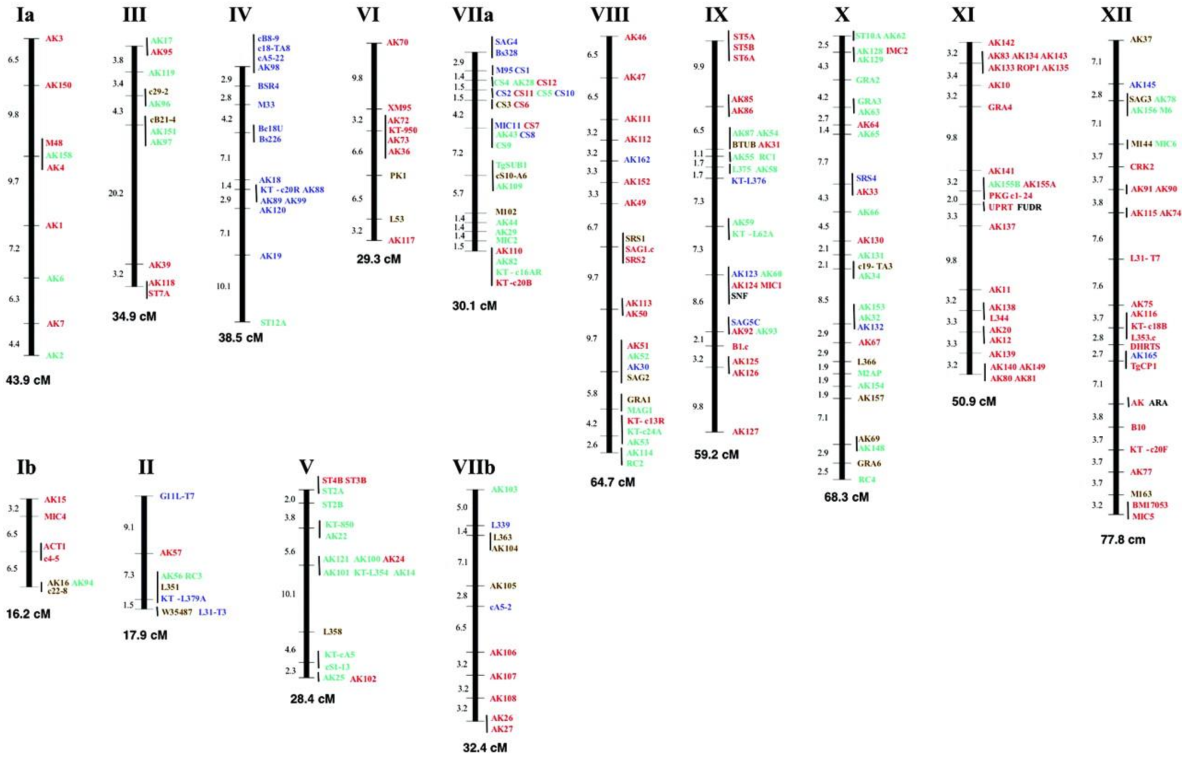


Figure 1.5 Genetic linkage maps for the 14 chromosomes of *Toxoplasma gondii*. Individual markers are shown to the right of the vertical bar and chromosome numbers are given above each map. Markers that map to the same node are indicated to the right of a solid vertical bar. The corresponding genetic distances between each node are given to the left of each map and the total sizes in cM are shown at the bottom of each chromosome. Polymorphisms that are unique to type I are shown in red, those unique to type II are shown in green, those unique to type III are shown in blue and markers that contain multiple polymorphism are shown in yellow. Maps were constructed using MAPMAKER from the analysis of 71 recombinant progeny using 250 genetic markers. Markers that include data analyzed by Southern blot are followed by the suffix '.c'.
Image source: Nucleic Acids Research, https://doi.org/10.1093/nar/gki604 (Khan *et al.*, 2005).

1.1.6. Genetic variation and disease severity

T. gondii has not only the capacity to propagate asexually but also sexually in its feline definitive host. Therefore, sexual recombination should provide for a high genetic diversity between *T. gondii* strains worldwide. However, the population structure of this parasite was initially described as being highly clonal and showing a low genetic diversity. Genotyping studies of *T. gondii* started in the 1990s and were based on a single marker, predominantly *Sag*2 (Howe *et al.*, 1997; Fuentes *et al.*, 2001; Sabaj *et al.*, 2010) and GRA6 (Fazaeli *et al.*, 2000; Messaritakis *et al.*, 2008), but these methodologies didn't allow the identification of non-clonal strains. Thus these methodologies were optimized in order to determine more precisely the presence of polymorphisms in the population and with the addition of new PCR-RFLP markers (Su *et al.*, 2006) and by microsatellite analysis (Ajzenberg *et al.*, 2005, 2010), this was achieved. Genetic

studies of isolates from Europe and the United States suggested the presence of a clonal population structure stable in time and space (Darde *et al.*, 1988; Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Ajzenberg *et al.*, 2002a; Khan *et al.*, 2007) where the majority of isolates (> 94 %) are grouped into three main clonal multilocus genotypes I, II and III. This simple clonal structure is accompanied by a low level of genetic divergence among the three lineages (only 1 to 2 % divergence at the DNA sequence level between lineages). However, multilocus and multichromosome genotyping of isolates from other continents revealed a much more complex population structure with a greater genetic diversity, likely reflecting a history of more frequent genetic exchanges and genetic drift (Lehmann *et al.*, 2004; Ajzenberg *et al.*, 2004) (Table 1.1). The majority of isolates from South America, Africa and Asia are not included in the three major lineages (with the exception of type III, which is really cosmopolitan). These deferent genotypes lead to the description of new haplogroups, some of them largely distributed over continents, being considered other successful clonal lineages (Khan *et al.*, 2007). So far, 12 haplogroups (including the 3 initially described lineages, types I, II, and III) have been described (Khan *et al.*, 2007, 2011), based on sequence-based analyses, but these haplogroups are not totally homogenous, and more specific markers revealed subclustering that may be associated with geographical origins and phenotypic characteristics. Based on the classical genotyping, from Northern (Jokelainen *et al.*, 2011) to Southern Europe (De Sousa *et al.*, 2006), the population structure of *T. gondii* shows a clonal profile, with a predominance of the type II lineage strains. The other two clonal lineages are sporadically found in Europe. While the three clonal lineages predominate in North America and Europe, strains from other regions in the world appear to have genotypes that are more diverse. By analyzing isolates from South America, Asia and Africa by using PCR-RFLP or microsatellite markers, it was revealed that the majority of these isolates have the classical type I, II, and III alleles identical to those in the main three lineages, but some novel alleles were also detected. These 'new' genotypes were designated as atypical, exotic, recombinant or non-archetypal genotypes (Grigg *et al.*, 2001a; Ajzenberg *et al.*, 2004). The recombinant genotypes have mixtures of classical alleles, while atypical, unusual, non-archetypical or exotic strains are characterized by the existence of many unique polymorphisms and novel alleles (Grigg *et al.*, 2001a; Su *et al.*, 2003; Ajzenberg *et al.*, 2004). Phylogenetic analysis, based on microsatellites, suggests that these atypical genotypes are phylogenetically disseminated with no clear structure, or association with the main three lineages. Although there is clear divergence among these strains, essentially due to the mixture of alleles, the overall level of sequence polymorphism provided by single nucleotide polymorphisms is modest. (Grigg *et al.*, 2001a; Su *et al.*, 2003; Ajzenberg *et al.*, 2004). The isolation of atypical strains, which do not fit into these three major lineages, is rare in Europe and likely suggests contamination by non-European strains either during residence abroad or after the consumption of imported food

(Ajzenberg *et al.*, 2009). In North America, the population structure appeared similar to that observed in Europe, with a predominance of type II strains (Howe and Sibley, 1995). South America is an area with a high level of diversity for *T. gondii* and type II seems to be very rare (Dubey *et al.*, 2007a). The high level of genetic diversity observed in this continent is maximal in the wild Amazonian area, with many unique polymorphisms (Ajzenberg *et al.*, 2004). It is believed that, in the Amazonia area, the interpenetration of anthropized and wild rainforest environments leads to hybridization between strains that may represent a potential risk for human health. In Asia, it has been shown that strains have more limited genetic diversity compared to South America (Dubey *et al.*, 2007b). Isolates from Cameroon that were analyzed via microsatellites revealed the existence of fixed combination of type I and III alleles, suggesting a unique clonal African type (Ajzenberg *et al.*, 2004).

Epidemiological studies have shown that type I strains are rare in human and animal infections, however has been linked to reactivation of the parasite in immunocompromised individuals (Khan *et al.*, 2005) and type II strains are considered to be the most common source of human toxoplasmosis (Howe and Sibley, 1995). In North America and Europe, most cases of human toxoplasmosis in AIDS and congenital infections are associated with type II strains (Howe and Sibley, 1995; Howe *et al.*, 1997; Ajzenberg *et al.*, 2002a). However, a study in Spain reported dominance of type II strains in AIDS patients, while type I strains were associated with congenital infections (Fuentes *et al.*, 2001). Another study from the USA revealed the relationship between severe ocular toxoplasmosis in immnunocompetent patients and type I strains and new recombinant genotypes (Grigg *et al.*, 2001a). The severity of *T. gondii* acute infection is considered to be one of the most significant phenotypes among *T. gondii* strains. Very little is known concerning circulating strains in Portugal, a small scale study was performed in France with Portuguese sera that reported a majority of type II humans strains (Sousa *et al.*, 2008; Ajzenberg *et al.*, 2009) and other European study that enrolled only two Portuguese isolates (Ajzenberg *et al.*, 2009) revelled that were both type II.

The technological development accompanied with the cost reduction of the Whole Genome Sequence (WGS) methodology will allow in mid-term the sequencing of multiple complete genomes of *T. gondii*, allowing understanding of the real degree of genetic variability of this protozoan, frequency of recombination and potential genotype - phenotype associations.

Table 1.1 Geographical distribution of *Toxoplasma gondii* genotypes and potential relationships with human disease.
Table Source: Adapted from Clinical Microbiology Reviews (Robert-Gangneux and Dardé, 2012).

Geographical area	Genotypes	Clinical features of human Toxoplasmosis
Europe	Type II (haplogroup 2), highly predominant; Type III, more in South; Other genotypes sporadically observed	Asymptomatic or benign disease in immunocompetent individuals associated with type II or III; retinochoroiditis in immunocompetent patients
North America	Type II (haplogroup 2); Haplogroup 12; Type III (haplogroup 3); Other genotypes	Asymptomatic or benign disease in immunocompetent individuals associated with type II or III
South and Central America	Africa (haplogroup 6); Type II occasionally; Type I rarely; Highly atypical genotypes in the Amazonian forest	Higher rate and severity of retinochoroiditis in immunocompetent patients and in those with congenital toxoplasmosis; disseminated, potentially lethal, cases observed with the most atypical genotypes
Africa	African 1, 2, 3 (haplogroup 6); Type III (haplogroup 3); Type II	Higher rate of retinochoroiditis than in Europe
Asia	Type III (haplogroup 3);	No data

1.1.7. Assessment of strain virulence

Experimental virulence is usually defined with the mouse model after the intraperitoneal inoculation with a given number of tachyzoites. Type I strains are highly virulent, have a lethal dose (LD100) (the minimal dose which causes 100 % mortality) of a single infectious organism, leading to the death of mice less than 10 days after the inoculation of about 10 tachyzoites. On the other hand, mouse non virulent strains, normally from type II and III, have a lethal dose of > 10 infectious organisms and usually generate chronic infection in the mouse (Sibley *et al.*, 2002). Isolates from other clonal lineages or from atypical strains range from the highly virulent to the intermediate or non-virulent phenotype, according to differences in the combination of genes that they have inherited. Genotypes with a majority of type I alleles are usually more virulent (Saeij *et al.*, 2005a) The mouse-virulent strains exhibit some characteristics that may explain the rapid dissemination of the parasite and the higher tissue burden observed in mice and other susceptible hosts: improved migration across polarized epithelia or across the extracellular matrix, higher rates of the ex vivo penetration of the lamina propria of mucosa and submucosa (Barragan and Sibley, 2002), and in cell culture, higher growth rates and lower rates of interconversion from tachyzoites to bradyzoites. Experimental crosses between strains with different virulence patterns facilitated the identification of several polymorphic genes coding for

secreted factors of *T. gondii* associated with differences in the expression of virulence in mice (Taylor *et al.*, 2006; Reese *et al.*, 2011). These key virulence factors are secretory proteins discharged from apical organelles, the rhoptries. The proteins of this rhoptry family (ROP5, ROP16, and ROP18) exert kinase or pseudokinase activity. They are injected directly into the host cell and play a role during the process of parasite invasion or in the induction of interleukin-12 (IL-12) secretion by mouse macrophages (Robben *et al.*, 2004).

T. gondii is considered to be a successful organism because it has the ability to cross biological barriers such as blood-brain barrier, placenta, or gut epithelium (Saeij *et al.*, 2005a). However, the expression of virulence in humans is a complex phenomenon due to many other factors that may influence the pathogenicity of a given strain, namely: parasitic factors such as the infectious stage, the inoculums and the genetic background of the strain and immune status of the host. (Robert-Gangneux and Dardé, 2012).

1.2. Toxoplasmosis, the human disease

1.2.1. Pathophysiology

Toxoplasmosis is the infectious disease caused by *T. gondii*. The pathophysiology of this infection results from the dissemination of tachyzoites throughout the body. Tachyzoites speedily invade monocytes and gain access to the blood flow (after trans-epithelial passage across the intestinal barrier) and from there to all organs (Robert-Gangneux and Dardé, 2012). In acute toxoplasmosis, a host may die due to necrosis (caused by intracellular growth of tachyzoites) of the intestine and mesenteric lymph nodes before severe damage of other organs. Focal areas of necrosis may develop in many organs; the clinical picture is determined by the extent of injury to these organs, especially vital organs such as the eyes, heart, and adrenals (Dubey, 2010). If the host survives, the invasive stages (tachyzoites) convert into a latent form (bradyzoites) within cells and persist as cysts, generally in muscles, retina, and brain, for a lifetime following the onset of an efficient immune response. The immunity is predominantly cell mediated and activated macrophages and T cells play a central role, while interferon gama (y) and other cytokines induce an effective immune response. The specific antibody in the presence of complement eliminates extracellular parasites (Holliman *et al.*, 2003).

1.2.2. Transmission

Human infection is acquired by ingestion of tissue cysts in raw, poorly cooked or cured meat, notably lamb and pork. Consumption of meat from warm blooded animals is considered the major source of infection in Western countries (Cook *et al.*, 2000). The transmission can occur

also by or ingestion of sporulated oocysts derived from contaminating soil, water or inadequately washed vegetables / fruit or directly from cat faeces. Once sporulated, oocysts are resistant to adverse environmental conditions, it can remain viable in a moist environment for more than a year, in very low (icy) or high water temperatures for long periods of time and can resist to chemical and physical treatments currently applied in water treatment plants, including chlorination and ozone treatment (Dumètre *et al.*, 2008). When primary infection is acquired by a pregnant woman, tachyzoites can colonize placental tissues during the dissemination process and access the foetus. The risk of transmission and the severity of disease depend on the gestational stage at which the mother first becomes infected. Congenital infection is the most important part of the disease burden due to *T. gondii* infection in humans.

Rarely, infection can be acquired via an organ transplant, as cysts can be found potentially in any organ *T. gondii* infection can be transmitted through a cyst-containing organ from a donor with infection acquired in the past to a nonimmunized recipient (Robert-Gangneux and Dardé, 2012). One the other hand toxoplasmosis associated with bone marrow transplant is a rare event and is usually due to reactivation of the recipients previously quiescent chronic infection. (Holliman *et al.*, 2003). As well as the transmission by blood transfusion because the duration of parasitemia following acute infection is limited. The eye can be reached by *T. gondii* via the bloodstream in the form of free tachyzoites or as tachyzoites that exist in circulating leukocytes (Roberts and McLeod, 1999). These establish in the retina and form cysts. Pathology occurs due to the release of tachyzoites as a consequence of the rupture of tissue cysts which result in invasion and inflammation of the retina. Another rarely event and not significant from an epidemiological point of view is the transmission by the consumption of unpasteurized goat’s milk (Tenter, 2000).

1.2.3. Clinical Features

1.2.3.1. The immonocompetent patient

Primary infection of *T. gondii* is asymptomatic and passes unnoticed in the majority of immunocompetent patients. (Holliman *et al.*, 2003) Nevertheless in some cases the symptomatic infection can occur being the most-common presentation fever, muscle weakness and painless cervical or occipital lymphadenopathy in the form of lymph node enlargement without tenderness or suppuration for about 4 - 6 weeks or even months in some cases (Ho-Yen, 2009). Infected humans remain infected for their whole lives and cysts persist a life-long threat to the individual. Parasite reactivation can occur as a result of immunosuppressive factors such as AIDS, or medication for inflammatory disease or transplantations (Porter and Sande, 1992).

1.2.3.2. The immunosuppressed patient

When for any reason an immunosuppression happens, the opportunistic reactivation of a latent *T. gondii* infection can occur. AIDS patients and transplant recipients constitute examples of this. Toxoplasmosis is the most common cause of focal brain lesions and one of the most frequent opportunistic infections in AIDS patients leading to a fatal outcome and representing a life-threatening disease. Central Nervous System (CNS) lesions are caused by tissue destruction as a result of tachyzoite proliferation yielding immunopathologic effects caused by inflammatory response (Denkers and Gazzinelli, 1998). It has been shown that the existence of encysted bradyzoites in the CNS is associated with high incidence of toxoplasmic encephalitis in these individuals (Denkers and Gazzinelli, 1998). In the beginning of HIV emergence, prior to the development of anti-viral therapy, it was found that toxoplasmic encephalitis developed in about 10-50 % of AIDS patients with chronic *T. gondii* infection (McCabe and Remington, 1988). In addition, the parasite can reactivate in the eye causing retinochoroiditis, or in the lung causing pneumonitis and acute respiratory failure (Luft and Remington, 1992; Ho-Yen, 2009).

Toxoplasmosis represents a life threading complication to organ graft recipients being the most common transplanted organs, the heart, lung, liver and kidney. The infected recipients develop fever, deterioration of consciousness and signs of respiratory failure usually 3-5 weeks after the surgery.

1.2.3.3. Ocular disease

Ocular toxoplasmosis is one of the most common sequelae of chronic toxoplasmosis and presents in the form of retinochoroiditis with the presence of typical lesions, which are white focal lesions often associated with a vitreous inflammatory reaction. It can occur in congenitally or post-natal acquired infection resulting from acute infection or reactivation of dormant infection. However, ocular toxoplasmosis is more commonly associated with congenital infection (Brézin *et al.*, 1994). Reactivation of infection and retinal disease in individuals with acquired, rather than non-congenital, toxoplasmosis is associated in some cases with reduction of immunity (Nicholson and Wolchok, 1976; Holland *et al.*, 1988). In addition, it has been suggested that ocular toxoplasmosis can occur through transmission from the brain to the eye through the optic nerve (Mets *et al.*, 1996). Furthermore, the development of tissue cysts that contain bradyzoites inside the eye can be involved in the reactivation of toxoplasmosis and is considered to be a significant feature in toxoplasmic retinochoroiditis pathogenesis which causes acute inflammation and can result in retinal scars that might cause blurred vision or blindness (Roberts and McLeod, 1999). Ocular infection can be manifested as pain, tearing, photophobia and finally loss of vision (Montoya and Remington, 1996; Holland, 2003, 2004; Ho-Yen, 2009).

