Pork as a source of *Toxoplasma gondii* infection in humans: a molecular approach.

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List of abbreviations

Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
AMA	Apical Membrane Antigen
CI	Confidence Interval
C_p	Crossing Point
CT	Congenital Toxoplasmosis
DALY	Disability Adjusted Life Years
Dpi	Days post infection
EFSA	European Food Safety Agency
ELISA	Enzyme-Linked ImmunoSorbent Assay
GRA	Dense Granule Protein
IFA	Immunofluorescence Assay
IMS	Immuno Magnetic Separation
IP	Intraperitoneal Injection
IPB-G	Gangji strain
IPB-LR	LR strain
LD	Lethal Dose
LAMP	Loop-Mediated Isothermal Amplification
MAT	Modified Agglutination Test
MbP	Mega base pair
MC-qPCR	Magnetic Capture Real Time PCR
Min	Minute
MLE	Multilocus enzyme electrophoresis
MS	Microsatellite
NRC	National Reference Center
NT	NeuroToxoplasmosis
ON	Overnight
OT	Ocular Toxoplasmosis
P.i.	Post infection
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	Real Time PCR/Quantitative PCR

PTM	Post Translational Modifications
PV	Parasitophorous Vacuole
QALYs	Quality-Adjusted Life Years
ROP	Rhoptry neck protein
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
RFLP	Restriction fragment length polymorphism
RT	Room Temperature
SAG	Surface Antigen
SD	Standard Deviation
TAT	Turnaround Time
TgERP	*T. gondii* embryogenesis-related protein
USDA	United States Department of Agriculture

Part I: Literature study

Taxonomic Classification

Toxoplasma gondii is a worldwide prevalent, zoonotic non-flagellated apicomplexan parasite. As a member of the Phylum Apicomplexa, *T. gondii* has an apicoplast, a non-photosynthetic organelle, which is homologous to the chloroplast. Moreover, as a member of the Coccidia class, *T. gondii* is an obligate intracellular and cyst forming parasite that infects its hosts through the gastrointestinal tract, see Figure 1.1 for an overview of the taxonomic classification.

Domain: Eukaryota

Kingdom: Alveolata

Phylum: Apicomplexa

Class: Coccidia

Order: Eucoccidiorida

Family: Sarcocystidae

Genus: *Toxoplasma*

Species: *Toxoplasma gondii*

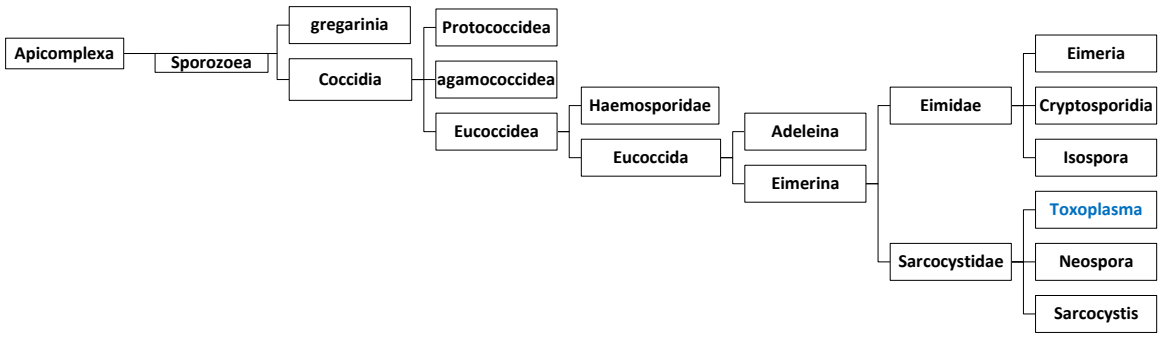

Fig 1.1: Schematic representation of the *Toxoplasma gondi's* taxonomic classification

Chapter I:

Toxoplasma gondii the ‘‘perfect’’ parasite

Toxoplasma gondii (*T. gondii*), considered as one of the most successful parasites worldwide, was concurrently discovered by Nicolle and Manceaux and Splendore in 1908 (Nicolle and Manceaux, 1908; Splendore, 1908). The parasite was isolated from two different hosts, a rodent species named gundi and a rabbit in which the organism was wrongly identified as *Leishmania* (Splendore, 1908). The isolated specimen took its name based on its morphology (toxo: arc-shaped, plasma: life) and on one of the first described hosts (gondii: gundi). However, the elucidation of the complete life cycle of *T. gondii* with felids as definitive hosts was not concluded till the late sixties and early seventies due to the different infectious stages and the wide range of intermediate hosts (Hutchison et al., 1969; Dubey et al., 1970; Sheffield and Melton, 1970). This discovery was a major breakthrough in medical and veterinary sciences, which led to the identification and the elucidation of the life cycle of different economically very important related parasites such as, *Neospora caninum, Sarcocystis* spp, etc. (Dubey, 2010).

1.1 Infectious stages

Toxoplasma gondii has three well-differentiated infectious stages: i) Tachyzoites, ii) Bradyzoites and iii) Sporozoites. The three stages are haploid and have the characteristic apicoplast, a non-photosynthetic organelle, which is homologous to the chloroplast shared by all the members of the Phylum Apicomplexa. A graphical scheme of the morphology of the three infectious stages is shown in Figure 1.5.

1.1.1 Tachyzoites:

Tachyzoites are the free fast replicating form of the parasite present during the acute phase of infection. They have the typical arched shape that gave the name to the species, with a size of 2-4 µm wide and 6-8 µm long (see Figure 1.2). Although tachyzoites lack any visible motility structures they move through undulating, gliding and rotating movements and they are able to penetrate and replicate in several types of host cells (Chiappino et al., 1984; Dubey, 1998). Active penetration of the cells is mediated by secretion of different micronemes, rhoptries and dense granule proteins, which are also responsible for the formation of the parasitophorous vacuole (PV) in which the parasite replicates by endodyogeny, protected from the host immune system (Carruthers and Sibley, 1997; McFadden, 2011) (Figure 1.5).

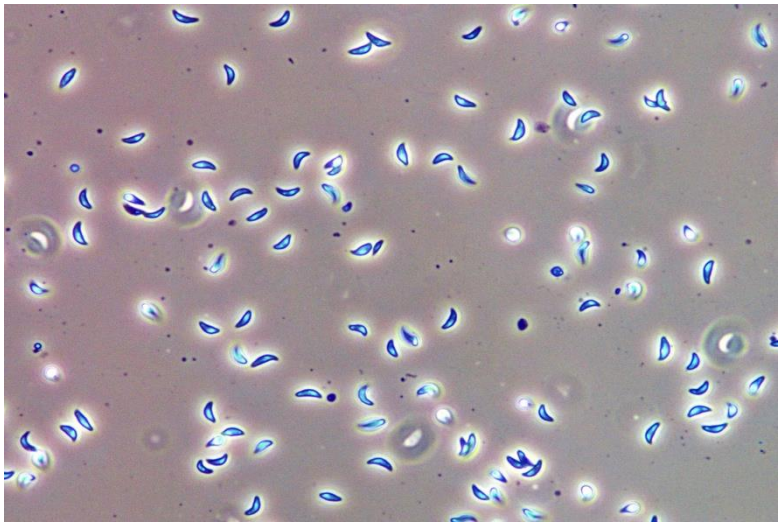

Figure 1.2: Purified tachyzoites of the RH strain. Source: National Reference Center (NRC) for Congenital infections, *Toxoplasmosis*, WIV-ISP, S. De Craeye.

1.1.2 Bradyzoites:

Bradyzoites are the slow replicating stages of the parasite encapsulated in intracellular cysts within the host tissues. They share the bowed shape with the other two infectious stages of the parasite (tachyzoites and sporozoites) and they are 5-8.5 µm long and 1-3 µm wide (Dubey, 1998). Tissue cysts can contain from a few bradyzoites to thousands of them depending on the strain and/or the time post infection (Figure 1.3). Tissue cysts are mainly present in neural, cardiac and skeletal muscular tissues although they can also be found in other visceral or muscular tissues (Dubey, 2010).