1.2.3.4. Congenital toxoplasmosis

T. gondii infection can be transmitted congenitally if the mother acquires the infection during pregnancy, as parasites cross the placenta and infect the foetus. The risk of infection being passed to the foetus increases as gestation progresses, however, inversely, the severity of disease decreases with an increase in gestation period (Dunn *et al.*, 1999; Cook *et al.*, 2000). Without treatment of the mother during pregnancy, the incidence of acquired foetal infection during the first trimester is 10 % - 15 %, in the second trimester is 30 % and in the third is 60 % (Wong and Remington, 1994). If the mother receives treatment with spiramycin, these incidences decrease to 4.5 %, 17.3 % and 28.9 %, respectively (Wong and Remington, 1994). Congenital infection can occasionally result from reactivation of infection in immunosuppressed women if acquired before pregnancy (Wong and Remington, 1994). The variety of manifestations of congenital infection that occur in the foetus and in infants include spontaneous abortion, still-birth, a live infant with classic signs of congenital toxoplasmosis such as hydrocephalus or microcephalus, cerebral calcifications, mental retardation, seizures and retinochoroiditis (Hill and Dubey, 2002). The majority of cases are asymptomatic at birth, but most will develop neurological or ocular manifestations later in their lives. (Figure 1.6)

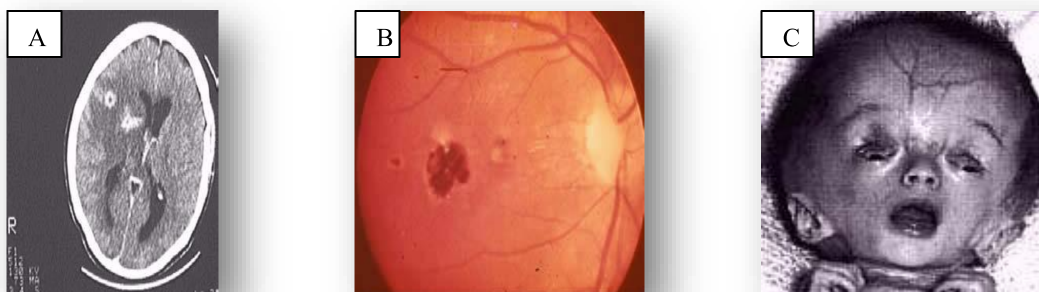


Figure 1.6: Clinical signs A) cerebral calcifications, B) Retina lesions due to ocular toxoplasmosis ,C) Newborn with hydrocephalus.
Image source http://wiki.ggc.edu/wiki/toxoplasma_gondii

1.2.4. The Diagnosis

In most instances the non specific nature of the signs and symptoms of toxoplasmosis do not permit reliable diagnosis based only on the clinical findings. Its detection depends mainly on biological, serological, or histological methods or on the combination of some of these methods (Hill *et al.*, 2005). However due to the diversity of *T. gondii* infection, investigations must be selected which are appropriated to that patient group. (Holliman *et al.*, 2003)

1.2.4.1. The immonocompetent patient

The diagnosis of *T. gondii* infection in immunocompetent subjects relies on serology. As the infection is often asymptomatic, serologic diagnosis is usually retrospective and is used to determine the immune status. Available serologic procedures for the detection of *T. gondii* humoral antibodies include; the Sabin-Feldman dye test (DT), the modified agglutination test (MAT), the indirect hemagglutination test (IHAT) the indirect fluorescent antibody assay (IFA), the direct agglutination test (DAT), the latex agglutination test (LAT), the enzyme-linked immunosorbent assay (ELISA), and the immunosorbent agglutination assay test (IAAT). The IFA, IAAT, and ELISA tests have been modified to detect IgM antibodies (Remington *et al.*, 1995). Immunosorbent agglutination assays (ISAGAs) are also suitable for IgM, IgA, or IgE detection. The methylene blue dye test for the detection of antibodies, introduced in 1948 by Sabin and Feldman, is maintained as a gold standard for serology tests by reference laboratories, but is labor-intensive and requires a continual supply of live organisms.

Immunoglobulin A and IgM are produced during the first week following infection and reach a plateau within one month, these antibodies appear sooner than the IgG antibodies and disappear faster than IgG antibodies after recovery (Remington *et al.*, 1995). Specfic IgE antibodies are also produced early and rapidly disappear. Specific IgM antibodies typically decrease after one to six months and disappear in 25 % of patients within less than seven months but commonly remain detectable for a year or longer with the most sensitive methodologies such as the ISAGA. Concerning that IgG antibodies are synthesized 1 to 3 weeks after the initial rise in IgM levels, IgG synthesis reaches a plateau within 2 or 3 months, then decreases and then persists lifelong as residual titers. Since IgG can persist for the life time, IgM, which typically persists for 1 - 6 months, is used as a marker of recent infection, (Wilson and McAuley, 1999).

1.2.4.2. The immunosuppressed patient

Diagnosis of organ donor or transplant recipient and HIV patient

Since acute toxoplasmosis in immunocompromised patients can be rapidly lethal, its diagnosis is an emergency. Whether serology is essential to estimate whether the patient is at risk for a reactivation of infection, evidence of evolutive infection is provided by the demonstration of tachyzoites in fluids or tissues by PCR or microscopic examination. In spite of be a highly sensitive method, in this case mice inoculation is not the first line of diagnosis because the time consuming. Several samples can be collected according to clinical signs and the type of immunosuppression. In transplant patients, where disseminated toxoplasmosis is frequent, parasites can be detected in bronchoalveolar lavage fluid (BAL), blood, bone marrow aspirate, cerebrospinal fluid (CSF), or virtually any biopsy specimen from a deep site. In HIV patients the most frequent presentation of this infection is the cerebral toxoplasmosis and the most effective approach is to perform PCR in CSF and blood.

1.2.4.3. Ocular disease

The diagnosis of retinochoroiditis is based on an ophthalmological examination together with PCR detection of *T. gondii* DNA in aqueous humor or vitreous fluid.

1.2.4.4. Congenital Toxoplasmosis

Prenatal diagnosis and follow-up of pregnancy

Maternal infection during pregnancy is confirmed by serological methods and performed a puncture of amniotic fluid (AM) after 16 / 18 weeks of gestation and at least 4 weeks after maternal infection. Prenatal diagnosis relies mostly on the PCR based detection of parasite, DNA targets include the 110 copies ITS1 region of *T. gondii* (Tenter *et al.*, 1994; Hurtado *et al.*, 2001), the 35 copies B1 region (Burg *et al.*, 1989) and the 300 copies 529 bp repeat element (Homan *et al.*, 2000) but in most reference laboratories, amniotic fluid is also inoculated in mice. This *in vivo* assay relies on the animal inoculation with the AM and then the detection of an antibody response in the animals by the examination of mice serum samples by direct agglutination on 10 days, 3 weeks and 6 weeks after inoculation. Finally, microscopic identification of the parasite tissue cysts in the mouse brain is performed in order to confirm the presence of brain cyst i.e. a positive result.

Postnatal diagnosis and follow-up of newborns

At birth, the neonate undergoes a complete clinical and neurological health check. In order to detect cerebral calcifications a transfontanellar ultrasound examination is performed followed by a computed tomography in the case of observed abnormalities. During the first week of life an examination of the ocular fundus is performed and is repeated every 3 or 4 months. Follow- up of newborns is essential and is based on (i) parasite detection in placenta or newborn blood and (ii) serologic analysis of newborn serum to chek the presence of specific antibodies which would be evidence of a congenital infection. Serologic diagnosis commonly relies on the detection of specific antibody produced by the newborn. The detection of specific IgM or IgA antibodies, which cannot cross the placental barrier (to the exception of low molecular weight IgM) is a key marker of fetal infection. As maternal IgG is passively transferred in utero, this single assay can't be a way to diagnosis of fetal infection. Consequently, a qualitative analysis is needed to differentiate between maternal antibodies and antibodies synthesized by the infected newborn, a comparative analysis of mother- and neonate-specific IgG/ low molecular weight IgM may help provide serologic evidence of fetal infection. This is allowed by the Immumoblot (IB) that perform a qualitative analysis of specific IgG or IgM by a comparison of band patterns, respectively, from paired mother-newborn sera in the first 2 or 3 months after birth. The parasitological examination of placenta and newborn blood at birth, is performed as in prenatal diagnosis, using mice inoculation, once more this *in vivo* assay relies on the animal inoculation with the placenta/ newborn blood and then the detection of an antibody response in the animals by the examination of mice serum samples by direct agglutination on 10 days, 3 weeks and 6 weeks after inoculation. The detection of parasites in the placenta and/or newborn blood is the only evidence of congenital toxoplasmosis at birth (Robert-Gangneux *et al.*, 2010).

1.2.5. Prevention and Treatment

Women during pregnancy or seronegative after conception and immunosupressed individuals should be given guidance to reduce the risk of acquiring toxoplasmosis. Fruit and vegetables should be washed properly, meats should be well cooked and hand wash after preparing raw meet should be emphasized. Gloves should be used when gardening or managing cat litters or cats directly.

Treatment drugs for toxoplasmosis target the tachyzoite stage of the parasite and do not eradicate encysted parasites in the tissues. Pyrimethamine, considered the most effective drug against toxoplasmosis, is a folic acid antagonist and can cause suppression of the bone marrow, thus is administered with another folinic acid leucovorin that protects the bone marrow from the toxic effects of pyrimethamine. A second drug, such as sulfadiazine or clindamycin, should also be included.

1.2.5.1. The immonocompetent patient

The treatment is rarely indicated in adults with lymphadenopathic toxoplasmosis because this form of the disease is usually self-limited. If visceral disease is clinically evident or symptoms are severe or persistent, treatment may be indicated for 2 to 4 weeks (CDC).

1.2.5.2. The immunosuppressed Patient

Toxoplasmosis in immunodeficient patients is often fatal if not treated. Treatment is recommended for at least 4 to 6 weeks beyond resolution of all clinical signs and symptoms, but may be required for 6 months or longer. Relapses are known to occur in AIDS patients and maintenance therapy is recommended until a significant immunologic improvement is achieved in response to antiretroviral therapy. Pyrimethamine, folinic acid (leucovorin), and sulfadiazine are standards of therapy for immunodeficient patients (CDC).

1.2.5.3. Ocular disease

The treatment is dependent of acuteness of the lesion, degree of inflammation, visual acuity and size, location and persistence of the lesion. The classic therapy for ocular toxoplasmosis consists of pyrimethamine, sulfadiazine and folinic acid. (CDC)

1.2.5.4. Congenital toxoplasmosis

In general, spiramycin is recommended for women whose infections were acquired and diagnosed before 18 weeks gestation and infection of the fetus is not documented. Spiramycin acts to reduce transmission to the fetus and is most effective if initiated within 8 weeks of seroconversion. Pyrimethamine, sulfadiazine and folic acid are recommended for infections acquired after 18 weeks of gestation and infection in the fetus is a reality. Congenitally infected newborns are generally treated with pyrimethamine, a sulfonamide and folic acid for 12 months (CDC).

1.2.6. Epidemiology and Burden of disease

The prevalence of specific antibodies to *T. gondii* is directly proportional to the age of the individual and or a population, indicating that infection is acquired throughout life. The incidence of infection shows marked geographical variations and these differences are associated with diet, level of hygiene, host susceptibility, cat contact and climate (humidity of the soil). Consequently, toxoplasmosis is most common in warm, wet, with a large cat population areas and where meat is eaten light cooked or raw. Moist conditions can increase oocyst survival during long periods of

heat, which likely accounts for the high prevalence in tropical countries of South America and Africa. According to a review study (Pappas *et al.*, 2009) the global status of *T. gondii* seroprevalence in women of reproductive age or pregnant ranged from above 60 % in countries such as Brazil, Gabon, Indonesia, Germany, and Iran, to less than 10 % in United Kingdom and Korea. Another recent review study which encompassed 88 countries (Flegr *et al.*, 2014) showed that the lowest seroprevalence (1 %) was found in some countries in the Far East and the highest (90 %) in some parts of European and South American countries. In European countries, the prevalence ranges between 10 % to 60 % and in some regions as high as 90 %. Ingestion of raw, undercooked or cured meat is the primary risk factor in Europe (Holliman *et al.*, 2003). Concerning the United States, the Center for Disease Control and Prevention (CDC) reported an overall seroprevalence of 11 %. In many developed countries, *T. gondii* seroprevalence declined sharply over the past three decades. (Cook *et al.*, 2000; Gargaté *et al.*, 2016) which may be associated with the practice of freezing meet, introduction of intensive farming techniques separating cats from livestock, and prevention strategies that includes elucidation of pregnant women and immunocompromised individuals about the sources of infection. According to the last European Centre for Desease Prevention and Control (ECDC) Annual Epidemiological Report for 2016 of congenital toxoplasmosis data (ECDC, 2017), 242 confirmed cases of congenital toxoplasmosis were reported in the EU/EEA, with France accounting for 81% of all confirmed cases due to the active screening of pregnant women. The notification rate was 6.7 cases per 100 000 live births, with the highest rates in France (24.9) followed by Poland (5.2). In 2016, gender was reported for 98 % of the congenital toxoplasmosis cases, with a male-to-female ratio of 1.1:1. Of 203 cases with known outcome, five were reported to have died, giving a case fatality of 2.5 %. No seasonal pattern was observed for this infection disease.

Symptoms of toxoplasmosis can be lifelong, namely, children whose born with congenital toxoplasmosis or individuals with ocular toxoplasmosis (whether acquired postnatally or congenitally) which, at its most severe, can cause blindness. A small number of studies within Greece, Netherlands and the USA have studied the disease burden of foodborne pathogens, comparing organisms such as *Campylobacter* spp (*Campylobacter*), *Salmonella enteric* (*Salmonella*), *Listeria monocytogenes* (*Listeria*), *Cryptosporidium parvum* (*C. parvum*), *Escherichia coli* O157:H7 with *T. gondii* (Gkogka *et al.*, 2011; Hoffmann *et al.*, 2012; Havelaar *et al.*, 2012; Batz *et al.*, 2012). This research has taken into account the annual cost or disability of illness linking it to either quality-adjusted life-years (QALYs) or disability-adjusted life- years (DALYs). These findings showed that *T. gondii* ranked third, after Salmonella and Campylobacter, based on QALYs in the USA, and from representative data taken in 2009 from Netherlands, *T. gondii* ranked highest among 14 other foodborne pathogens when DALY's were used to classify these pathogens. Other results from another study (Flegr *et al.*, 2014) suggest that

the prevalence of toxoplasmosis in 88 countries is correlated with various disease burden measured with age standardized Disability Adjusted Life Years (DALY) or with age standardized mortality being the highest among all foodborne pathogens. According to a WHO study the global annual incidence of congenital toxoplasmosis was estimated to be 190 100 cases, this was equivalent to a burden of 1.20 million DALYs and high burdens were seen in South America and in some Middle Eastern and low-income countries (Torgerson and Mastroiacovo, 2013). The high disease burden reported in these publications highlights the lifelong effects that *T. gondii* can have on infected individuals.

1.3. Sate of the Art before this work

In Portugal, the last epidemiological data concerning *T. gondi* seroprevalence reported to 1979, on behalf of the 1st National Serological Survey, and showed a global prevalence of 47 %, where the highest rates were obtained in the north and central Portuguese regions, and was higher in individuals above 15 years old (Ângelo, 1983). The small-scale serological studies conducted in the last years enrolled solely pregnant women and showed prevalence rates of approximately 30 %. The deficit of the current epidemiological situation of toxoplasmosis in Portugal was recognized by the Directorate General of Health, which stated in Guideline Nº 37/2011 of 30/09/2011 DGS that screening of toxoplasmosis should be performed in surveillance low risk pregnancies and that there was few data of the immune status of women in Portugal for toxoplasmosis. Thus, we believe that the evaluation of seroprevalence of this infection in Portuguese population is of great importance in public health because it allows us to infer the risk of infection, namely of pregnant women and of women in childbearing age, allowing health authorities to perform an effective prevention for this life threat infection.

Genetic studies integrating intermediary and definitive hosts were lacking in Portugal, in spite of the three genotypes have already been described in animals, revealing a majority of type II strains (De Sousa *et al.*, 2006; Dubey *et al.*, 2006, 2007a). In humans there were only two evaluations regarding the genetic variability of *T. gondii* strains in Portugal (Sousa *et al.*, 2008; Ajzenberg *et al.*, 2009) which were limited and only one used the classical typing method, which hampers the comparison of the molecular epidemiology data with the scenarios from other countries. Thus, the understanding of the molecular epidemiology of *T. gondii* circulating strains in Portugal would certainly be useful to highlight the transmission routes and preview clinical presentation of the infection.

According to the World Health Organization and Health Protection Agency guidelines, the gold standard methodology for the laboratory diagnosis of congenital toxoplasmosis is inoculation in mice of the diverse biological products. This methodology is also used for

maintaining/propagating the isolated strains in reference laboratories. However, this technique presents several disadvantages for the latter purpose. Indeed, it requires the sacrifice of many animals, which is badly seen in the light of animal welfare and is highly laborious and time consuming. In this way, it would be important to reduce the number of mice sacrificed in the *in vivo* model.

1.4. Aims of this thesis

According to this, the general goal of this PhD thesis is to contribute for the knowledge of toxoplasmosis in Portugal, by means of three specific objectives, each one constituting one distinct chapter of this thesis:

i) to evaluate the prevalence and evolution of antibodies anti *T. gondii* in the Portuguese population by comparing three cross - sectional studies spanning three decades, 1979/80, 20/2003 and 2013 (Chapter II);

ii) to perform a genetic and virulence characterization of *T. gondii* strains isolated from the several biological samples from the population with suspected toxoplasmosis that attended to the NIH to perform the laboratorial diagnosis of *T. gondii*. And also to describe the demographic characteristics of the referred patients and estimated the rate of new cases in the last 10 years (Chapter III);

iii) to evaluate the use of an *in vitro* system based on a cell-line for strain propagation, in order to replace, or at least reduce, the use of the demanding animal model and to assess the potential genotypic alterations throughout cell line propagation by New Generation Sequencing (Chapter IV).

We believe the achievement of these objectives will contribute to enhance the knowledge of toxoplasmosis in Portugal, which will be ultimately beneficial for the control of *T. gondii* infections and its potentially tragic outcome for the newborns and immunosupressed patients.

Chapter II

Toxoplasma gondii seroprevalence in the Portuguese population: comparison of three cross-sectional studies spanning three decades

This chapter corresponds to a manuscript (with discrete changes) with the following reference:

BMJ Open. 2016; 6(10): e011648.
Toxoplasma gondii seroprevalence in the Portuguese population: comparison of three cross-sectional studies spanning three decades.
Maria João Gargaté, Idalina Ferreira, Anabela Vilares, Susana Martins, Carlos Cardoso, Susana Silva, Baltazar Nunes and João Paulo Gomes
Published online 2016 Oct 5. doi: 10.1136/bmjopen-2016-011648

Personal contribution

Maria João Gargaté designed the study, performed most of the experimental work, analyzed the data and wrote the manuscript.

2. *Toxoplasma gondii* seroprevalence in the Portuguese population: comparison of three cross-sectional studies spanning three decades

2.1. Abstract

Toxoplasma gondii is an obligate intracellular protozoan infecting up to one-third of the world's population, constituting a life threat if transmitted from mother to child during pregnancy. In Portugal, there is a lack of knowledge of the current epidemiological situation, as the unique toxoplasmosis National Serological Survey was performed in 1979/1980. Methods: We studied the seroprevalence trends in the Portuguese general population over the past 3 decades, by assessing chronological spread cross-sectional studies, with special focus on women of childbearing age, by age group, region and gender. Results: The *T. gondii* overall seroprevalence decreased from 47 % in 1979/1980 to 22 % (95 % CI 20 % to 24 %) in 2013. Generally, we observed that the prevalence of *T. gondii* IgG increased significantly with age and it decreased over time, both in the general population and in the childbearing women (18 % prevalence in 2013). Conclusions: The scenario observed for the latter indicates that more than 80 % of childbearing women are susceptible to primary infection yielding a risk of congenital toxoplasmosis and respective sequelae. Since there is no vaccine to prevent human toxoplasmosis, the improvement of primary prevention constitutes a major tool to avoid infection in such susceptible groups.

Keywords

Toxoplasma gondii; epidemiology; pregnancy; congenital toxoplasmosis; laboratory surveillance.

2.2. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to phylum apicomplexa, which infects most mammals worldwide. It undergoes a sexual cycle in the intestinal epithelium of the definitive host, members of the cat family, therein transforms into to oocysts which are subsequently shed in the environment, and an asexual cycle in an intermediate hosts, such as birds, rodents and other mammals including human beings. (Holliman *et al.*, 2003) Oocysts are extremely stable in the environment, and are transmitted to other hosts through inadvertent ingestion. Humans acquire *T. gondii* through ingestion of tissue cysts in the undercooked meat of intermediate hosts, mainly pork and lamb, or by the ingestion of water or food contaminated by faeces containing oocysts from the definitive host, (Cook *et al.*, 2000) and rarely through transplantation of a infected organ (Hill and Dubey, 2002). While toxoplasmosis

is often benign in immunocompetent individuals (revealing no symptoms or may experience swollen lymph glands), it induces major complications in immunocompromised individuals and during pregnancy, constituting a life-threatening disease; congenital toxoplasmosis (transmission to the foetus when a pregnant woman acquires *T. gondii* infection for the first time during pregnancy) can result in abortion or lead to severe malformation of the foetus, or to visual or neurological injuries in the newborn, such as hydrocephalus, cerebral calcification and/or chorioretinitis. Such patients may require prolonged, sometimes life-long, therapy. Also, recent studies suggest that subtle behavioural or personality changes may occur in *T. gondii* infected humans, (Flegr, 2012) and toxoplasmosis has recently been associated with neurological disorders, particularly schizophrenia (Holub *et al.*, 2013) and bipolar disorder (Tedla *et al.*, 2011). However, evidence for causal relationships remains limited (Webster *et al.*, 2013).

T. gondii has a wide spectrum of prevalence across the globe and infects up to one-third of the world's population (Tenter, 2000). Several decades ago the reported prevalence among general population elsewhere in the world varied from 0 % in Eskimos to 94 % in Costa Rican and Guatemalan (Gibson and Coleman, 1958; Feldman, 1982). Cultural habits in regard to cooking food are likely the major cause of differences in the frequency of infection with *T. gondii* in many areas of the world. Consequently, there are large differences in the incidence of congenital infection that may vary from 1:1000 live births in France, to 1:10 000 in countries with a lower seroprevalence, and in the USA, it has been estimated that ∼3500 infants are born each year with congenital toxoplasmosis (Feldman, 1982). According to a study review (Pappas *et al.*, 2009) the global status of *T. gondii* seroprevalence in women of reproductive age or pregnant ranges from above 60 % in countries such as Brazil, Gabon, Indonesia, Germany, and Iran, to less than 10 % in United Kingdom and Korea. In many countries, *T. gondii* seroprevalence declined sharply over the past three decades (Cook *et al.*, 2000). The surveillance schemes of toxoplasmosis are very heterogeneous in European countries, which hinders the burden of congenital toxoplasmosis estimates comparison (ECDC, 2013). The first National Serological Survey was conducted in continental Portugal between 1979 and 1980 and showed *T. gondii* overall seroprevalence of 47 % (Ângelo, 1983). Since then, no National Serological Surveys enrolling *T. gondii* antibodies were performed, but only small-scale studies (Ângelo, 2003), (Machado, 2005) (Lopes *et al.*, 2013). Thus, Portuguese health national authorities consider that there is a lack of knowledge of the current epidemiological situation of toxoplasmosis in Portugal, and the 2011 guidelines of the General Directorate of Health for toxoplasmosis screening establish the surveillance of low-risk pregnancy based on three monthly retesting of susceptible women (Ministério da Saúde: Direção-Geral da Saúde, 2011). Of note, congenital toxoplasmosis is a mandatory notifiable disease in Portugal (Saúde, 2014), and its diagnosis should be performed in our laboratory at the National Reference Laboratory of Parasitic and Fungal Infections of the Portuguese National Institute of

Health. In this regard, considering our data, there are about three cases of congenital toxoplasmosis per year in Portugal (data not published). Considering that the unique overall population study performed in Portugal dates back to 35 years ago, the aim of this study was to describe the seroprevalence trends in the Portuguese general population (with special focus on women of childbearing age) over the past three decades (1979 – 1980 to 2013), by age group, region and gender.

2.3. Material and Methods

2.3.1. Study design and sampling

In order to fulfil the defined objectives, three cross-sectional seroprevalence studies (1979/1980, 2001/2002 and 2013) were used. All of them were based on opportunistic sampling. Nevertheless, few biases are associated with this strategy in our study, as sera belonged to individuals seeking diverse blood analysis rather than specific *T. gondii* evaluation, which also reflects the vast majority of the general population. Furthermore, the same methodology was applied to all three surveys, ensuring their internal validity by increasing their comparability. The starting point was the *T. gondii* serological data released on behalf of the First Portuguese National Serological Survey in 1979/1980. This sample enrolled 1675 individuals of both genders, which were homogeneously distributed by the following stage groups: 8 months - 5 years, 6 - 15, 16 - 30, 31 - 45 and ≥ 46 years. Each age group included individuals from each of the 18 districts of Portugal (Portuguese islands were not included) that were representative of the population of each district (Ângelo, 1983). Then, in this study, we processed two distinct samples. The first one was composed of sera belonging to the Second Portuguese National Serological Survey 2001 - 2002 that aimed to estimate the prevalence of antibodies to vaccine-preventable diseases, and for this reason, the determination of antibodies for toxoplasmosis was not performed at that time. This sample enrolled 3525 individuals of both genders, and covered all age groups. Similar to the first serological survey, each age group included a number of individuals representative of the population of each of the 18 districts. For the participation of individuals, a document was prepared with the objectives and benefits of the study and informed consent was obtained either from the participants themselves or from their legal representatives (Freitas and Paixão, 2004). From these sera, we used a subsample of 1657 sera, which was established to detect the difference in antibodies prevalence between the 1979/1980 and 2001/2002 Portuguese National Serological Surveys of at least 5 %, with a power of 80 % and a significance level of 5 %. This subsample comprehended individuals of both genders, homogeneously distributed in five age groups: 8 months - 5 years, 6 - 15, 16 - 30, 31 - 45 and ≥ 46 years. Each age group included individuals from each of the 18 districts (as in previous surveys, proportion within districts was ensured). It

served as baseline for the establishment of the sample size of the 2013 *T. gondii* serological survey, which was composed of 1440 individuals of both genders, homogeneously distributed in the same five age groups as the previous survey. In each age group, for both surveys, individuals were distributed by 18 districts of Portugal (Portuguese islands were not included) proportionally to the resident population. For the 2013 survey, individuals were selected (to fulfil the above cited requisites of age, gender and geography) from the attendees of the private clinical laboratory of Dr Joaquim Chaves during the period of January to December 2013. This is the largest Portuguese laboratory comprehending more than 40 sampling collection units dispersed throughout the whole country. No informed consent was obtained from each participant as, besides the information regarding gender and age, no further information was available to the laboratory. This procedure is in agreement with the Portuguese law No. 12/2005.

2.3.2. Serological analysis

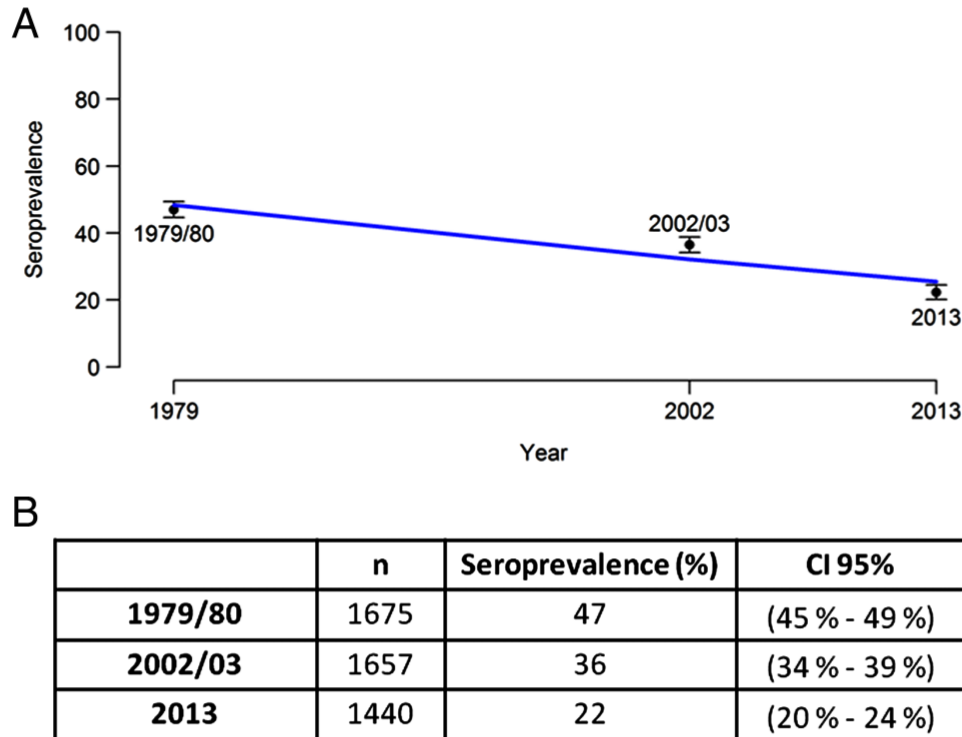
Sera regarding the 2001/2002 seroprevalence study were analysed for the presence of *T. gondii*-specific antibodies in 2012, whereas the analysis for the 2013 survey was performed in 2013, both at the National Reference Laboratory of Parasitic and Fungal Infections of the Portuguese National Institute of Health (NIH). *T. gondii* IgG-specific antibodies were detected by using the automated methodology enzyme linked fluorescent assay-sensibility 99.65 % (interval confidence (CI) 94.55 % to 97.39 %), specificity 99.92 % (CI 99.58 % to 100 %) and cut-off 4 ≤ titre < 8 IU/mL, with the VIDAS TOXO IgGII commercial reagents (bioMérieux SA, Marcy-l'Étoile, France) according to the manufacturer's instructions. For the resolution of *equivocal* samples (ie, 4 ≤ titre < 8 IU/mL), we retested them using a manual methodology of the direct agglutination test-sensibility 96.22 % (CI 94.55 % to 97.39 %), specificity 98.80 % (CI 96.46 % to 99.60 %) and cut-off 4 IU/mL, by using the TOXO-Screen DA commercial reagents (bioMérieux SA, Marcy-l'Étoile, France), according to the manufacturer's instructions. Most of the epidemiological surveys are based on the IgG titre because IgG antibody positive titres can be detected 2 - 3 weeks after infection, reaching a maximum titre within 2 months. It then declines to a baseline level that persists throughout the remainder of one's life. These methodologies were not available at the time the first National Epidemiological Survey was performed (1979/1980), which used an *in house* indirect immunofluorescence method (Ângelo, 1983).

2.3.3. Statistical analysis

Statistical analysis consisted of the estimation of the seroprevalences among the categories of the variables sex, age groups and region. Differences between the estimated seroprevalences were analysed using the χ^2test, considering the significance level of 5 %. The stats package of R software (V.3.0.3) was used (R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2013. ISBN 3–900051–07–0, URL. http://www.R-project.org).

2.4. Results

In the 2013 serological survey (n = 1440), we observed an overall prevalence of *T. gondii* antibodies of 22 % (95 % CI 20 % to 24 %), whereas in the 2001/2002 survey (n = 1657) the overall prevalence of *T. gondii* antibodies was 36 % (95 % CI 34 % to 39 %) (Figure 2.1A). The seroprevalence in the Portuguese population decreased by 11 % (95 % CI 7 % to 14 %) between 1979/1980 and 2001/2002, 14 % (95 % CI 11 % to 17 %) between 2001/2002 and 2013, and 25 % (95% CI 22% to 28%) between 1979/1980 and 2013. The significant decreasing trend during this 34 - year period is illustrated in Figure 2.1B. Since there were no statistically significant differences between the 18 districts (data not shown), we grouped them into four regions: north, centre, Lisbon area and south. In the 2013 survey, the seroprevalences ranged from 13 % (CI 95 % 10 % to 17 %) in the north of Portugal to 33 % (CI 95 % 29 % to 37 %) in the south region (Table 2.1).



	n	Seroprevalence (%)	CI 95%
1979/80	1675	47	(45 % - 49 %)
2002/03	1657	36	(34 % - 39 %)
2013	1440	22	(20 % - 24 %)

Figure 2.1 (A and B) (A) shows the evolution of *Toxoplasma gondii* seroprevalence in Portugal over the past three decades; (B) shows the sample sizes for the three National Serological Surveys, the precise estimated seroprevalences and respective 95 % confidence interval (CI).

Table 2.1 Trends of *Toxoplasma gondii* seroprevalence in Portugal by region calculated with an interval confidence of 95 %.

	n	1979/80 Seroprevalence (%)	n	2001/02 Seroprevalence (%)	n	2013 Seroprevalence (%)
North	405	51 % (46% - 56%)	426	45% (41%- 50%)	464	13% (10% -17%)
Centre	590	47% (43%- 51%)	426	38 % (0,33% -42%)	344	29 % (25%- 34%)
Lisbon	302	47 % (41%- 52%)	630	33 % (29% -37%)	504	23% (20% -27%)
South	378	43% (38% - 48%)	171	25 % (19% - 32%)	128	33 % (25% - 41%)

This finding is in opposition to the ones observed for the serological surveys of 1979–1980 and 2001/02, where the seroprevalence was higher in the north, centre and Lisbon area. In particular, the north region presented the highest levels in the first two surveys but the lowest in the last survey, in opposition to the south. In general, a decreasing trend was observed for the prevalence of *T. gondii* antibodies over the studied years in the four regions, with the exception of the south region that showed an increase of 8 % (95 % CI 2 % to 19 %) between 2001/2002

and 2013 (Tables 2.1 and 2.2). In particular, the decrease observed for the north region between these two surveys was statistically significant (Table 2.2)

Table 2.2 Multivariate analysis adjusted for gender, age group and region.
The values represent the prevalence ratios between the 2013 and the 2001 / 2002 serological surveys. Data are not available for the 1979/1980 survey.

	Prevalence ratios from 2002 to 2013
North	**0.37 (0.28 – 0.50)**
Centre	0.93 (0.72 – 1.20)
Lisbon	0.84 (0.67 – 1.06)
South	1.27 (0.82 – 1.95)
8 months - 5 years	0.60 (0.13 – 2.80)
6 – 15 years	0.21 (0.07 – 0.64)
16 – 30 years	0.52 (0.35 – 0.76)
31 – 45 years	0.70 (0.52 – 0.94)
>= 46 years	0.83 (0.70 – 0.99)

Regarding the association between seroprevalence and age, we observed that the prevalence of specific *T. gondii* IgG increased significantly with this variable for each age group (eg, for the 2013 survey, χ^2 test for trend in proportions gives a p value < 0.001) and it generally decreased over time (Figure 2.2). The only exception occurred for the age group ≥ 46 that revealed an increase from the 1979/1980 to the 2001/2002 survey. Considering solely the last two surveys (no data are available for the 1979/1980 survey, except for females between 15 and 45 years of age), the prevalence of specific *T. gondii* IgG decreased significantly for all age groups (except for 8 months – 5 years) (Table 2.2).

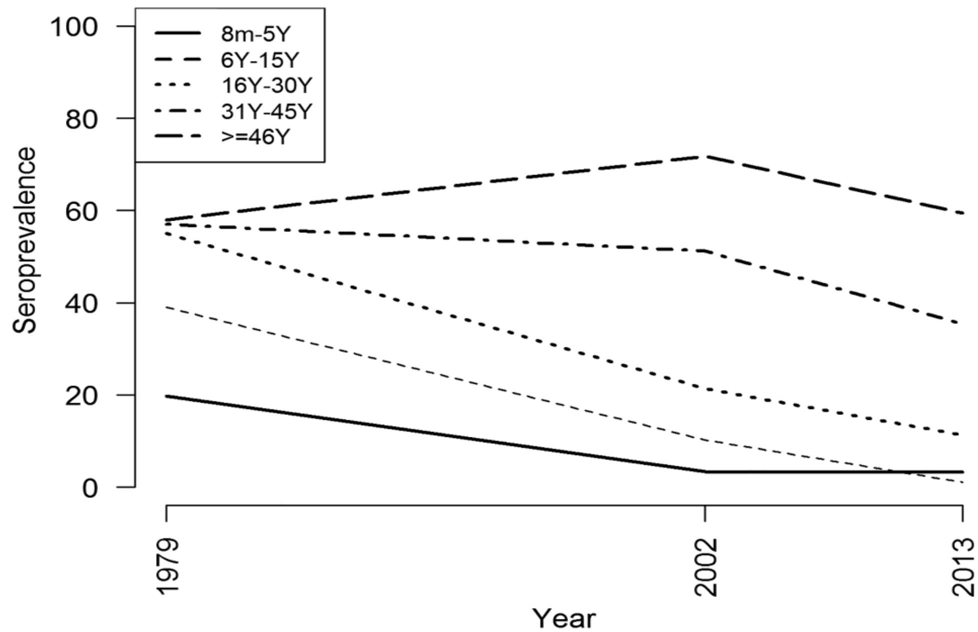


Figure 2.2 Comparison of *Toxoplasma gondii* seroprevalence trends, according to the data from three National Serological Surveys, by age groups.

Concerning the seroprevalence distribution by gender, we observed no significant differences, either for the 2001/2002 or the 2013 survey. Both genders showed a significant decrease in prevalence between these two surveys (Table 2.3).

Table 2.3 *Toxoplasma gondii* seroprevalence by gender and year of analysis calculated with an interval confidence of 95 %.

	Male		**Female**	
	n	**Prevalence %**	**n**	**Prevalence %**
2002	641	35% (31% -39%)	1016	38 % (35% - 41%)
2013	638	22 % (19% -25%)	802	23 % (20% - 26%)

Among the female population, we considered childbearing women aged 15–45 years, and observed a significant decreasing trend in the seroprevalence in this group: 53 % (95 % CI 48 % to 59 %) in 1979–1980, 35 % (95 % CI 32 % to 38 %) in 2001/2002 and 18 % (95 % CI 14 % to 22 %) in 2013. We also subdivided this population into five groups: 15 - 20, 21 - 26, 27 - 32, 33 - 38 and 39 - 45 years and observed that, like in the general population, there was a significant increase of *T. gondii* seroprevalence with age (except for a unique age group within the 1979 - 1980 survey), and a significant decreasing trend over time (Table 2.4).

Table 2.4 *Toxoplasma gondii* seroprevalence in childbearing women by age group calculated with an interval confidence of 95 %

	1979	2002	2013
"15-20"	51% (41%-60%)	19% (14%-25%)	4% (2%-10%)
"21-26"	56% (44%-67%)	23% (18%-30%)	10% (4%-19%)
"27-32"	46% (35%-58%)	34% (28%-41%)	17% (10%-28%)
"33-38"	60% (45%-72%)	48% (40%-56%)	32% (20%-49%)
"39-45"	60% (47%-72%)	62% (53%-69%)	50% (37%-63%)

2.5. Discussion

In this study, we aimed to perform a cross-sectional *T. gondii* seroprevalence study in the Portuguese population. We acknowledge the following limitations in this serological study, namely: (1) the lack of more complete personal and socioeconomic and demographic information, hampering an evaluation involving risk factors; (2) the probable lower sensitivity and/or specificity of the serological tests used in the first national survey when compared with the ones used in the latest surveys; (3) the use of different serological methods (1979/1980 vs the two most recent surveys); and (4) the use of both fresh (1979/1980 and 2013 surveys) and frozen (2001/2002) sera. We believe that some of these limitations could impact some false-negative and/or false-positive results and quantitative data (ie, the determination of serological titres), but less significantly impacted the determination of the immune status of the enrolled people, which was beyond the scope of this study. Also, sensitivity and specificity of the conventional methods most likely changed little over the years, as demonstrated, for instance, by the maintenance of the gold standard (Sabin-Feldman dye test) over the past 60 years. Finally, interassay variability is very unlikely to explain the large changes in seroprevalence observed from 1979/1980 to the 2013 survey. The 2013 serological survey established an overall prevalence of *T. gondii*-specific IgG of 22 %. This value indicates a prevalence that is similar to what has been described for other Mediterranean countries, such as Spain, Italy and Greece (Gutiérrez-Zufiaurre *et al.*, 2004; Diza *et al.*, 2005; de Ory Manchón, 2009; Pappas *et al.*, 2009; Pinto *et al.*, 2012; Mosti *et al.*, 2013), which could be associated with the similarity between these countries in terms of: (1) climate: seroprevalence may depend on the appropriate conditions for sporulation and oocysts survival in the environment, as oocysts maturation and transmission to a new host is faster at mild and wet climates (Tassi, 2007), which are not characteristic of these countries; (2) cat contact: cats are one of the major sources of infection, as they shed a large amount of oocysts via faeces, indoors and

outdoors, even after ingestion of a single bradyzoite or one tissue cyst (Hill and Dubey, 2002; Dabritz and Conrad, 2010). More, sporulated oocysts can survive for years in water, gardens, beach sands and farms, which constitute environmental features that are typical of these countries, enabling similar means of transmission due to the contact with the abundant stray and domestic cats in these environments (Hill and Dubey, 2002; Dabritz and Conrad, 2010), and (3) diet, the ingestion of raw, undercooked or cured meats is the primary risk factor in Europe for acquiring toxoplasmosis (Holliman *et al.*, 2003), and these countries present the same nutritional behaviour and eating habits, namely the traditional consumption of cured meats and raw vegetables. Although the frequent cat contact and typical diet would support higher seroprevalences than those observed, other factors, such as the climate and population education (discussed below), most likely balance this trend. We observed a huge decrease in the *T. gondii* seroprevalence in Portugal, from 1979/1980 to the 2013 survey, as the first survey revealed a 47% rate (Ângelo, 1983), which was low compared with the values reported in France, but sharply high when compared with north European countries. Curiously, the 2013 prevalence (22 %) remains low compared with France but is now sharply lower compared with some countries from the north of Europe such as Germany, Poland, Belgium, the Netherlands and Switzerland (Pappas *et al.*, 2009). We speculate that a possible explanation for those striking differences may rely on cultural habits in regard to eating and cooking practices. The sharp decreasing trend of *T. gondii* seroprevalence observed in Portugal during the past 34 years has been also reported in many studies performed in several developed countries (Nowakowska *et al.*, 2006c). Since the consumption of raw or undercooked meat is considered to be the major source of *T. gondii* infection, we believe that this decreasing trend may be associated with: (1) the practice of freezing meat; (2) the changes in nutritional habits that took place in Portugal and Europe during the past years related to the wide access to fast foods and pre-prepared meals, including frozen meat meals and the decreasing number of home-prepared meals (Nowakowska *et al.*, 2006c); (3) the introduction of intensive farming techniques involving the separation of cats from livestock, coupled with the reduction of breeding cattle in backyards (Holliman *et al.*, 2003; Nowakowska *et al.*, 2006c); (4) the release of legislation for toxoplasmosis, concerning sanitary inspection in the slaughterhouses; and (5) the improvements in health education and information by health professionals, as the lack of awareness of disease sources of transmission is a crucial factor in the risk of infection.