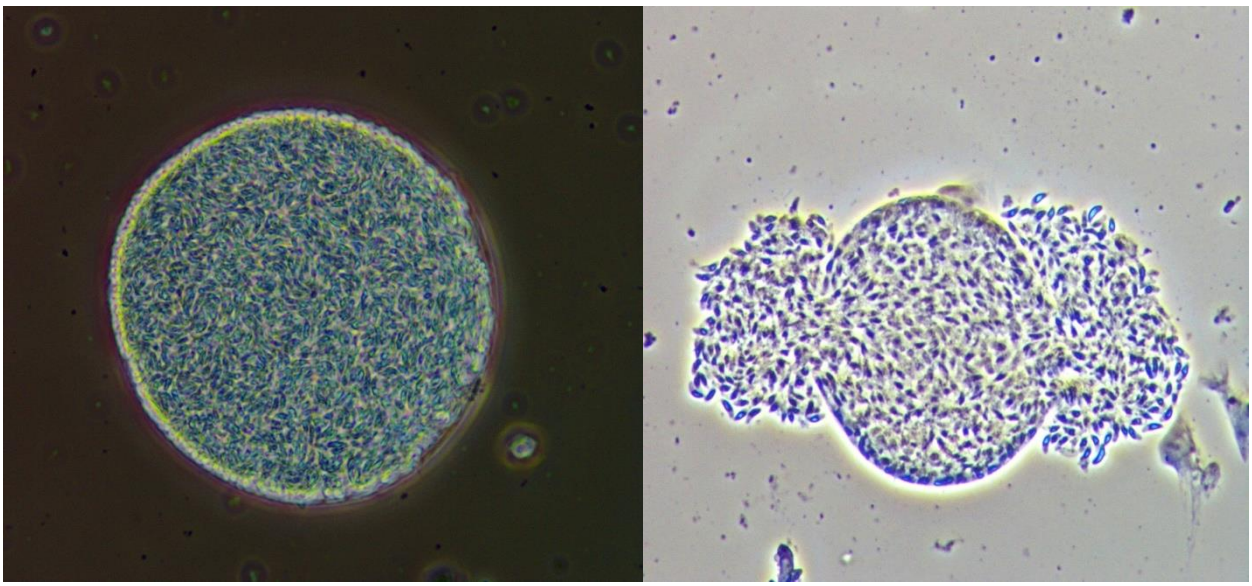

Figure 1.3: A tissue cyst and a ruptured tissue cyst with freed bradyzoites using phase contrast microscopy .Source: National Reference Center (NRC) for Congenital infections, Toxoplasmosis, WIV-ISP, S. De Craeye.

1.1.3 Sporozoites:

Sporozoites are the result of the sexual replication in the definitive host; each sporozoite measures approximately 2 µm wide and 6-8 µm long. They are contained inside the sporulated oocyst; each oocyst contains 2 differentiated sporocyst and each sporocyst contains 4 sporozoites (Figure 1.4). The sporozoites are protected by 3 different layers: an outer electron-dense layer, an electron-lucent middle layer and a moderately electron-dense inner layer (Dubey,2010). The oocysts are sphere-shaped stages with 10-12 µm of diameter (Figure 1.4). Remarkably, the oocysts are the resistant stage of the parasite and they remain viable in the environment for at least one year after excretion (Torrey and Yolken, 2013) and they can remain viable for at least 12 months in 2% sulphuric acid (Frenkel et al., 1975). It is generally accepted that sporulated oocysts survive well in a moist environment while they are quickly destroyed in dry conditions. There is not a maximum survival time known. Sporulated oocysts were still viable after 18 months in faeces buried in soil (Frenkel et al., 1975).

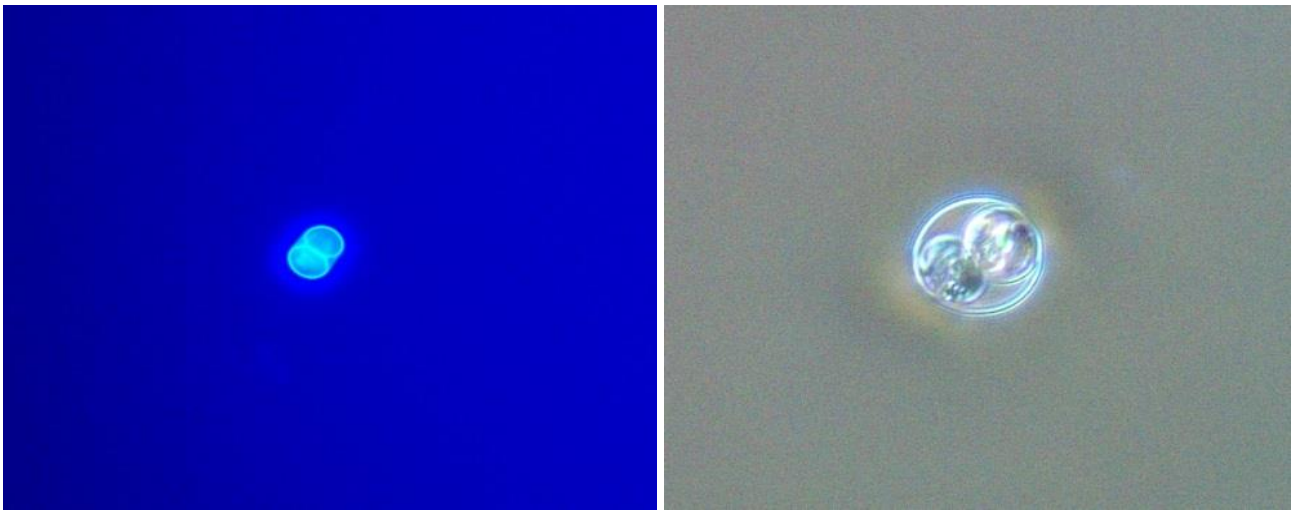

Figure 1.4: Pictures of a sporulated oocyst Tg-SweF2 using fluorescent microscopy (left) and phase contrast microscopy (right) Source: NCR for Toxoplasmosis, WIV-ISP, S. De Craeye.