Regarding the distribution of *T. gondii* seroprevalence by geographic region, we observed an intriguing phenomenon as, contradicting the continuous decrease detected in the north, centre and Lisbon area, there was an 8 % increase (table 2.1) of the seroprevalence in the south, from 2001–2002 to the 2013 survey. Also, in the 2013 survey, this region was the one with the highest *T. gondii* seroprevalence (33 %), which was significantly higher (about 2.5 – fold) than the region

showing the lowest values (north – 13 %). The variation in the geoseroprevalence of this parasite may be due to the local rainy conditions and altitude (Flegr *et al.*, 2014). Nevertheless, this would explain the lower values observed for the south region in the first two surveys when compared with the other regions, but contradicts the scenario observed in the 2013 survey, as the south of Portugal presents a prominent lower altitude and is clearly drier and warmer than the remaining regions.

In most human populations, the *T. gondii* seroprevalence increases with age, indicating that infection is acquired throughout life (Holliman *et al.*, 2003). Our study was no exception and showed a higher seroprevalence in older age groups (for all geographical regions), most likely due to their longer exposure to the risk factors.

Concerning childbearing women, we observed a significant decreasing trend in the *T. gondii* seroprevalence over time (Table 2.4). This observation is corroborated by other studies, which show that the prevalence of *T. gondii* infection in women of childbearing ages has decreased over the past 30 years (Bobić *et al.*, 2011), and consequently, more women are now susceptible to the infection. Besides the factors stated above that most likely justify the seroprevalence decrease in the general population, the major factor associated with the decline observed for this target group most likely relies on the promotion of educational programmes yielding an improvement of the primary prevention. The currently observed seroprevalence of 18 % indicates that about 80 % of Portuguese women are not immune against the *T. gondii* infection. Thus, the majority of potential pregnant women are susceptible to primary infection and the risk of congenital toxoplasmosis is high, a scenario that mirrors the one observed for several cities in Spain (Bartolomé Alvarez *et al.*, 2008). The low prevalence observed among women of childbearing age should not be neglected. In fact, a recent study from the WHO (Torgerson and Mastroiacovo, 2013), which estimated the global burden of congenital toxoplasmosis by using a previously described prediction model (Larsen and Lebech, 1994), alerts that the global burden of the disease is considerably higher than that suggested by the congenital toxoplasmosis data. The incidence of infection is dependent on the general seroprevalence, determining the population susceptibility and the frequency of risk factors for toxoplasmosis acquisition. Therefore, seroprevalence should be considered the indicator to establish screening policies. Regarding this issue, several countries have no surveillance of the infection, whereas others focus solely on severe cases and few have surveillance targeted at congenital toxoplasmosis (European Food Safety Authority Panel on Biological, 2007). In Portugal, there is a surveillance system, which includes both the screening of pregnant women with follow-up during pregnancy of those who are not immune, in order to detect seroconversion, and the mandatory laboratory

notification of congenital toxoplasmosis cases detected during this process (Ministério da Saúde: Direção-Geral da Saúde, 2011).

This study reinforces that prenatal screening for toxoplasmosis is necessary due to the high rate of seronegative women exposed to infection and the probability of a high number of primary infections in the childbearing period. Targeted information should be crucially provided to childbearing and pregnant women by the health professionals regarding consumption of uncooked or cured meat, raw vegetables, contaminated drinking water and contact with cats. With one exception (Toxovax for sheep), there is no approved vaccine to prevent human or animal toxoplasmosis; therefore, primary prevention is the major tool to prevent the infection in the general population, mainly in high-risk individuals, such as immunocompromised, pregnant and childbearing women. Besides these primary prevention measures, there are several actions that will determine the prevention and control of *T. gondii* infection, including the governmental inspection of slaughterhouses and food production industries, the improvement of hygienic standards of abattoirs, the promotion of public educational schemes and the establishment of serological screening programmes.

Chapter III

Genetic and virulence characterization of *Toxoplasma gondii* isolates causing human infection in Portugal

Part of this chapter corresponds to a manuscript where Maria João Gargate is second author with the following reference, so some sections constitute a faithful reproduction of what was published.

Parasitol Res (2017) 116:979–985
Molecular and virulence characterization of *Toxoplasma gondii* strains isolated from humans in Portugal
Anabela Vilares, Maria João Gargaté, Idalina Ferreira, Susana Martins, João Paulo Gomes
DOI 10.1007/s00436-017-5374-5

Personal contribution

Maria João Gargate participated in the design of the study, performed most of the experimental work, analysis, interpreted data and wrote part of the article.

3. Genetic and virulence characterization of *Toxoplasma gondii* isolates causing human infection in Portugal

3.1. Abstract

Toxoplasma gondii is an apicomplexan parasite responsible for toxoplasmosis which infects all warm-blooded vertebrates, including humans, this infection is mostly asymptomatic in immunocompetents but in immunocompromised individuals, may lead to death when associated with reactivation of cysts both in situations of congenital and acquired infection. The laboratorial notification of congenital toxoplasmosis cases is mandatory in our country being the pre and postnatal diagnosis of toxoplasmosis carried out in Portugal exclusively at National Institute of Health Doctor Ricardo Jorge (NIH). The majority of studies conducted in Europe have revealed that more than 80 % of strains isolated from human infections belong to genotype II, whereas genotypes I and III are responsible for a small number of cases. Atypical and recombinant strains are generally associated with more severe infections. In Portugal, there is a lack of data concerning genetic diversity as the classical typing studies in humans have never been performed. We aimed to determine the *Sag*2 and microsatellite-based (TUB2, TgM-A, W35, B17, B18) genotypes of *T. gondii* strains as well as to study their virulence in mice. These strains were isolated from the biological samples of the patients with suspected toxoplasmosis whose attented NIH, also we estimated the demographic characteristics of the referred patients in the last ten years. We analyzed 48 strains from congenital and acquired toxoplasmosis context. Sag2-based genotyping of *T. gondii* was achieved in all 48 strains where 35 (73 %) were classified as type II and 13 (27 %) were type I. The multilocus PCR of five microsatellites allowed the classification of 10 strains (21 %) as recombinant strains that had been previously identified as type II or I by *Sag*2 typing. Seven out of the 48 strains, including three types I, three recombinant, and one type II, were virulent in mice. This study constitutes the first evidence of recombinant strains circulating in Portugal in humans from congenital infection, highlighting the need for a better evaluation of these strains as their phenotype is still barely understood.

Keywords *Toxoplasma gondii*, Genotype, Recombinant strain, Congenital toxoplasmosis

3.2. Introduction

The *Toxoplasma gondii* is an apicomplexan protozoan parasite and one of the most successful parasites worldwide due to its ability to infect all warm blooded animals including humans. One third of the world's human population is assumed to be infected with *T. gondii* (Weiss and Dubey, 2009; Innes, 2010). Toxoplasmosis is the infectious disease caused by this parasite and is mostly asymptomatic in immunocompetent individuals; however retinitis is often a cause of severe eye disease in healthy adults (Gilbert *et al.*, 1999). In immunocompromised individuals, the infection may be severe or even lead to death when associated with reactivation of cysts in situations of congenital and acquired infection. During pregnancy, this infection can cause miscarriage or serious congenital diseases namely encephalitis, chorioretinitis, and lymphadenopathy. In the majority of newborns the infection may be asymptomatic; these children, when deprived of treatment, may develop recurrent toxoplasmosis which can lead to blindness and neurological problems during childhood and adolescence (Zhou *et al.*, 2011), such as schizophrenia (Torrey *et al.*, 2012), bipolar disorder (Del Grande *et al.*, 2017), depression and attempts to suicide (Groër *et al.*, 2011). In Portugal, there is a guideline for screening of pregnant women with follow-up during pregnancy of those who are not immune (Ministério da Saúde: Direção-Geral da Saúde, 2011). According to the document of the Ministry of Health, the laboratorial notification of congenital toxoplasmosis cases is mandatory (Saúde, 2014) also this document establishes that a case of congenital toxoplasmosis is confirmed by the existence of at least one of the following four criteria: a) confirmation of the presence of *T. gondii* in tissues or body fluids of the newborn; b) detection of nucleic acids of *T. gondii* in a biological sample (body fluids of the newborn); c) antibody specific response to *T. gondii* (Ig M, IgG and IgA) in a newborn; d) serum stable titers of *T. gondii* IgG every two months in children less than twelve months old. The first line of diagnosis of an infection acquired during pregnancy is the serology. Then, for the suspected cases, mice inoculation and PCR are performed in the amniotic fluid (AF) finally, after baby's birth the later two procedures are also applied to placenta (PL) and newborn blood (NBB). Thereby the whole pre and postnatal diagnosis of toxoplasmosis is composed by serological, molecular and mice inoculation approaches and is carried out in Portugal exclusively at National Institute of Health Doctor Ricardo Jorge (NIH), where we have a collection of biological products and strains that likely constitutes a national representation of this infection. Thus, the biological products from all over the country are routinely sent to NIH laboratory to confirm the presence of this parasite as well as to perform the molecular characterization of the isolates. It is believed that the number and the genetic diversity of parasites that are causing the infection play an important role in pathogenesis of *T. gondii* (Sarvi, 2019). Although the determinants of disease severity are not well understood, several factors such as host genotype, nutrition, immune status, infection load, and parasite genotype have been suggested to influence

the outcome of infection (Sibley *et al.*, 2002). The distribution of *T. gondii* genotypes varies by geographic region (Lehmann *et al.*, 2006). Genetic studies of isolates from Europe and the United States, suggested the presence of a clonal population structure stable in time and space (Darde *et al.*, 1988; Sibley and Boothroyd, 1992; Dardé *et al.*, 1992; Howe and Sibley, 1995; Ajzenberg *et al.*, 2002a). The majority of the isolates (> 94 %) are linked into the three main clonal multilocus genotypes I, II and III, where the population structure of *T. gondii* in Europe (De Sousa *et al.*, 2006; Jokelainen *et al.*, 2012) belongs predominantly to the type II lineage. In the south of Europe (Mediterranean countries), genetic diversity seems to be higher, revealing genotypes I and III and recombinant (Messaritakis *et al.*, 2008; Mancianti *et al.*, 2014; Vilares *et al.*, 2014; Bacci *et al.*, 2015). A fourth clonal lineage (haplotype 4) has been described as the most common type in wildlife in North America (Dubey *et al.*, 2011; Rajendran *et al.*, 2012). These findings are based, not only on the classical typing procedures (*Sag*2 and microsatellite typing), but also on PCR-RFLP by using other loci (Waap *et al.*, 2008; Jokelainen *et al.*, 2012; Vilares *et al.*, 2014; Verma *et al.*, 2015). In both animal and human samples, atypical and recombinant strains have been rarely reported (Bossi and Bricaire, 2004; Ajzenberg *et al.*, 2004; Lehmann *et al.*, 2006; Cavalcante *et al.*, 2007; Vilares *et al.*, 2014). Several studies revealed that recombinant (Dubey *et al.*, 2007a; b) and atypical strains are associated with more severe symptoms in immunocompromised hosts, the atypical genotypes are frequently associated with severe toxoplasmosis also in the immunocompetent human patients (Grigg *et al.*, 2001a; Bossi and Bricaire, 2004; Carme *et al.*, 2011; Demar *et al.*, 2012). In Portugal, there is a lack of data regarding the *T. gondii* genotype distribution in humans, and this issue gains special relevance since Portugal has a long history of trade and social interaction with Brazil and African countries, involving human and animal migrations and also import/export of food products. In fact, only one study has been published involving human samples (Sousa *et al.*, 2008), but it was based on serotyping (antibodies anti-GRA6) rather than on molecular approaches. That study revealed a majority of type II strains but also an unexpected high prevalence of non-type II strains that could not be typed as type I or III, suggestive of a recombinant profile. The existence of recombinant strains was later reported in animals (Vilares *et al.*, 2014). These results suggested the existence of a typing profile different from the one found in the other European countries (Sousa *et al.*, 2008). Considering this, our study aims to evaluate the *T. gondii* genotyping distribution and the virulence in mice of strains isolated from the biological samples of the patients with suspected toxoplasmosis. Also we describe the demographic characteristics of the referred patients, whose laboratory diagnosis was confirmed in the National Reference Laboratory of Parasitic and Fungal Infections of the NIH.

3.3. Materials and methods

Sample

We performed a retrospective analysis of the laboratory results of all cases with clinical suspicion of congenital and acquired toxoplasmosis, which have recourse to NIH for confirmation of infection, between January 2009 until December 2018. A total of 5722 cases were analysed (see Results for details) and we proceeded to study the molecular and virulence characterization of the strains isolated from the several biological samples of the positive cases.

For the descriptive analysis of the demographic data of the confirmed cases we used the calculation of absolute and relative frequencies.

Human biological products

Different biological products, namely blood (mother and child), amniotic fluid and placenta were analysed for the laboratory confirmation of congenital infection (pre and postnatal). For the laboratory diagnosis of acquired infection were analysed cerebrospinal fluid (CSF), cerebral biopsy (CB), vitreous humor (VH) and aqueous humor (AH).

***T. gondii* isolates**

All strains belong to the strain collection of the National Reference Laboratory of Parasitic and Fungal Infections at the Portuguese National Institute of Health (NIH) which is the responsible laboratory in Portugal for the pre - and postnatal diagnosis of toxoplasmosis at national level. We studied 48 *T. gondii* strains being 47 isolated in a congenital toxoplasmosis context from the follow biological samples: 31 strains were isolated from placentas (PL), 9 from umbilical cords blood (UCB), 5 from amniotic fluids (AF) and 2 from new born blood (NBB). In scope of aquireded toxoplasmosis, a single strain was isolated from a cerebral biopsy (CB) of a positive HIV with cerebral toxoplasmosis. The 48 strains revealed the following geographic distribution: 66.7 % were collected from the central region of Portugal, 25 % from the north, 6.3 % from the south, and 2 % from the Azores islands.

Laboratory confirmation

For the laboratory confirmation of congenital infection (pre and postnatal) we performed: Serological methods such as Direct Agglutination test (DAT) (bioMérieux SA, Marcy-l'Étoile, France), Enzyme-Linked Fluorescent Assay (ELFA), bioMérieux SA, Marcy-l'Étoile, France), Immunoglobulin Immunosorbent Agglutination Assay (ISAGA) (bioMérieux SA, Marcy-

l'Étoile, France), and Imunoblot (IB) (LDBIO Diagnostics, Lyon, France); molecular methods (real-time PCR for amplification of the REP-529bp repeat region) (Kasper *et al.*, 2009) and mice inoculation.

Mice Inoculation

The inoculation, maintenance, and euthanasia of mice were performed according to the Portuguese standards of the NIH, established for the pre- and postnatal diagnosis of toxoplasmosis, which are in accordance with the Protocol of International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences. Briefly, all clinical samples were inoculated by intraperitoneal injection (1 ml/mouse) in female mice [Hsd: ICR (CD-1®); Harlan Ibérica, Barcelona], two each for the AF, BB, UCB, and CB, and four for PL samples with negative serology to endoparasites. AF were centrifuged and resuspended in 1 ml, and PL were pretreated with trypsin (1:250) and antibiotics (250 UI / ml penicillin and 500 µg / ml streptomycin) before the inoculation. The remaining biological samples were not subjected to any pretreatment protocol.

The inoculated animals were monitored serologically by DAT at 10, 21, and 42 days post-inoculation (p.i.), in order to check the presence of *T. gondii*-specific antibodies. At the end of this period, mice were euthanized by inhalation of 5 % halothane and confirmed death by physical examination. The general morphological state of organs was observed, and sample tissues were collected from the brain and were visualized by optical microscopy in order to search for *T. gondii* cysts.

Although tissue cysts may develop in visceral organs, including the lungs, liver, and kidneys, they are more prevalent in the neural tissues, including the brain (Suzuki *et al.*, 2010). Intact tissue cysts are believed not to cause any harm and can persist for the life of the immunocompetent host without causing a host inflammatory response (Dubey *et al.*, 1998). We defined virulence at isolation (without knowledge of infecting dose) based on the mortality of mice within 4 months of infection and categorized isolates into two groups: virulent (death of 100 % of mice) and nonvirulent (< 30 % death).

T. gondii DNA extraction

T. gondii DNA was extracted from two different mice samples, namely, the ascitic fluid or the brain, depending on the concentration of tachyzoites in microscopy of ascetic fluids. The *T. gondii* DNA extraction from ascitic fluids was performed by boiling 1 ml of the sample for

30 min. From tissue cysts (± 1 cm3), DNA was extracted by using the QIAamp DNA mini kit (Qiagen, Chatsworth, USA) according to the manufacturer's tissue protocol, with slight modifications. These consisted of the homogenization of samples with 200 µl of extraction buffer and 40 µl of proteinase K and the DNA elution with 50 µl of elution buffer.

Genotype analysis of *T. gondii*

For genotyping purposes, all DNA samples from the inoculated mice were subjected to amplification and sequencing of the two ends of *Sag*2 gene and to a multiplex PCR of five microsatellites. The later included the beta-tubulin (TUB2) and the myosin A (TgM-A) genes as well as three expressed sequence tag (W35, B17, B18) markers (Howe et al. 1997; Ajzenberg et al. 2002, 2004). For *Sag*2, the PCR products were visualized in GelRED (Biotium Inc., Fremont, USA) stained 2 % agars gel electrophoresis, purified, and sequenced with an ABI 3130xl Genetic Analyzer (Applied Biosystems). For microsatellite analysis, capillary electrophoresis of PCR products was performed on the ABI 3130xl Genetic Analyzer (Applied Biosystems), and data were stored and analyzed with Gene Mapper (version 3.7; Applied Biosystems). MEGA5 software (Tamura *et al.*, 2007) was used to identify the genotypes by comparing the obtained sequences with the ones from reference strains available in GenBank (ME49-XM_018781602.1, VEG-LN714498.1, and RH-AY941252.1 and AY895019.1, RUB-AF357581.1, and MAS-AF357580.1).

3.4. Results

Sample characterization

During the ten years study period, 1459 cases of suspected toxoplasmosis were confirmed in a total of 5722 analyzed. Of these, 1436 (98.4 %) were patients with acquired infection and 23 (1.6 %) were patients with congenital infection (Figure 3.1).

Concerning the acquired infection, 93.4 % (1341/1436) were females, 5.5 % (79/1436) males and in 1.1 % (16/1436) of cases the gender was not known (Figure 3.2).