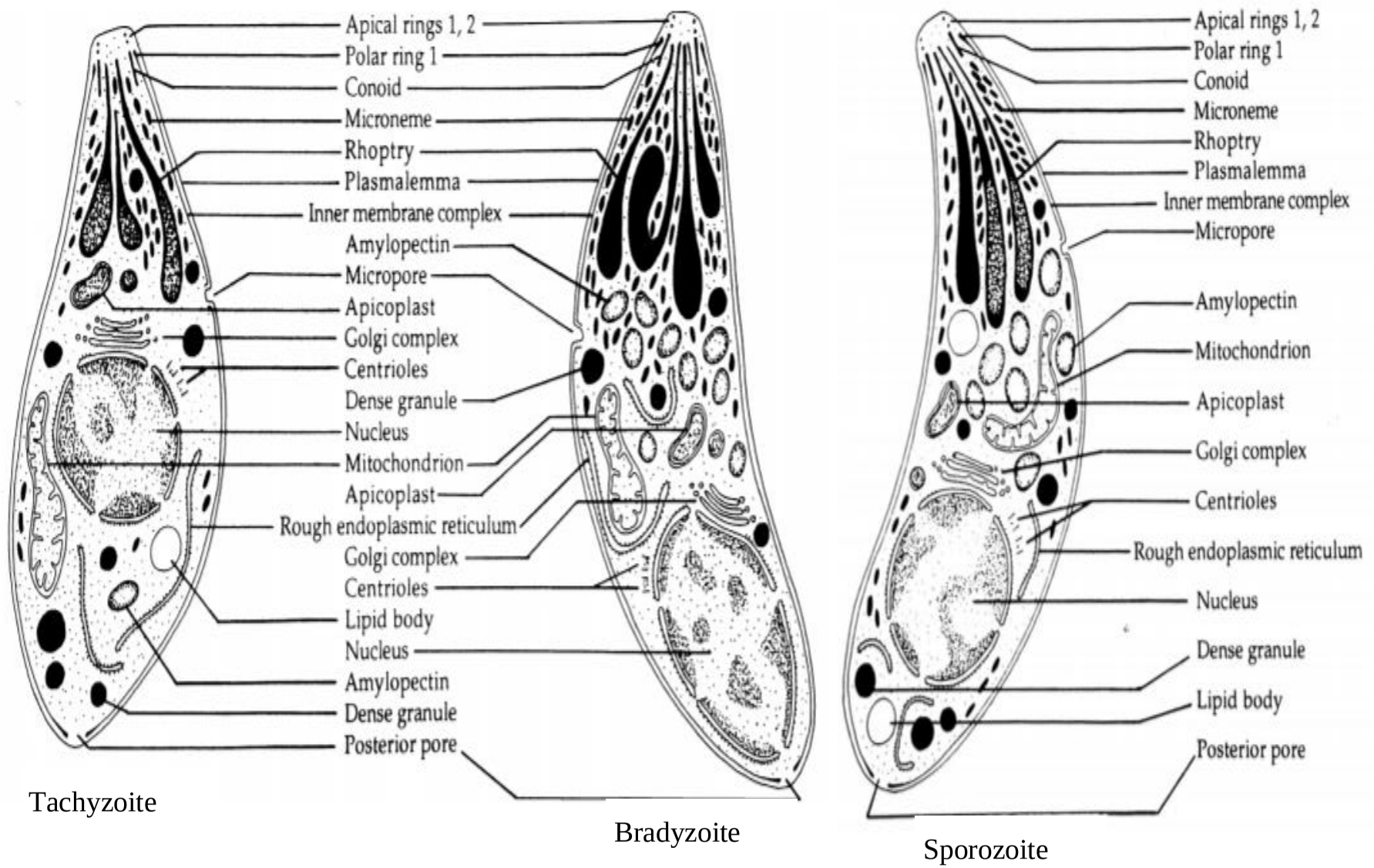

Figure 1.5: General overview of the morphology of the different infectious stages adapted by De Craeye et al., 2012

1.2 Life cycle

T. gondii has a facultative heteroxenous life cycle with members of the Felidae family as the definitive hosts and practically all warm-blooded animals as the intermediate hosts. An overview of the life cycle is shown in Figure 1.6. Two clearly differentiated phases are found in the definitive host, the intra-intestinal and the extra-intestinal phases, while in intermediate hosts only the extra-intestinal phase occurs.

The life cycle starts with the ingestion by the definitive host, mostly cats, of a chronically infected prey. Once in the stomach, the proteolytic enzymes will initiate the digestion of the cyst wall resulting in the release of the bradyzoites in the cranial part of the small intestine and the later invasion of the enterocytes from the intestinal epithelium by the freed bradyzoites. After numerous rounds of asexual replication, five different morphological stages designated from A-E will develop exclusively in the enterocytes of the final host. The described stages (schizonts type A-E) will multiply for generations before gametogony follows. The gamete formation will start between 3-15 days after the ingestion of tissue cysts, probably when merozoites released from schizonts type D and E will initiate gametogony in the small intestine, commonly in the ileum. Macrogametes (female) will then be fertilized by bi-flagellated microgametes (male) resulting in the formation of a zygote, discharged into the lumen after the rupture of the infected cells. The zygote is protected by 5 layers of oocyst wall and shed into the environment by the definitive host (Hutchison et al., 1969).

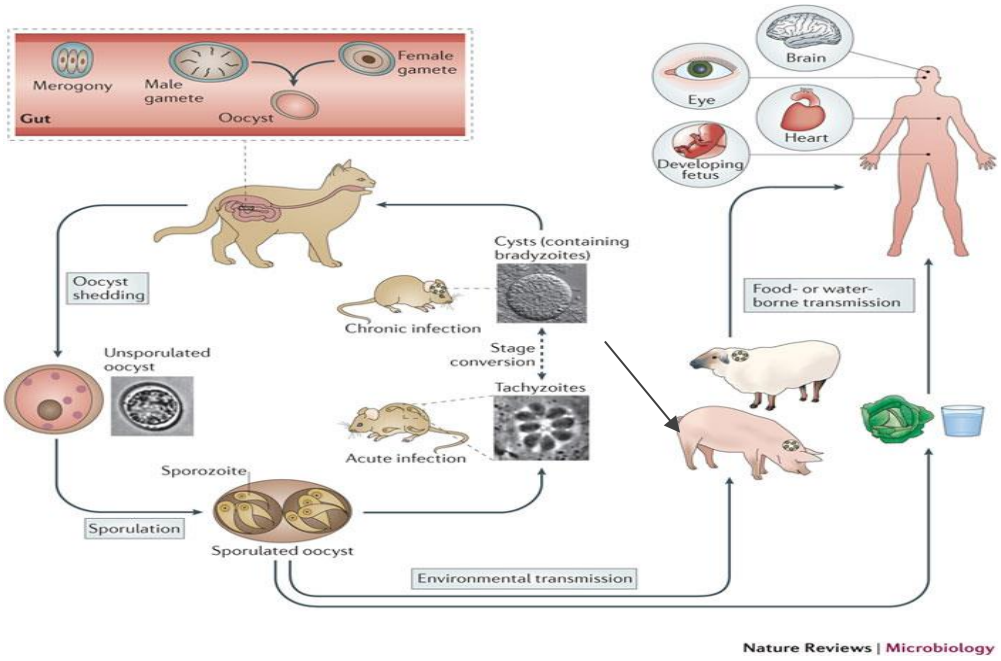

Figure 1.6: Schematic representation of the *T. gondii*'s life cycle adapted from Hunter and Sibley, 2012

In the environment, the oocysts will sporulate forming two sporocysts that will further develop into a total of 8 sporozoites per oocyst within 3-5 days. The sporulated oocysts can then be "picked up" by a "new" definitive host re-initiating the life cycle with less efficiency or by an intermediate host continuing the cycle.

When ingested by an intermediate host, sporozoites will penetrate the enterocytes and goblet cells and will be carried to the lamina propria where they will transform into tachyzoites, "jump" to the blood stream and disseminate through the neural, visceral, and muscular tissues of the host (acute phase). Once in the tissues, some tachyzoites will transform into bradyzoites and encapsulate inside of a tissue cyst that can remain present life-long (chronic phase). Interferon gamma (IFN-ɣ) is thought to be responsible for the transformation of the tachyzoites into bradyzoites and the formation and maintenance of the tissue cyst; however, all the factors regulating the formation of tissue cysts *in vivo* are not fully understood yet (Dubey, 2010; Hunter and Sibley, 2012; Jennes, 2017). This dissemination and encapsulation process (the extra-intestinal phase) occurs also in the definitive host in parallel to the intra-intestinal phase described previously. (Dubey, 2010).

1.3 Molecular epidemiology

Toxoplasma gondii is a haploid eukaryotic organism, which possesses only one set of chromosomes in all its different stages except for the unsporulated oocysts. These oocysts are the result of sexual replication, which occurred in the gastrointestinal tract of the definitive host and they are present for a short period of time before the sporulation starts. With a total of 14 chromosomes, 7793 genes and an approximate size of 63.5 mega base pairs (Mbp), the *T. gondii* genome is longer than these of closely related parasite species such as *Plasmodium falciparum* or *Cryptosporidium parvum* (Khan et al., 2007).

Initially, *T. gondii* was thought to be a highly clonal organism, showing almost no genetic diversity. However, in the early 90's molecular epidemiology started to be taken into consideration and for the first time, different strains were grouped into three main lineages using the restriction fragment length polymorphism (RFLP) technique (Figure 1.7) (Howe and Sibley, 1995). Although this manuscript was first refused for publication due to a lack of editor interest and the simplicity of the classification, the manuscript was finally published in a different journal and since then has been cited many times settling the bases for the molecular epidemiology of *T. gondii* (Howe and Sibley, 1995) .