Regarding the distribution of cases of acquired infection by age group, it was observed that the majority of the women belonged to the group 25-44 years old 75.8 % (588/1341), followed by the group aged 15 - 24 years 16.9 %, (226/1341), with the majority of men belonging to the age group between 25 and 64 years old. Of the 23 cases of confirmed congenital toxoplasmosis during the 10 years under analysis it was found that 22 were children under one year of age and one were a child belonging to 1- 4 years old (Table 3.1). The frequency of cases of congenital infection all over these ten years of study was 1.6 %, (23/1459).

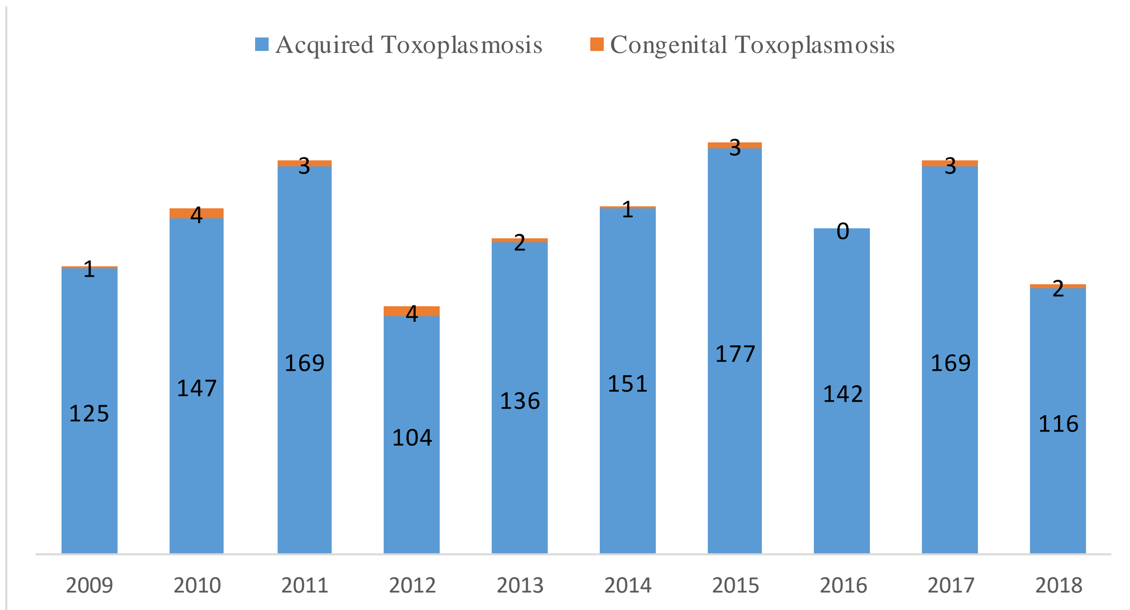


Figure 3.1. Annual distribution of acquired and congenital *Toxoplasma gondii* infection, 2009-2018. Number of cases by year of diagnosis.

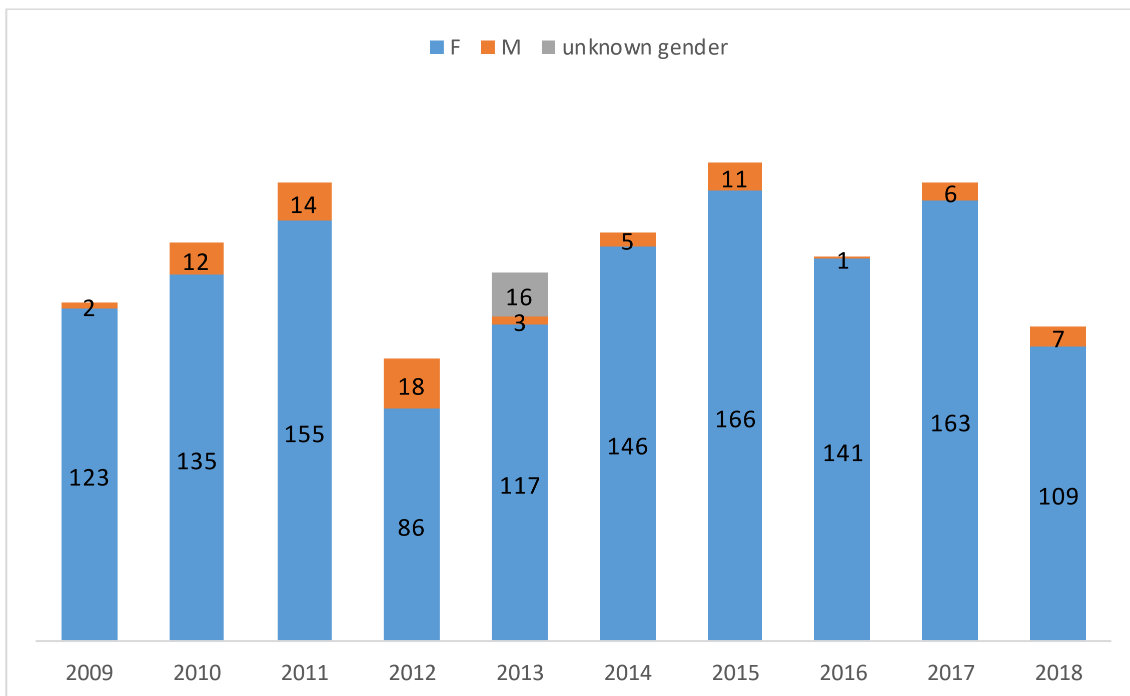


Figure 3.2 Annual distribution of *Toxoplasma gondii* infection acquired by gender, 2009-2018. Number of cases by year of diagnosis.

Table 3.1. Annual distribution of acquired and congenital *Toxoplasma gondii* infection by age group, 2009-2018. Number of cases by year of diagnosis.

Age (years)	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
< 1	1	4	3	4	2	0	3	0	3	2	**22**
1 – 4	0	0	0	0	0	0	0	0	1	0	**1**
5 – 14	4	0	0	0	0	2	3	1	0	2	**12**
15 – 24	32	41	25	19	21	18	23	26	16	19	**240**
25 – 44	66	106	115	75	88	124	147	112	140	80	**1053**
45 – 64	1	0	9	7	3	6	4	2	6	2	**40**
> 64	1	0	4	3	0	0	0	0	1	3	**12**
AU	21	0	16	0	24	2	0	1	5	10	**79**
Total	**126**	**151**	**172**	**108**	**138**	**152**	**180**	**142**	**172**	**118**	**1459**

Analysis of virulence in mice

Seven (Str_1, Str_3, Str_6, Str_11, Str_13, Str_18, Str_28) out of the 48 strains showed to be virulent in mice (Table 3.2), as they killed the mice after the first inoculation. Considering the location of tissue cysts, we searched for the presence of cysts in mice brain and found that, except for ten cases, all were positive. Two of the strains (Str_11, Str_3) that did not yield cysts in mice brain were virulent in mice. In this work, we did not observe moderate virulence (30% to less than 100 % mortality).

Genotype analysis

*Sag*2 genotyping was achieved in 100 % of samples and allowed the differentiation among the three "classical" *T. gondii* strain types. Thirty-five (73 %) strains were classified as type II, where 23 were isolated from mice inoculated with PL, four with AF, two with BB, and six with UCB. Thirteen (27 %) belonged to type I, where eight strains were isolated from mice inoculated with PL, one with AF, one with CB, and three with UCB (Table 3.2). PCR multiplex microsatellites analysis was applied to all samples and was highly efficient, it allowed the identification of 35 (73 %) strains as type II (25 PL, 3 AF, 1 BB, and 6 UCB), six (12.5 %) as type I (1 PL, 1 AF, 1 CB, and 3 UCB), one (2 %) as type III (1 PL), and six (12.5 %) as recombinant strains (4 PL, 1 AF, and 1 BB). Ten strains (21 %) identified by microsatellites analysis did not confirm the type previously identified by *Sag*2 typing. In fact, from these 10 samples, three were identified as type II (previously classified as type I by *Sag*2), one as type III (previously classified as type I by *Sag*2), three as I/III recombinant strains (previously classified

as type I by *Sag*2), and three more as I/II recombinant strains (previously classified as type II by *Sag*2) (Table 3.2).

Except one because we have no data, all the virulent strains were isolated in biological samples whose the newborns showed positive serology for IgG and IgM. Regarding PCR findings was negative or we had no data. Concerning newborn clinical features and treatment there is a lack of information from the clinicians to the laboratory (Table 3.2).

Table 3.2. Virulence and genotyping analysis of *Toxoplasma gondii* strains and laboratorial and clinical features of the biological samples.

Strain Number	Biological sample	Mice virulence /death day after inoculation	Genotype	NB Serology IgG/IgM	NBB PCR	NB clinical features	Treatment
1	AF	V/9	I	+/+	nd	-	-
2	UCB	NV	I	+/-	-	-	-
3	UCB	V/9	I	+/+	I	-	-
4	AF	NV	II	nd	nd	-	-
5	AF	NV	II	+/+	-	-	-
6	PL	V/11	I	nd	nd	-	-
7	PL	NV	II	+/+	-	-	-
8	PL	NV	II	+/+	nd	-	-
9	PL	NV	REC I/II	+/+	-	-	-
10	PL	NV	REC I/III	+/-	-	-	-
11	PL	V/7	REC I/III	+/+	-	-	-
12	PL	NV	II	+/+	nd	-	-
13	PL	V/42	REC I/III	+/+	-	-	Rov
14	PL	NV	II	+/-	+	-	-
15	UCB	NV	II	+/-	-	-	-
16	PL	NV	II	+/+	-	-	-
17	PL	NV	REC I/II	nd	+	-	-
18	PL	V/120	II	+/+	-	-	-
19	UCB	NV	II	nd	-	-	-
20	PL	NV	II	+/+	-	-	Spir
21	UCB	NV	II	+/+	I	-	-
22	PL	NV	II	+/-	I	-	Spir
23	PL	NV	II	+/+	nd	-	Rov
24	UCB	NV	II	+/+	-	-	No
25	PL	NV	REC I/II	+/+	I	IC	Rov
26	PL	NV	II	+/+	nd	-	Spir
27	AF	NV	REC I/II	Nd	I	-	Spir

28	PL	V/10	REC I/II	+/+	nd	-	Rov
29	PL	NV	II	nd	nd	-	-
30	AF	NV	II	+/-	+	-	Sulf+Pyr
31	CB	NV	I	NA	NA	NA	NA
32	PL	NV	II	+/+	-	-	No
33	UCB	NV	II	+/-	-	-	
34	UCB	NV	I	+/+	+	-	-
35	PL	NV	II	nd	nd	IC+C	No
36	UCB	NV	II			IGR	-
37	PL	NV	II	+/-	-	-	-
38	PL	NV	II	+/+	+	-	Rov
39	PL	NV	II	+/-	-	-	Spir
40	PL	NV	II	+/-	-	-	-
41	PL	NV	II	nd	-	-	No
42	NBB	NV	REC I/II	+/-	+	-	-
43	PL	NV	REC I/II or II/III	+/-	-	-	-
44	PL	NV	II	+/-	nd	-	-
45	PL	NV	II	nd	-	-	-
46	PL	NV	II	+/+	-	-	Spir
47	PL	NV	II	nd	nd	H	Rov
48	NBB	NV	II	+/-	-	-	Spir

AF amniotic fluid, UCB umbilical cordon blood, PL placenta, CB cerebral biopsy, NB newborn, NBB newborn blood, NV nonvirulent, V virulent, REC recombinant , Sulfadiazine (Sulf) and pyrimethamine (Pyr), Spiramycin (Spir), Rovamycin (Rov), Not applicable (NA), Intracranial calcifications (IC), Chorioretinitis (C), Intrauterine growth retardation (IGR), hydrocephalus (H) No data (nd), Inhibition (I) Positive (+) and (-) Negative.

3.5. Discussion

According to the laboratory criteria applied to the diagnosis of congenital toxoplasmosis, the 23 cases of infection in this study obtained a positive result for IgG and IgM antibodies, molecular detection and mice inoculation, leading to their validation as congenital toxoplasmosis cases.

As in other European countries, this study showed that that the greatest number of cases occurred in the 25 - 44 years of age and predominantly in women, what is expected because Portugal is one of the countries of the European Union where it is recommended to carry out the laboratory diagnosis of toxoplasmosis to all pregnant women and childbearing women who want to get pregnant (Ministério da Saúde: Direção-Geral da Saúde, 2011). With the exception of France, Germany and Poland that showed highest rates of new cases the new cases rates of

congenital toxoplasmosis found in this study is similar to the ones observed in the remain European coutries that range 0-4 cases per year (ECDC, 2017). In spite of the frequency of cases of congenital infection is low all over these ten years of study (1.6 %, 23/1459), it is important to emphasize that the majority of the acquired infections occurred in pregnant women, subsequently may have originated congenital infections. This fact demonstrates, for itself, the importance of laboratory toxoplasmosis surveillance in the pre-conception period and, even more, during pregnancy. According to the national seroprevalence study (corresponding to the study presented in Chapter II) (Gargaté *et al.*, 2016) the 18% of seroprevalence in childbearing women indicates that about 80% of portuguese women are not immune against the *T. gondii* infection. Thus, the majority of potential pregnant women are susceptible to primary infection and the risk of congenital toxoplasmosis is high. Thus, the identification of cases of toxoplasmosis in the present study corroborates the results referenced in previous studies and demonstrates the importance of active surveillance and systematic diagnosis of this infection, particularly in pregnant women and immunocompromised individuals, because they are population groups where this parasite is responsible for high morbidity and lethality rates.

In Portugal, as in most European countries, *T. gondii* isolates collected from animals (which enroll the majority of the studies) with chronic infections have shown remarkably little genetic diversity, where type I or recombinant strains have been rarely found (Herrmann *et al.*, 2010, 2013; Herrmann, 2012; Vilares *et al.*, 2014). Concerning strains collected from human beings, type II predominates in Europe (Howe *et al.*, 1997; Ajzenberg *et al.*, 2005), mirroring the scenario found in animals. However, there is a lack of knowledge of the genetic diversity of *T. gondii* strains from human samples in Portugal. The only previously published data concerns the report of type II strains in two HIV seropositive individuals (Ajzenberg *et al.*, 2009). In the present study, we enrolled 48 *T. gondii* strains collected throughout about two decades and observed that 67 % (32/48) of strains were classified as type II simultaneously by *Sag*2 and microsatellite procedures, which was quite similar to the genotypes distribution found in other European and US studies (Herrmann *et al.*, 2014). The results of multilocus genotyping were in agreement with the *Sag*2 genotyping for 38/48 isolates. The ten discrepant strains reinforce the interest of using a multilocus approach. Particularly, seven *Sag*2 type I isolates exhibited a mixture of type I/III or I/II alleles and three type II isolates exhibited a mixture of type I/II, being considered as recombinant strains. In this regard, the microsatellite typing method, which uses one multiplex PCR to perform multilocus typing with five markers (Ajzenberg *et al.*, 2005), was an efficient and essential tool for the identification of a molecular epidemiology scenario where 21 % (10/48) of the investigated strains were found to be recombinant strains. These were described in Portugal for the first time in 2008 and also in 2014 (both in animals) (Waap *et al.*, 2008; Vilares *et al.*, 2014). We speculate that the higher proportion of type I and recombinant

isolates in the human Portuguese sampling compared to other European studies (Howe *et al.*, 1997; Nowakowska *et al.*, 2006a; Ajzenberg *et al.*, 2010) may be explained by the fact that Portugal has a long history of trade and social interaction with South American countries (such as Brazil) and China (where atypical and recombinant strains are not uncommon), which involve human migrations, the import of food products (like meat or vegetables with the potential of transmitting the parasite), and also the presence of rats and cats (*T. gondii* intermediate and definitive hosts, respectively) in trading ships. Concerning the inoculation in mice, the virulence rate in vivo was quite low, 15 % (7/48), which is similar to the one observed in other countries (Ajzenberg *et al.*, 2002a; Gebremedhin *et al.*, 2014a). Two recombinant type I/III (Str_11, Str_13) and one recombinant type I/II (Str_28) were lethal to mice at the first inoculation and two of these (Str_11, Str_28) had the ability of transplacental transmission to the fetus. Unexpectedly, two type I strains were not lethal to mice, and mice death inoculated with one type II strain was observed. One could speculate that these strains may not be typical type I and II strains but instead recombinant strains that could not be identified with the limited number of molecular markers used. Another explanation for the observed virulence of the type II strain could be an elevated parasite load in the inocula. In fact, the biological samples are inoculated in mice according to the standard clinic diagnostic procedures, preventing the a priori determination of the number of parasites.

As concluding remarks, this study presents the first data in Portugal concerning *T. gondii* genotyping from human samples and it reveals genetic variations in the predominant clonal lineages and more specifically the existence of a considerable proportion of recombinant strains. It is imperative to better characterize the genetic background of these unusual strains as unusual genotype-phenotype associations (e.g., implications in specific disease outcomes) may eventually be found for some recombinant profiles. In this regard, it will be important to strength the genetic characterization of *T. gondii* strains by analyzing several other loci that are polymorphic and genome dispersed, in order to better understand the degree of genomic mosaicism displayed by the circulating strains.

Chapter IV

Parallel propagation of *Toxoplasma gondii* *in vivo*, *in vitro* and alternate models: towards less dependence on the mice model

This chapter corresponds to a recently submitted manuscript for publication in a *peer reviewed* international journal (Parasitology Research):

Parallel propagation of *Toxoplasma gondii in vivo*, *in vitro* and alternate models: towards less dependence on the mice model.
Maria João Gargaté, Anabela Vilares, Idalina Ferreira, Tania Reis, Susana Martins, Vitor Borges, João Paulo Gomes.

Personal contribution

Maria João Gargaté designed the study, performed most of the experimental work, analyzed the data and wrote the manuscript

4. Parallel propagation of *Toxoplasma gondii* ***in vivo***, ***in vitro*** and alternate models: towards less dependence on the mice model

4.1. Abstract

Toxoplasma gondii is an obligate intracellular protozoan able to infect most mammals worldwide. In pregnant women it can lead to severe birth defects or intrauterine death of the fetus. In immunocompromised patients, reactivation of latent infection may lead to life-threatening encephalitis. Almost everything we currently know about cell biology, immunology and genetics of *T. gondii* was achieved by using the RH virulent strain propagated in mice, as a model in the majority of the national reference laboratories for toxoplasmosis. According to the new recommendations concerning the animal welfare on behalf of their use in laboratory, we aimed to evaluate the potential of an *in vitro* system based on *T. gondii* propagation in cell-line, to replace, or at least reduce, the demanding animal model for strain propagation.

We evaluated the genetic and phenotypic stability of the *T. gondii* RH strain throughout its parallel continuous propagation in mice, in human foreskin fibroblasts, and in an alternate fashion of these two procedures. We also assessed the virulence impact of RH strain after different periods of long-term propagation strictly in cells.

Concerning the phenotypic impact of long-term tachyzoite passage in HFF we observed that the RH strain completely lost its virulence. On the other hand, we obtained a successful outcome with the alternate passaging of the parasite in HFF and in mice as this approach enabled *T. gondii* to maintain the virulence potential while keeping a putative stable genome. Although this strategy was very encouraging and putatively allows the reduction of sacrificed mice in more than 80 %, future studies are needed to evaluate its applicability to clinical strains, which are typically less virulent than the reference RH strain.

Keywords: genetic stability . phenotypic stability . RH strain . mice inoculation . Tachizoites

4.2. Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan able to infect most mammals worldwide. The parasite exists in three stages: *i)* oocysts, the product of sexual recombination in the intestine of members of the feline family; *ii)* tachyzoites, the invasive, rapidly replicating intracellular stage of the parasite; and *iii)* bradyzoites, the slowly replicating stages found in cysts in latently infected hosts (Holliman *et al.*, 2003). *T. gondii* infects up to a third of the world's human population and infection is mainly acquired by ingestion of food or water that is contaminated with oocysts shed by cats, or by eating undercooked or raw meat containing tissue cysts (Weiss and Dubey, 2009). This parasite crosses the intestinal, the placenta and the blood–brain barriers, and persists in its latent form in the central nervous system and muscle tissue. Primary infection is usually subclinical but in some patients it varies from a flu-like syndrome to lymphadenopathy and retinochoroiditis; infection is mostly asymptomatic in pregnant women but can lead to severe birth defects or intrauterine death in the fetus. In immunocompromised patients, namely AIDS and organ transplantation patients, reactivation of latent infection may lead to life-threatening encephalitis (Weiss and Dubey, 2009).

Almost everything we currently know about cell biology, immunology and genetics of *T. gondii* has been discovered using the RH strain as a model, which is a strain that was isolated in the USA by Albert Sabin from a 6-year-old boy who died of encephalitis in 1939 (Sabin, 1941).

Strain genotyping revealed that more than 95 % of strains belonged to one of three major clonal lineages, known as types I, II and III which predominate in North America and Europe (Howe and Sibley, 1995). More recent genetic analyses identified a fourth lineage in North America (Khan *et al.*, 2011). The RH strain and most of other highly virulent strains for mice were classified into lineage I, whereas the non-virulent strains mostly clustered into lineages II or III. The three lineages are all genetically identical, differing only by approximately 1–2% at the nucleotide level (Sibley *et al.*, 2009). Still, despite their expectedly high genetic similarity, they can induce strong phenotypic differences in mice. Type I (high virulence) strains are mortal at all doses, are more motile than the less virulent strains and are able to migrate across cellular barriers, which may explain how they disseminate so rapidly *in vivo* and cause high mortality (Barragan and Sibley, 2002), whereas types II (intermediate virulence) and III (low virulence) strains are much less pathogenic(Sibley and Boothroyd, 1992).

Population genetics and epidemiological studies have indicated a correlation between the geographic variations of this parasite genotype and disease manifestation in humans. For example, severe symptoms associated with ocular toxoplasmosis are more frequently reported in Brazil than in European countries (Grigg *et al.*, 2001a; Holland, 2003), and numerous incidences of severe systemic toxoplasmosis in immunocompetent adults from French Guiana have been

reported, in some cases resulting in the deaths of the afflicted individuals (Ajzenberg *et al.*, 2009); Darde et al., 1998; (Demar *et al.*, 2012). Taken together, virulence of different *T. gondii* strains in mice appears to be generally correlated with disease manifestations in human cases (Yolken, 2015). Therefore, determination of *T. gondii* virulence in mice could be invaluable in predicting the potential outcome of human infections.

The legislative regulations introduced in the last years by several countries had a huge impact on the development and evolution of the laboratory animal manipulation. The book of Russell and Burch of 1959, entitled *The Principles of Humane Experimental Technique,* became a central theme in laboratory animal science because it highlights how animal experimentation can be diminished or removed (Russell and Burch, 1959). These authors introduced the *Three Rs concept*, designating the terms Reduction (i.e., decrease the number of animals used), Refinement (i.e., diminish in the incidence or severity of painful or distressing procedures) and Replacement (i.e., substitution of living animals by *in vitro* techniques, computerized models, etc.), as a main guideline for the responsible use of animals in experiments. This concept is particularly applicable to *T. gondii* given the very high number of mice used in reference laboratories for strain maintenance and diagnosis. According to the new recommendations concerning the animal welfare on behalf of their use in laboratory, we aimed to evaluate the potential of an *in vitro* system based on *T. gondii* propagation in cell-line, to replace, or at least reduce, the demanding animal model for strain propagation. To reach this goal, we evaluated the genetic and phenotypic stability of the virulent *T. gondii* RH strain throughout its parallel propagation in mice, in human foreskin fibroblasts, and in an alternate fashion of these two procedures.

4.3. Material and Methods

The RH strain used in the present study is routinely maintained in mice by syringe intraperitoneal inoculation passages of ascitic fluid at 2-day intervals at the National Reference Laboratory of Parasitic and Fungal infections from the National Institute of Health since 1985.

General Workflow

We prepared six suspensions of the RH strain obtained from the mice ascitic liquid (Figure 1), each containing about 1×10^5 of tachyzoites in 0.2 ml. The concentration of tachyzoites was determined by using a Neubauer chamber under light microscope (40 x magnification) and its viability was accessed after staining a cover slip with metilen blue at 0.1 % (v/v). These suspensions were used to simultaneously inoculate in two mice ("experiment *A"*), in two cell culture flasks ("experiment *B"*) and in two additional cell culture flasks ("experiment *C")*. As represented in Figure 1, the experiment *A* consisted of continuous passages of the RH strain in mice, the experiment *B* consisted of continuous passages of the RH strain in a cell line, and finally, the experiment *C* consisted of intercalate passages of the RH strain in the cell line and in mice. This study was developed during a period of 10 months. At the harvesting steps (detailed below), a 500 µl aliquot was taken and frozen at – 20 ºC and – 80 ºC. From this large set, 30 samples (10 samples from each experiment *A, B*, and *C*), corresponding to one sample per month, were subjected to genetic characterization of the passages strain by Next Generation Sequencing (NGS) (also detailed below). Additionally, two control mice were injected with DMEM only and two cells culture flasks were used as control as well.

In order to set up the time points enrolled in Experiment C (i.e., the decision of a suitable period regarding the maintenance of the tachyzoites in cells before passing them to mice), we preliminarily evaluated the time at which the tachyzoites revealed a considerable number and motility decrease and shape alteration (through microscopic evaluation). This time consisted of approximately three weeks (data not shown), so we opted for a highly conservative approach in Experiment C by designing it with periods of RH passaging in the cell line not exceeding one week. Concerning experiment *B,* which is a challenging mice-independent procedure, to avoid the culture loss, whenever a strong decrease on the number of harvested tachyzoites was observed (after approximately 12 days), the aliquot of the immediate previous passage was thawed and was used to proceed the continuous cell line culture.

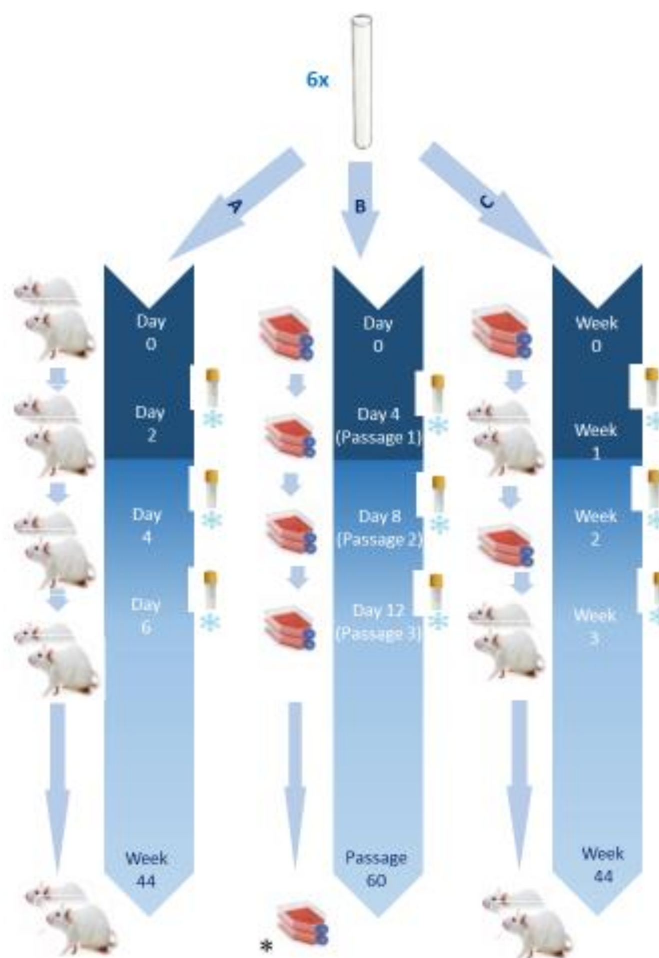


Figure 4.1 Workflow of RH strain inoculations. Experiment A: continuous mice inoculation, which took place every two days; Experiment B: continuous cell line (Human foreskin fibroblasts) passages, taking place every 72 - 96 h; Experiment C: intercalate cell line passages and mice inoculation. Before each inoculation in mice, tachyzoites were propagated for two periods of 72 - 96 h (designated as "week" in the Figure) in the cell line. The study duration was about 44 weeks (about 10 months).
* Although the experiment B consisted of a continuous passaging of the RH strain exclusively in the cell line, an aliquot taken from the harvesting procedure at week 3, 22 and 44 was used for a mice inoculation in order to evaluate the phenotypic impact after different periods of long-term propagation of *T. gondii* strictly in cells.

Mice propagation

We used female CD1 mice with 6-8 weeks old and weighted 16 - 18 grams [Hsd: ICR (CD-1®); Harlan Ibérica, Barcelona]. Mice inoculation, maintenance and euthanasia were performed under the standards of the Portuguese NIH, which are in accordance with the Protocol of International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences. Animals were housed in cages

and maintained under controlled conditions (21 ± 2 °C, 65 - 70 % humidity) and standard food and water *ad libitum* during the experiments.

A suspension of the RH strain (0.2 ml, corresponding to 1×10^5 tachyzoites**)** was inoculated in mice by syringe intraperitoneal inoculation and after 48 hours mice were euthanized. Then, tachyzoites were harvested from the peritoneal cavity of infected mice by flushing with 5 ml of sterile phosphate buffer saline (PBS; 0.01 M, pH 7.2), and were centrifuged at 2000 rpm for 10 min at room temperature to remove peritoneal cells and cellular debris. The pellets enriched with tachyzoites were recovered with PBS (PBS; 0.01 M, pH 7.2) and 0.2 ml of a 1×10^5 / ml suspension were intraperitoneally inoculated in another animal. Following this procedure, brain tissue was harvest from the mouse and observed in the light microscope in order to search for *T. gondii* cysts. Visual inspection was used to identify the phenotypic effect of *T. gondii* RH strain in mice, namely, the general condition of the animal, hair condition and stool consistency. This entire procedure was rigorously followed in experiment A, and, with exception of the timelines, also followed in experiment C for mice inoculation and harvesting.

Cell culture propagation

Human foreskin fibroblasts (HFF-1ATCC - SCRC-1041) obtained from ATCC were grown in 10 ml of culture medium using 25cm^2 flasks (Sarstedt, 831810.002). For maintenance purposes, this cell line was grown in Dulbecco's Modified Eagle Medium DMEM (Gibco 42430-25) with 10 % of heat inactivated fetal calf serum FCS (Gibco, 10270106), penicillin (12 ug/ml), streptomycin (10 ug / ml) (Gibco 15140 - 122) and (1 %) fungizone (Gibco 15290-018) and was incubated in 5 % CO_2 at 37 °C and > 80 % humidity. Then, when a confluent monolayer was obtained, a maintenance medium (the same as above but supplemented with FCS 5 % instead of 10 %) was used. Cells were routinely subcultured every 3 days by trypsination, 0.25 % trypsin (Gibco 25300 - 062) with 0.03 % EDTA solution and washing with phosphate-buffered saline (pH = 7.2).

For the inoculation with the RH strain, after 70 % confluency of the cell line, 1ml of the suspension of tachyzoites (~ 1×10^5) were added to the cell line followed by an incubation at 37 °C, > 80 % humidity and 5 % CO_2 for 72 - 96 h. Two 25 cm^2 flasks were always simultaneously inoculated. After this period, the medium was aspirated and the cells monolayer was washed with 5 ml of sterile phosphate buffer saline (PBS; 0.01 M, pH 7.2) and scraped. The amount and the viability of tachyzoites harvest from the cell culture were determinate with a Neubauer chamber under light microscope (40 x magnification) after staining with metilen blue 0.1 %. These tachyzoites were then diluted to a final concentration of 1x10^5 per ml and constitute the new inoculum for a new set of two 25 cm^2 flask with 70 % confluency of HFF cells. From each passage,

a 500 ul aliquot was taken and freezed with 1ml of 50 % FBS and 1 ml of 20 % DMSO in cryovials with a cooling rate of 1 ºC per minute until reaching - 80 ºC and then they were placed in a - 80 ºC freezer or in a liquid nitrogen tank. Whenever the need to use a frozen aliquot arose (see "general workflow" above), the frozen aliquot to be used for inoculation purposes, were placed in a water bath at 37 ºC with shaking, until completely thawed. In order to identify the phenotypic effect of the *T. gondii* RH strain on the inoculated cells, direct examination with a phase contrast optics microscope (40 x) was used. This entire procedure was rigorously followed in experiment B, and, with exception of the timelines, also followed in experiment C for cells inoculation and harvesting.

Although the experiment B consisted of a continuous passaging of the RH strain exclusively in the cell line, an aliquot taken from the harvesting procedure at week 3, 22 and 44 was used for a parallel mice inoculation in order to evaluate the phenotypic impact after different periods of long-term propagation of *T. gondii* strictly in cells.

DNA extraction, PCR and Next Generation Sequencing (NGS) of RH *T. gondii* strain

RH *T. gondii* DNA was extract directly from the ascitic fluid of tachyzoites suspension and from the tachyzoites harvest from HFF cells suspension by using the QIAamp DNA mini kit for tissues (Qiagen, Chatsworth, USA) according to the manufacturer's tissue protocol.

In order to perform a brief evaluation of the genetic stability of *T. gondii* on the course of the three described experiments, the following loci, which are spread by different *T. gondii* chromosomes (http://toxomap.wustl.edu/verticalmap08_01-2005high.jpg), were enrolled in this study: i) *Sag*2 (the classical typing gene); ii) three loci traditionally used for microsatellites-based typing (TgM-A, B18, W35); and, iii) five polymorphic loci potentially involved in adaptation/virulence (CB21-4, PK1, *Gra*6, *Sag*3, M102). Target regions and PCR primers for all loci are described in Table 4.1, and are divided in two panels (with and without 5' adapters for amplicon-based NGS Illumina protocols), according to the amplicon size and the position of the target microsatellites within some amplicons. In order to generate high quality amplicons for subsequent high-throughput amplicon-based Next Generation Sequencing (NGS), different PCR were conducted. Briefly, a multiplex PCR was applied for loci M102, B18 and W35, while *Sag*2, TgM-A, CB21-4, PK1, *Gra*6, *Sag*3 were targeted by independent PCR. All PCR products were visualized in GelRED (Biotarget, Lisbon, Portugal) stained 2% agarose gel electrophoresis. For each sample selected for NGS (see above), PCR products, with and without Illumina adapters, were pooled separately, before being purified and and subjected to the Nextera XT DNA Library Preparation protocol (Illumina Inc, San Diego, CA, USA), according to manufacturer's

instructions. Finally, libraries obtained from amplicons with and without Illumina adapters were independently sequenced (2 x 150bp paired-end reads) using a MiSeq (Illumina) equipment.

Table 4.1 Loci, amplicon length and primers used for PCR.

Locus	Chr	Foward Primer	Reverse Primer	Box 1[c]	Box 2[c]	Amplicon length (bp)
Pk 1	VI	FI 5' TCA TCG CTG AAT CTC ATT GC 3'	RI 5' CGC AAA GGG AGA CAA TCA GT 3'	NA	NA	873
Gra 6	X	FI 5' TTT CCG AGC AGG TGA CCT 3'	RE 5' TCG CCG AAG AGT TGA CAT AG 3'	NA	NA	314
*Sag*2 5'	VIII	F4E 5' GCTACCTCGAACAGGAACAC 3'	R4E 5' GCATCAACAGTCTTCGTTGC 3'	NA	NA	305
*Sag*2 3'	VIII	F3E 5' TCTGTTCTCCGAAGTGACTCC 3'	R3E 5' TCAAAGCGTGCATTATCGC 3'	NA	NA	297
Sag 3	XII	FI 5' TCT TGT CGG GTG TTC ACT CA 3'	RI 5' CAC AAG GAG ACC GAG AAG GA 3'	NA	NA	211
M 102	VIIa	F 5' GAG CGA CGC CCG TAT GAT AAG G 3'	R 5' CGC GCT GAG AAG CTG ACA TAC AG 3'	NA	NA	427
CB21-4	III	F 5' CCA GGT GTT TCG ATA TTG AT 3'	R 5' GCC TGT GTG GTG TTC GAA TC 3'	TACGCATACA	GTACATTCTT	469
TgM-A	X	F[a] 5' GGCGTCGACATGAGTTTCTC 3'	R[b] 5' TGGGCATGTAAATGTAGAGATG 3'	CGTGTTTCCA	TTTGTAAGTC	207
B18	VIIa	F[a] 5' TGGTCTTCACCCTTTCATCC 3'	R[b] 5' AGGGATAAGTTTCTTCACAACGA 3'	TGCCTGTAGC	GGATTCCGCA	160
W35	II	F[a] 5' GGTTCACTGGATCTTCTCCAA 3'	R[b] 5' AATGAACGTCGCTTGTTTCC 3'	TCTTGGCTTT	GTGTCGCTGT	248

[a] Primers with adapters - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
[b]Primers with adapters - GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
[c]Sequences contiguously flanking each side of the tandem repeat region that are used for in silico extraction of the microsatellite size.
NA - not applicable

Bioinformatics analyses

For Single Nucleotide Polymorphisms (SNPs) / indel screening, NGS data was processed using the mapping-based bioinformatics pipeline implemented in INSaFLU (https://insaflu.insa.pt/), which is a web-based platform for amplicon-based NGS data analysis (Borges *et al.*, 2018). Briefly, the core bioinformatics steps involved: i) raw NGS reads quality analysis and improvement using FastQC v. 0.11.5; (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and Trimmomatic v. 0.27 (http://www.usadellab.org/cms/index.php?page=trimmomatic), respectively; and, ii) reference-based mapping, consensus generation and variant detection using the multisoftware tool Snippy v. 3.2-dev (https://github.com/tseemann/snippy), using a multi-FASTA file with representative sequences of RH strain of each one of the targets amplicons as reference sequence. Mapping results were inspected and confirmed through visual inspection using the Integrative Genomics Viewer (http://www.broadinstitute.org/igv).

For *in silico* microsatellite-size analysis, we took advantage of a previously applied bioinformatics script (Pinto *et al.*, 2016) that allows capturing, directly from NGS reads (after quality improvement with Trimmomatic), the repeat number profile within the microsatellite region (i.e., the microsatellite size). This strategy consists on the extraction and counting DNA

sequences (i.e., the microsatellites sequences) that are flanked by two conserved, small DNA strings. The defined strings for the microsatellite-containing loci are detailed in Table 4.1.

All raw sequence reads generated in the present study were deposited in the European Nucleotide Archive (ENA) (BioProject PRJEB34235). Detailed ENA accession numbers are described in Supplementary Table S1.

4.4. Results

Throughout the entire 10-month study, in experiment A, we observed that RH strain always killed the mice in two days and presented tachyzoites-rich ascites, with a median of 7.50 x 10^7 / ml (mean of 9.15 x 10^7 / ml SD 4.30 x 10^7 / ml) tachyzoites recovered in the harvest procedure (Figure 4.2). During these two days, the animal showed great prostration, completely bristly hair and soft stools (Figure 4.3). In the necropsy of the more than 200 mice enrolled in experiment A, no *T. gondii* cysts were observed in mice brain tissues.

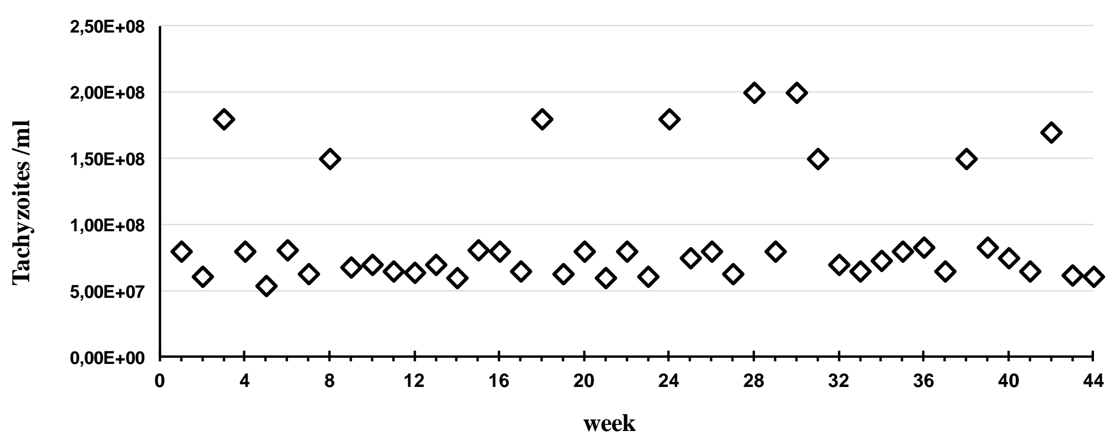


Figure 4.2 Experiment A: Continuous RH strain mice propagation. The squares represent the tachyzoites concentration harvested during the 10-month study period.

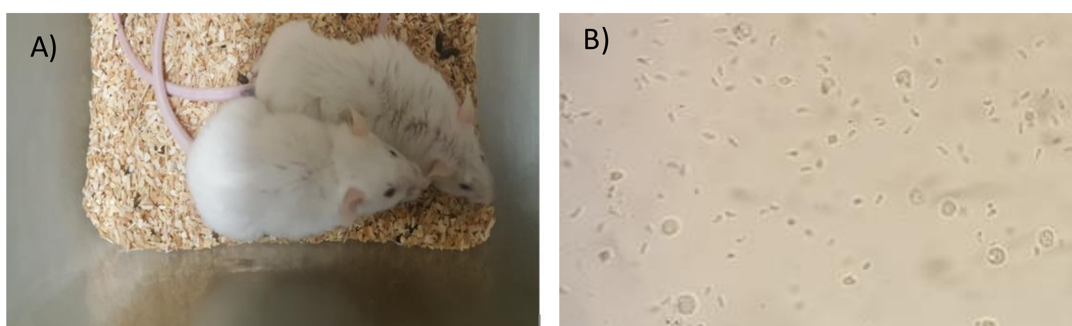


Figure 4.3 Phenotypic evaluation on the course of experiment A. Panel **A** shows the phenotypic effect of RH strain propagation in mice, where mice can be observed totally prostrate in a corner of the cage and with bristly hair; Panel **B** represents the phenotypic effect of RH strain propagation in HFF, showing tachyzoites with a thin shape and motile rich ascites (40x magnification).
Image source: National Reference Laboratory of Parasitic and Fungal Infections.

Regarding experiment B, we observed an increase in the number of RH strain tachyzoites inside the cells within 6 to 9 h, and after approximately 24 h parasites formed rosettes with the apical ends directed towards the parasitophorous vacuole membrane and some of them were dispersed in the cytoplasm of the host cell. After 72 h, most of the cells were infected and some of them detached from the flask. After 96 h, the monolayer was destroyed and a large number of tachyzoites could be observed in the supernatant. These observations stood for the entire study period. (Figure 4.4)

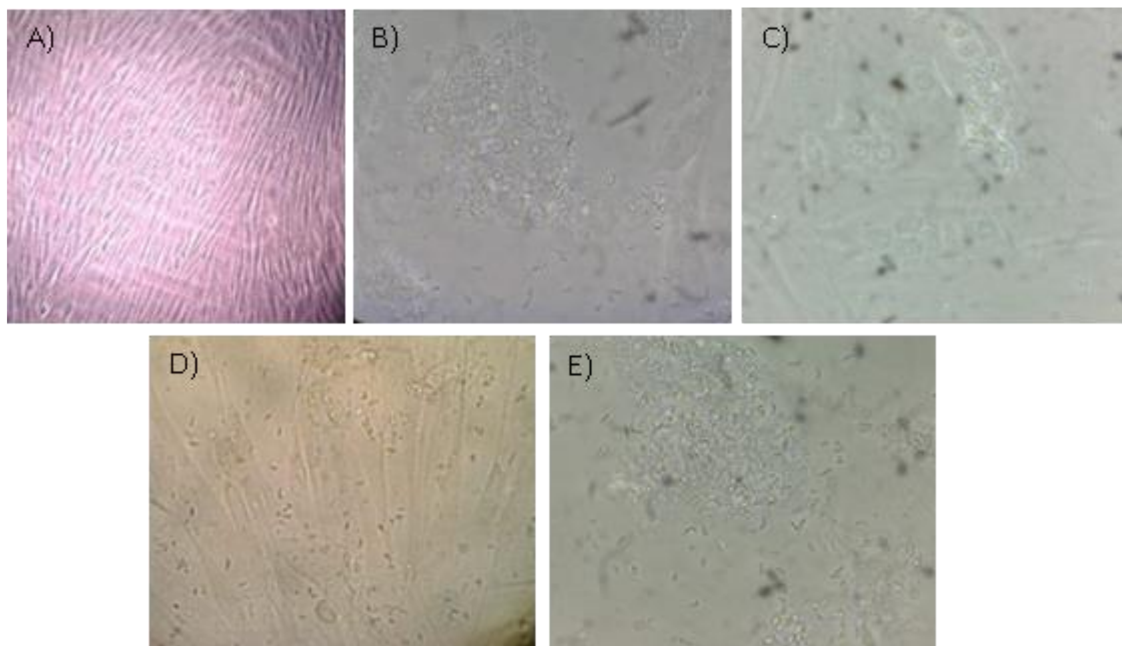


Figure 4.4 Phenotypic evaluation on the course of experiment B. Phenotypic effect of RH strain inoculation in HFF (40x magnification). Panel **A** - HFF monolayer; Panel **B** - Tachyzoites replicated in the cells within 6 to 9 h; Panel **C** - Tachyzoites formed rosettes with the apical ends directed towards parasitophorous vacuole membrane within 24 h; Panel **D** - Most of HFF are infected and some of them detached from the flask within 72 h and Panel **E** - Monolayer is destroyed and a large number of tachyzoites are in the supernatant within 96 h.
Image source: National Reference Laboratory of Parasitic and Fungal Infections.

We also observed that, in each cycle of three passages (approx. 12 days), the amount of tachyzoites that were harvested in each passage progressively decreased, from a median of 3.36×10^7 / ml (3.58×10^7 / ml SD 7.16×10^6 / ml) in the first passage of each cycle, to a median of 1.05×10^7 /ml (mean of 1.08×10^7 / ml SD 1.28×10^6 / ml) in the last passage (Figure 4.5). Therefore, in order to avoid losing the strain, the aliquot of the immediate previous passage (i.e., second passage) was thawed and used for the subsequent inoculation. This yielded a highly homogeneous fluctuation dynamic of the tachyzoites recovery during the entire experiment B period (Figure 4.1) Also, within each of these three-passages cycles, the size of the tachyzoites

diminished and their shape became rounded, although these phenotypes were hardly seen after the first of the three passages.

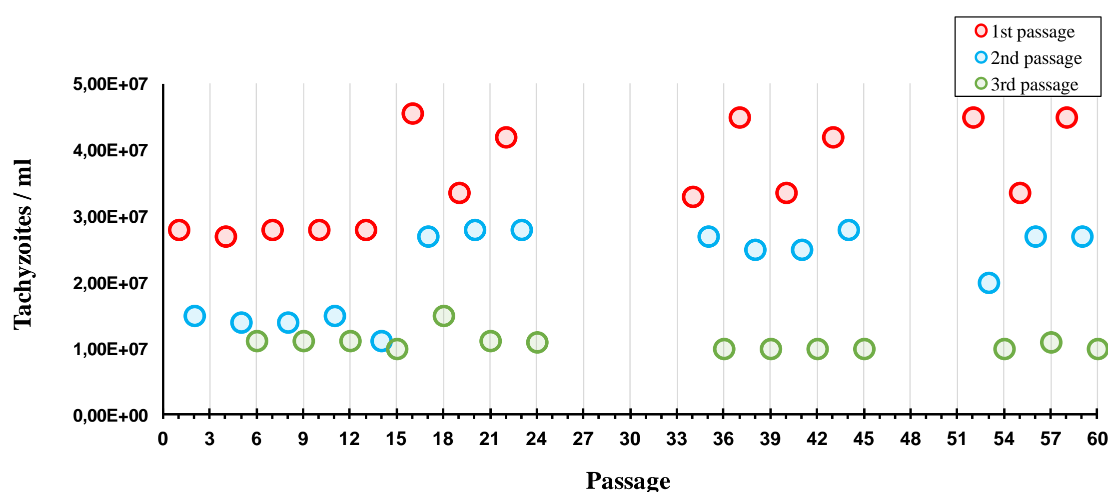


Figure 4.5 Experiment B: Continuous RH strain cell line propagation. The circles represent the tachyzoites concentrations that were harvested during the study period (60 passages of 72 - 96 h). The different colours represent the three passages within each cycle of ~12 days.

We observed that the virulence of the RH strain after long periods of passages in the cell line strongly decreased. In fact, after different periods of long passaging in HFF (namely, after 3, 22 and 44 weeks from the first inoculation), the two mice that were inoculated with the harvested tachyzoites did not die in short periods of time. In fact, one of them was euthanized for observation after seven days and the other lived for 11 months. The ones that were euthanized showed no tachyzoites in the ascitic liquid.

Finally, regarding experiment C, during the entire 10 - month period, we observed a microscopic-based phenotypic profile of tachyzoites that was similar to the one described above for the first passage within each cycle of three passages of experiment B (i.e., timelines, rosettes formation and tachyzoites shape), i.e., with only sporadic and modest alterations when compared with the ones observed in the tachyzoites from the mice ascites.. We observed quite homogeneous values of the tachyzoites concentration that were harvested from the mice (median 7.05 x 10^7, mean of 7.03 x 10^7 / ml SD 8.43 x 10^6), which were in the same range as in experiment A. For the harvested tachyzoites from the cell line, the values obtained (median 2.60 x 10^7 / ml, mean of 2.35 x 107 / ml SD 5.50 x 106 / ml) fitted the interval observed for the three passages within each 12-day cycle of experiment B (Figure 4.6). For better visualization purposes, Figure 4.7 shows the comparison of tachyzoites yield between the three propagation experiments.

We observed that, after each period of 72-96 h in the HFF cell line, the tachyzoites showed a virulence decreased phenotype in mice as they caused mice death only after about seven days.

Curiously, we observed no obvious decrease in the number of tachyzoites (Fig. 4.6 and 4.7) that were recovered in the ascites, when compared with experiment A.

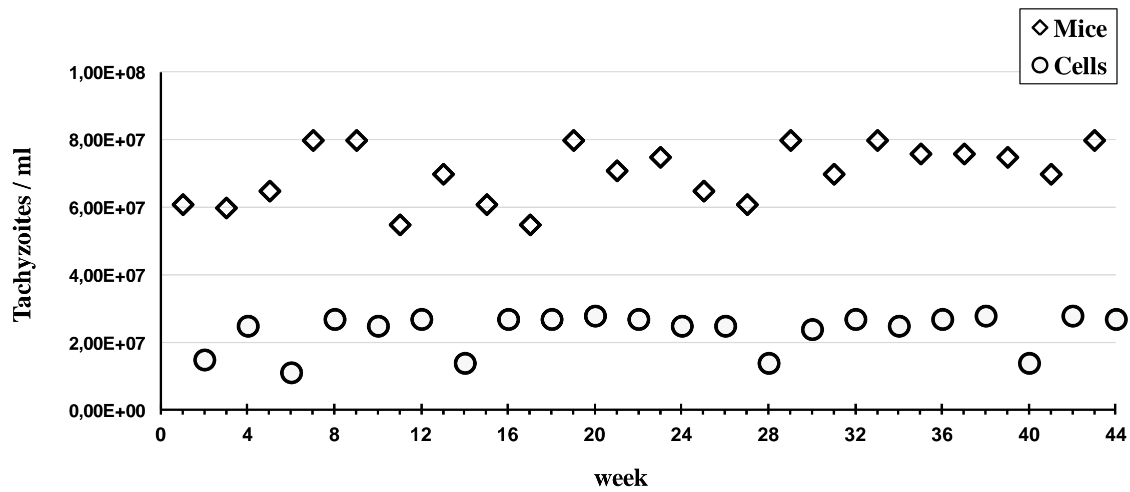


Figure 4.6: Experiment C: intercalate passages of the RH strain in the cell line and in mice. The circles and squares represent the tachyzoites concentration that were harvested from the cell line and mice, respectively, during the study period (44 weeks).

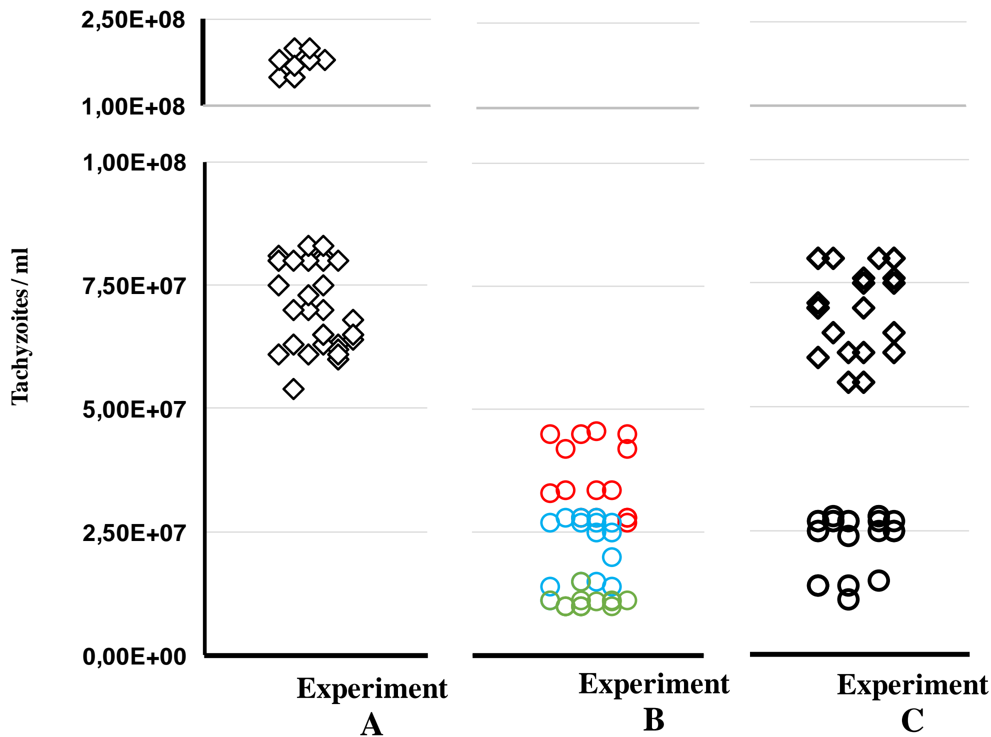


Figure 4.7 Comparison of tachyzoites yield between propagation experiments. This dispersion graph displays the concentration of *T. gondii* (expressed as tachyzoites/ml) that were harvested during the three parallel propagation experiments: For experiments A and C, each value reflects the tachyzoites concentration that was harvested each week (a total of 44 weeks), while, for experiment B, each value reflects the tachyzoites concentration obtained after each passage (a total of 60 passages). For experiment B, it also shows the yield obtained after the first (red circles), second (blue circles), and third passage (green circles) within each cycle of three passages (~12 days). Circles and squares represent concentration values obtained from harvested cells and mice, respectively.

Finally, regarding the evaluation of potential genomic changes arising throughout propagation, which enrolled ten samples (about one per month) from each "experiment" and targeted nine genome loci, we observed no SNP/indels both chronologically (for a given experiment) and between experiments. Also, for microsatellite-harbouring loci (B18, W35, TgM A and CB21-4), we observed the same consensus microsatellite length (and profile) for all samples. Of note, the consensus lengths retrieved by our NGS/Bioinformatics-based approach for the RH strain matched the ones previously reported (Vilares *et al.*, 2017) by traditional microsatellites-based analyses relying on electrophoresis-based DNA sizing.

4.5. Discussion

The maintenance of reference strains, namely the RH, in a National Reference Laboratory (NRL) is imperative since the Sabin- Feldedman dye test is the Gold Standard among serological test and requires fresh viable tachyzoites. While parasite propagation is obligatory for antigen production, it is also useful for tachyzoites enrichment for typing purposes, and can also provide vital information in studies focusing *T. gondii* lifecycle (and its particular stages) (Lambert *et al.*, 2006; Lachenmaier *et al.*, 2014), host invasion and host–parasite interactions. Although some of these goals may be achieved by using cell-lines for tachyzoites' propagation, such as antigen production (Evans R., 1999), the animal model remains crucial for studying the natural infection dynamics and parasite dissemination, and is required to confirm findings after initial *in vitro* investigations (Szabo and Finney, 2017). Also, the *in vivo* model has a vital role in a NRL because it is the gold standard method for the isolation of *T. gondii* strains in biological samples or body fluids (Derouin *et al.*, 1987) constituting, for instance, the reference methodology in pre- and post-natal diagnosis of toxoplasmosis. However, this *in vivo* procedure frequently involves the need to sacrifice hundreds of mice per year, and is also extremely laborious, requiring complex facilities and human skills either for the isolation of strains or for their maintenance process. Furthermore, the legislative regulations and the ethical measures that have been arising regarding the use of animals in the laboratory (EEC, 1986; Haywood and Carbone, 2009; 2010/63/EU, 2010) push the researchers towards the use of alternative approaches to diminish the animal manipulation. As such, recent technological advances rely, for instance, in the development of three-dimensional (3D) tissue culture models as well as on engineering specific tissues and organs for laboratory use. These improved models try to mimic *in vivo* host physiology and could replace current *in vivo* models, thus reducing the need for animal manipulation. For example, in the field of congenital toxoplasmosis, a recent study enrolling the development of a 3D culture system based on trophoblasts allowed to investigate the interaction between specialized cells of the embryonic epithelial that are in direct contact with maternal blood (syncytiotrophoblasts) (McConkey *et al.*, 2016) and *T. gondii*. However, in general, these developed models are in an embryonic stage and

still constitute a long shot for routine use in most laboratories. As such, on behalf of our role as the reference laboratory for toxoplasmosis at the Portuguese National Institute of Health, we aimed to investigate if we can replace, or at least reduce, the demanding animal model for strain propagation by using “feasible” methodologies. Therefore, we used three parallel approaches consisting of continuous passages of the RH strain in mice (Experiment A), in a cell line (Experiment B), and intercalate passages of the RH strain in the cell line and in mice (Experiment C).

In order to compare the outcomes of each propagation experiment, we started to evaluate the tachyzoites capacity to maintain an active multiplication in a cell line throughout continuous passaging. As expected, throughout continuous propagation in mice (Experiment A) we always obtained tachyzoites-rich ascites with values for tachyzoites contentration in the harvesting processes (median of 7.50 x 10^7 / ml) that were up to 7.5 - fold higher than the ones obtained for the approach enrolling the exclusive propagation in the cell line. (Experiment B). Curiously, in Experiment C, which enrolled 46 passages in the cell line intercalated with 14 passages in mice, we faced a very encouraging scenario. In fact, not only we obtained tachyzoite-rich ascites with harvesting values (median of 7.05 x 10^7 / ml), similar to the ones obtained in Experiment A, but we also obtained tachyzoites yields in the multiples harvesting processes of the cell line (median of 7.05 x 10^7 / ml) about 2.5 - fold higher than the ones obtained at the end of each cycle in Experiment B (Figures 4.6 and 4.7). This suggests that, although the tachyzoites capacity to maintain an active multiplication in a cell line throughout continuous passaging decreases over time (we observed a ~3-fold decrease from the first to the third passage within each cycle of ~12 days in Experiment B) (Figure 4.5), this capacity seems to be renewed when, after short periods in a cell line (e.g., one week, Experiment C), the tachyzoites are transferred to mice.

A second evaluation consisted of assessing potential phenotypic alterations upon propagation of the parasite in a cell line, such as tachyzoites shape and motility. Contrasting to Experiment A for which we observed tachyzoite-rich ascites with tapered shape and with motility, the continuous propagation of tachyzoites in the cell line lead to a decrease in their size and to an alteration of its shape, which was notably seen at the end of each 12-days cycle. This was consistently observed for all 12 - days cycles of Experiment B. Noteworthy, minor phenotypic changes were observed in the microscopic observation of tachyzoites harvested from the cell line in Experiment C, whereas a typical phenotype could be seen in the mice ascites.

Afterwards we evaluated the capacity to maintain a virulence phenotype in mice. Regarding Experiment A we observed that RH strain always killed the mice in two days, as is routinely observed in our laboratory for this strain. This is concordant with what is expected for *T. gondii* genotype I strains, which are usually responsible for lethal infections in mice, while types II and

III are significantly less virulent (Lambert *et al.*, 2006). Several studies showed (Saeij *et al.*, 2005b; Kim *et al.*, 2007) that a prolonged passage in mice of Type I RH strain leads to an attenuation of tissue cysts/bradyzoites formation, although this assumption is not consensual. There are still controversies on the potential of cyst formation of this strain but, similarly to several studies, (Asgari *et al.*, 2013) we observed that our RH strain lost its ability of cystogenesis possible due to the prolonged and interrupt mice passages since 1985. In particular for Experiment B, we also evaluated the impact of the tachyzoites long-term passaging in the HFF cell line at three time points (at week 3, 22 and 44) after the first inoculation, and observed that the RH strain completely lost its virulence. In fact, when we inoculated the harvested parasite into two mice and euthanized one of them (the other died of old age) for observation, there were neither tachyzoites in its ascite nor cysts in the brain. In Experiment C, we observed an intermediate scenario, as some virulence decreased phenotype in mice was observed after ~1 week in cell culture (throughout the entire study period) because RH strain consistently killed the mice after about seven days and not in two days as in Experiment A. This strongly suggests that, although a continuous passaging in cell lines may be useful for some specific proposes (e.g., antigen production), this approach cannot be successfully used for maintaining *T. gondii* strains for other reference purposes as they unequivocally loose the virulence phenotype. Nevertheless, hybrid approaches such as the here presented in Experiment C seem to have the potential to overcome the virulence issue, which is crucial in most *T. gondii* reference laboratories.

Finally in order to assess if the propagated RH strain underwent genomic changes throughout propagation (regardless the strategy), we conducted a humble genomic evaluation focused on both traditional typing loci and selected polymorphic loci that are genome-dispersed and are believed to play a role in adaptation. Whereas the hypothetical detection of genetic alterations throughout the study could suggest the putative inadequacy of using the selected approaches for strain maintenance, we observed no genetic changes throughout each experiment and between experiments. Thus, contrarily to the former hypothesis, no definitive conclusions about genetic stability can be taken, given the limited extent of the *T. gondii* genomic regions that were surveyed Nevertheless, broader evaluations at the genomic level are very challenging, considering both the large genome size (~65 Mb) and the high complexity of the 14 chromosomes of *T. gondii* . Genome-wide surveys would make this evaluation impractical and cost prohibitive. Of note, in this study, we unprecedentedly applied a robust NGS/bioinformatics approach for *in silico* extraction of the microsatellite size for three loci (TgM-A, B18, W35) traditionally used in microsatellites-based *T. gondii* genotyping. This technological innovation constitutes a proof-of-concept that the traditional microsatellites analyses (based on laborious and error-prone electrophoresis-based DNA sizing) might be straightforwardly transfer to more robust

NGS/bioinformatics-based methodologies, while keeping backwards compatibility with "historical" typing data.

As concluding remarks, the results of the present study demonstrate the feasibility of using tachyzoites propagation approaches based on alternate passages on mice and cell lines, strongly impacting the number of sacrificed mice. This would enable laboratories to maintain and propagate *T. gondii* strains and use them for virtually any purpose, as not only the replication capacity, shape and motility of tachyzoites seems to be maintained, but also the virulence appears not to be obliterated. Finally, although a humble genomic survey was performed, no apparent genetic alterations were observed as a consequence of *in vitro* propagation. Nevertheless, other evaluations will be needed, eventually enrolling other cell lines, propagation time lines and strains other than the reference strain RH (that is maintained in all reference labs) in order to reach more definite conclusions regarding the usefulness of *T. gondii* propagation approaches as the one presented here.

Chapter V

Final overview, concluding remarks and future perspectives

5. Final overview, concluding remarks and future perspectives

T. gondii is one of the most successful parasitic organismsis that infects over a third of the world's humanity. Humans remain infected for their whole lives and this infection constitutes a life-long threat. The high disease burden reported in the multiple studies performed worldwide (referred in chapter I of this dissertation) highlights the lifetime effects that *T. gondii* can have on infected individuals and the worldwide public health concern that this infection represents. The main goal of this PhD thesis was to contribute for the knowledge of toxoplasmosis in Portugal. We pursued this goal essentially due to the existence of significant knowledge gaps regarding toxoplasmosis in our country. Firstly, Portuguese national health authorities considered that there was a lack of knowledge on the epidemiological situation of toxoplasmosis in Portugal, even though congenital toxoplasmosis is a mandatory notifiable disease in our country. Secondly, there was also a lack of data regarding the *T. gondii* circulating genotypes in humans and this issue gains special relevance since our country has a long history of trade and social interaction with our ex colonies, involving human and animal migrations and also import/export of food products. Finally, on behalf of the animal welfare by imposition of legislative regulations introduced in the last years in Europe, we also developed laboratory approaches aiming at replacing, or at least reducing, the laboratory animal manipulation that is a daily requirement in a toxoplasmosis reference laboratory.

In chapter II, we focused our efforts in evaluating the *T. gondii* seroprevalence in the Portuguese population by a comparison of three cross-sectional studies spanning three decades (1979 / 80, 2001 - 2002 and 2013). We assessed the seroprevalence trends in the Portuguese general population, by age group, region and gender with special focus on women of childbearing age. We analysed the presence of *T. gondii* IgG specific antibodies in 1657 sera from the 2001 - 2002 survey and in 1440 sera from the 2013 survey, by using the automated methodology Enzyme Linked Fluorescent Assay for screening purposes and the Direct Agglutination Test to clarify the equivocal samples. Concerning the 1979/80 survey we didn’t perform laboratorial work because the data was already available. The *T. gondii* overall seroprevalence decreased from 47% in 1979/1980 to 22 % in the 2013 survey, and concerning childbearing women we observed a decrease from 53 % in 1979 – 1980 to 18 % in 2013. We also observed that the prevalence of *T. gondii* IgG increased significantly with age, indicating that infection is acquired throughout life. The observation that *T. gondii* seroprevalence decreased over time, both in the general population and in the childbearing women, is in agreement with what was observed in many developed countries where *T. gondii* seroprevalence declined sharply over the past three decades (Nowakowska *et al.*, 2006c). Of special relevance is the scenario obtained for childbearing women, indicating that about 80 % of Portuguese women are not immune against the *T. gondii*

infection, and that the majority of potential pregnant women are susceptible to primary infection yielding a risk of congenital toxoplasmosis and respective sequel. Thus, the low prevalence observed among women of childbearing age should not be neglected. The incidence of infection is dependent on the general seroprevalence, determining the population susceptibility and the frequency of risk factors for toxoplasmosis acquisition. Therefore, seroprevalence should be considered a strong indicator to establish screening policies. This study reinforces that prenatal screening for toxoplasmosis is necessary due to the high rate of seronegative women exposed to infection and the consequent probability of a high number of primary infections in the childbearing period. Since there is no vaccine to prevent human toxoplasmosis, the improvement of primary prevention constitutes a major tool to avoid infection in the susceptible groups. We believe that the evaluation of seroprevalence of toxoplasmosis in Portuguese population was of great significance in public health because it allowed us to infer the risk of infection, namely of pregnant women and of women in childbearing age, allowing health authorities to perform an effective prevention for this life threat infection. Of note, this study would have been enriched if we had the opportunity to evaluate other risk factors of infection. However, this could not be performed due to the lack of more complete personal, socioeconomic and demographic information. To overcome this limitation and also in the context of epidemiological studies, we intend to give a step forward and identify the sources of *T. gondii* infection. We will discuss this issue afterwards in the future perspectives item.

We then extended the focus of the study to the parasite, thus, on behalf of national reference laboratory mission, in chapter III we intended to genetically characterize the *T. gondii* strains isolated from the several biological samples from the population with suspected toxoplasmosis that attended to the NIH to perform the laboratorial diagnosis of *T. gondii.* We also studied the demographic characteristics of the referred patients and estimated the rate of new cases in the last 10 years. The complete pre and postnatal diagnosis of congenital toxoplasmosis is composed by serological, molecular and mice inoculation approaches and this laboratory work are carried out in Portugal exclusively at NIH. Considering this, it is reasonable to assume that the existing collection of biological products and strains likely constitutes a national representation of this infection. A total of 5722 cases were analysed, being 1459 (25.5 %) cases confirmed as toxoplasmosis, 98.4 %, (1436 cases) of these were new cases of acquired toxoplasmosis and 1.6 %, (23 cases) were new cases of congenital toxoplasmosis. With the exception of France, Germany and Poland that showed the highest rates of congenital toxoplasmosis in Europe, the number of new cases per year found in this study was similar to the ones observed in the other European countries (ECDC, 2013, 2015, 2017). This is somehow in line with the prevalence estimated in the Chapter II for the general Portuguese population in 2013 (22 %), which constitutes a low value when compared with the ones obtained in France, Germany and Poland

and some other countries from the north of Europe (Pappas et al., 2009). The policies of active screening of pregnant women in these countries, especially in France, can explain why these countries report the highest rates of congenital toxoplasmosis among reporting EU/EEA countries (ECDC, 2017). Concerning *T. gondii* genetic characterization, we analyzed 48 strains isolated in a congenital and acquired toxoplasmosis context from the biological samples of the referred positive cases. Similar to other European studies (Nowakowska et al., 2006b; Ajzenberg et al., 2015) we found a majority of type II strains (73 %; 35/48) using *Sag*2 classical PCR methodology. Curiously, we found 21 % (10/48) of recombinant strains by PCR of five microsatellites, although these strains had previously been identified as type II or I by *Sag*2 classical genotyping. This suggests that multi-loci approaches for typing purposes that may be developed in a near future may soon reveal a considerably higher recombination scenario in *T. gondii* than the one that is currently assumed. Regarding the virulence phenotype, we observed a virulence rate of 15 % (7/48) in mice inoculation, which was quite low but was similar to the one observed in other countries (Ajzenberg et al., 2002b; Gebremedhin et al., 2014b). An unexpected finding was that only three type I strains demonstrated virulence in mice, and, on the contrary, one type II strain killed the mice in 120 days after the first inoculation (and continued killing mice in less time in the subsequent passages). A possible explanation for this fact is that some of the strains classified as type I and II by classical *Sag*2 and 5 microsatellites may not be "true" type I and II but instead recombinant strains. The recombinant strains may be of great relevance, as it is believed that they are associated with more severe symptoms in immunocompetent and immunocompromised hosts and could pass through the placenta more easily than the archetypal strains (Grigg et al., 2001b). Nevertheless, revealing a recombinant character by simply using the traditional typing loci is certainly a too humble approach as the probability of finding recombinant strains increases if broader genomic regions are evaluated. Therefore it is imperative to better characterize the genetic background of these unusual strains as unusual genotype-phenotype associations (e.g., implications in specific disease outcomes) may eventually be found for some recombinant profiles. In this regard, it will be important to analyze several other loci that are polymorphic and genome dispersed, in order to better understand the degree of genomic mosaicism displayed by the circulating strains. Overall, this study presents the first data in Portugal concerning *T. gondii* genotyping from human samples and it reveals genetic variations in the predominant clonal lineages and more specifically the existence of a considerable proportion of recombinant strains.

The tasks enrolled in this previous study, which are strongly associated with the role of the laboratory at the Portuguese NIH as the national reference laboratory, deeply involve the use of the animal model, not only to propagate the prototype *T. gondii* strains (e.g. RH strain) and the clinical isolates, but also to enrich the parasitic load of the biological samples to increase the success of the typing procedures. Thus, in Chapter IV we aimed to give a step forward towards

the improvement of good practices regarding the use of the animal model in our reference laboratory. To reach this goal, we evaluated the genetic and phenotypic stability of the virulent *T. gondii* RH strain throughout its parallel propagation in mice, in a cell line, and in an alternating way of these two procedures. To fulfill this objective we performed three parallel approaches during a period of 10 months, namely, a continuous passage of the RH strain in mice (Experiment A), a continuous passage of the RH strain in human foreskin fibroblasts (Experiment B) and the use of intercalate passages of the RH strain in the cell line and in mice (Experiment C). We then evaluated multiple phenotypic aspects, such as the capacity to maintain an active multiplication, the tachyzoites shape, number, motility, the capacity to maintain a virulence phenotype in mice, and also evaluated potential genotypic alterations throughout cell line *versus* mice propagation. One of the highlights of this study is our finding in Experiment C, namely, the observation of tachyzoite-rich ascites with harvesting values similar to the ones obtained in Experiment A. To reinforce the success of this experiment we also obtained tachyzoites yields in the multiples harvesting processes of the cell line about 2.5 - fold higher than the ones obtained at the end of each cycle in Experiment B. This outcome indicates that while the ability of tachyzoites to maintain active multiplication in a cell line during continuous passage decreases over time, this ability appears to be renewed when, after short periods in a cell line (eg, a week, experiment C), tachyzoites are transferred to mice. We also assessed the impact of long-term tachyzoite passage in the HFF cell line at three time points (at weeks 3, 22, and 44) after the first inoculation (Experiment B) and observed that the RH strain completely lost its virulence. We observed no tachyzoites in the ascites and no cysts in the brain after scarification of the mice inoculated with the parasite that was harvested from the referred three time points. Also, the other mouse that was inoculated with the same harvested parasite died of old age. These data strongly suggest that a long-term passaging of the parasite exclusively in a cell line is highly detrimental in terms of tachyzoites virulence. Concerning the evaluation of potential genomic changes throughout propagation, which enrolled ten samples (about one per month) from each “experiment” and targeted nine genome loci, we observed no SNP/indels both chronologically (for a given experiment) and between experiments, thus no apparent genetic alterations were observed as a consequence of *in vitro* propagation. Nevertheless, it should be emphasized that, although we surveyed loci that are polymorphic and believed to be involved in adaptation (de Melo Ferreira *et al.*, 2006; Ajzenberg *et al.*, 2010; Su *et al.*, 2010), they represent just a tiny part of the large *T. gondii* genome, hampering strong conclusions about this specific evaluation.

Since the publication of *The Principles of Humane Experimental Technique*,by Russell and Burch in 1959 (Russell and Burch, 1959), scientists and government agencies all over the world have endorsed *replacement, reduction, and refinement* as essential tools for promoting the humane treatment of research animals. In the Principles, Russell and Burch proposed a new applied

science that would improve the treatment of laboratory animals while advancing the quality of science in studies that use animal manipulation. They introduced the definitions of the 3Rs (“replacement, reduction, and refinement”), which subsequently have become known as ‘alternative methods’ for minimizing the potential for animal pain and distress in biomedical research. It should be noted that it was not the use of animals in research that these scientist found problematic, but the infliction on research animals of unnecessary or avoidable pain, fear, stress, anxiety, bodily discomfort and other significantly unpleasant feelings (Tannenbaum and Bennett, 2015) They defended the principle that, when it is scientifically appropriate to use animals in research or testing, all reasonable efforts should be made to minimize and, when possible, eliminate the stress experienced by these animals. At the NRL of the Portuguese NIH we can't abandon the animal model because it constitutes both the serological and isolation reference method for diagnosis of congenital and acquired toxoplasmosis, playing also irreplaceable roles in studies focusing natural infection dynamics and parasite dissemination. Nevertheless, our data suggests that we can significantly reduce the use of the demanding *in vivo* model and we will now adopt the Experiment C procedure for RH strain propagation in our laboratory. This would strongly impact, not only the number of sacrificed mice in our routine procedure, (about 360 animals per year that we can reduce to about 70) but would also ensure the putative maintenance of the virulence of the passaged strain.

As concluding remarks, we believe that this PhD dissertation contributes to the better knowledge of toxoplasmosis in Portugal in an epidemiological, genetic and animal experimentation manner. In fact, not only we used a human-targeted approach and determined the *T. gondii* seroprevalence throughout the last decades, but we also focused on the parasite by assessing the genetic variability of the disease-causing clones and their virulence in mice, and finally established procedures aiming at reducing the number of sacrificed animals that are routinely used for both diagnostic and research purposes.

Future perspectives:

The future perspectives are drawn according to the results of this PhD dissertation and the laboratory's ability. Thus, we anticipate three major tasks to be developed in a near future:

To overcome the lack of the identification of the risk factors of infection in the study described in Chapter II we intend to go a step forward and identify the sources of *T. gondii* infection. Eating undercooked meat is currently considered a major source of *T. gondii* infections in Europe, moreover, and as we have already observed, there are major geographical differences in the epidemiology of the parasite as well as in consumption habits, which affects the importance of different transmission routes and specific product types. In 2011 the identification of a sporozoite specific antigen (TgERP) enabled researchers to detect infections caused through oocysts by a serological method (Dolores Hill, Cathleen Coss, 2011) but identification through cysts is still missing. To accomplish this propose we aim to develop a novel NGS-MLST typing method that can detect within-genotype patterns that are important for understanding transmission routes and source tracing, and may improve preparedness to identify outbreaks and imported emerging atypical strains. The lack of information on the attribution to specific infection sources has hampered the development of effective intervention strategies and science-to-policy translation.

On behalf of the data presented in chapter III, we will proceed with studies that are already ongoing in our laboratory towards the implementation of a multi loci NGS-based approach to perform a more robust genetic characterization of the *T. gondii* isolates. This will also allow us to better identify recombinant strains, which are believed to have specific phenotypic properties and likely geographical distribution. Ultimately, this may also be useful for future genotype-phenotype association studies, which may reveal genomic structures potentially associated with an increased virulence phenotype and specific disease outcomes, launching basis for the development of proper prophylactic or therapeutic measures.

Regarding the outputs of chapter IV, we aim to understand if the approach of Experiment C is also successfully adaptable to the collection of clinical strains in the Reference laboratory, and not only to the highly virulent RH strain that was enrolled in the pilot study. This would tremendously impact the routine laboratory procedures associated with the maintenance of the viable *T. gondii* strains, namely with the significative reduction of the sacrificed mice.

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Supplementary Material

Supplementary Table S 4.1 BioProject PRJEB34235 European Nucleotide Archive (ENA) accession numbers.

SAMPLE ID ENA	BioProject	READS ENA ACCESSION
Tg_RH_1_A	PRJEB34235	ERR3505173
Tg_RH_2_A	PRJEB34235	ERR3505211
Tg_RH_3_A	PRJEB34235	ERR3505219
Tg_RH_4_A	PRJEB34235	ERR3505218
Tg_RH_5_A	PRJEB34235	ERR3505214
Tg_RH_6_A	PRJEB34235	ERR3505175
Tg_RH_7_A	PRJEB34235	ERR3505196
Tg_RH_8_A	PRJEB34235	ERR3505188
Tg_RH_9_A	PRJEB34235	ERR3505191
Tg_RH_10_A	PRJEB34235	ERR3505210
Tg_RH_11_A	PRJEB34235	ERR3505195
Tg_RH_12_A	PRJEB34235	ERR3505202
Tg_RH_13_A	PRJEB34235	ERR3505183
Tg_RH_14_A	PRJEB34235	ERR3505208
Tg_RH_15_A	PRJEB34235	ERR3505201
Tg_RH_16_A	PRJEB34235	ERR3505162
Tg_RH_17_A	PRJEB34235	ERR3505161
Tg_RH_18_A	PRJEB34235	ERR3505169
Tg_RH_19_A	PRJEB34235	ERR3505213
Tg_RH_20_A	PRJEB34235	ERR3505174
Tg_RH_21_A	PRJEB34235	ERR3505198
Tg_RH_22_A	PRJEB34235	ERR3505160
Tg_RH_23_A	PRJEB34235	ERR3505178
Tg_RH_24_A	PRJEB34235	ERR3505205
Tg_RH_25_A	PRJEB34235	ERR3505204
Tg_RH_27_A	PRJEB34235	ERR3505171
Tg_RH_28_A	PRJEB34235	ERR3505212
Tg_RH_30_A	PRJEB34235	ERR3505203
Tg_RH_31_A	PRJEB34235	ERR3505159
Tg_RH_32_A	PRJEB34235	ERR3505166

Tg_RH_1_NA	PRJEB34235	ERR3505163
Tg_RH_2_NA	PRJEB34235	ERR3505189
Tg_RH_3_NA	PRJEB34235	ERR3505179
Tg_RH_4_NA	PRJEB34235	ERR3505206
Tg_RH_5_NA	PRJEB34235	ERR3505168
Tg_RH_6_NA	PRJEB34235	ERR3505190
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Tg_RH_11_NA	PRJEB34235	ERR3505182
Tg_RH_12_NA	PRJEB34235	ERR3505197
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Tg_RH_30_NA	PRJEB34235	ERR3505217
Tg_RH_31_NA*	PRJEB34235	ERR3505200
Tg_RH_32_NA*	PRJEB34235	ERR3505207

* RH positive controls