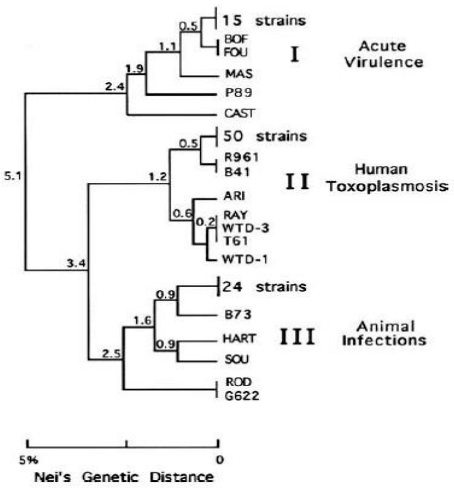

Figure 1.7: Representation of the first genotyping classification of *T. gondii* by Howe and Sibley, 1995

In parallel to Sibley, Dardé et al. (Dardé et al., 1992) conducted comparable results in Europe using multilocus enzyme electrophoresis (MLE) , confirming the presence of the same structured population in North America and Europe. Even though these three main lineages differ in less than 2% at a DNA sequence level (Grigg, 2001), strikingly, a difference in virulence of *T. gondii* strains depending on their genetic profile was established (Sibley, 2009; Su et al., 2002).

Since then, several molecular epidemiological studies using MLE, PCR-RFLP and microsatellite (MS) polymorphism-based techniques have shown the same highly structured population (clonal population) with three main clonal lineages (Type I, II and III) in Europe and North America (Howe and Sibley, 1995, Dardé et al., 1996, Ajzenberg et al., 2002; De Craeye, 2012). The existence of this structured population can easily be explained by different factors: i) *T. gondii* can be transmitted between intermediate hosts bypassing the sexual replication and hence the sexual recombination in the definitive host (Howe and Sibley, 1995; Ajzenberg et al., 2004); ii) During the sexual replication, many macrogametes are not fertilized but they still develop into oocysts by parthenogenesis (Ferguson, 2002); iii) Definitive hosts infected concurrently with more than one *T. gondii* strain are extremely rare, hence recombination between different strains is handicapped (Ajzenberg et al., 2004).

Nowadays, although highly clonal, the *T. gondii* population has also been revealed to show genetic diversity (Table 11. and Figure 1.8). Some strains do not fit in any of the three main lineages and are therefore considered as atypical since their alleles could not be found in any of the main lineages (Ajzenberg et al., 2004). These atypical strains are endemic in tropical areas of South America such as, French Guiana and Brazil, where the life cycle passes more often through wild felids. These strains can also be found in Africa and China and rarely in North America and Europe (Ajzenberg et al., 2004).

Table 1.1: Overview of the main *T. gondii* circulating strains in the world and their nomenclature by Shwab et al., 2014

Conventional genotype designations	ToxoDB PCR-RFLP Genotypes	Representative isolates	References
Type I, type 1	#10	GT1	Su *et al*. (2012)
Type II, type 2 (type 2 clonal)	#1	PTG	Su *et al*. (2012)
Type II, type 2 (type 2 variant)	#3	PRU	Su *et al*. (2012)
Type III, type 3	#2	VEG	Su *et al*. (2012)
Type 12, atypical, exotic	#4	B41	Khan *et al*. (2011); Su *et al*. (2012)
Type 12, atypical, exotic, includes Type X and Type A	#5	ARI	Khan *et al*. (2011); Su *et al*. (2012)
Type BrI, atypical, exotic, Africa 1	#6	FOU, TgCatBr2	Pena *et al*. (2008); Mercier *et al*. (2010); Su *et al*. (2012)
Type BrII, atypical, exotic	#11	TgCatBr1	Pena *et al*. (2008); Su *et al*. (2012)
Type BrIII, atypical, exotic	#8	P89 (TgPgUs15), TgCatBr3	Pena *et al*. (2008); Su *et al*. (2012)
Type BrIV, atypical, exotic	#17	MAS, TgCkBr147	Pena *et al*. (2008); Su *et al*. (2012)
Chinese 1, atypical, exotic	#9	TgCtPRC4	Dubey *et al*. (2007*b*); Chen *et al*. (2011); Su *et al*. (2012)

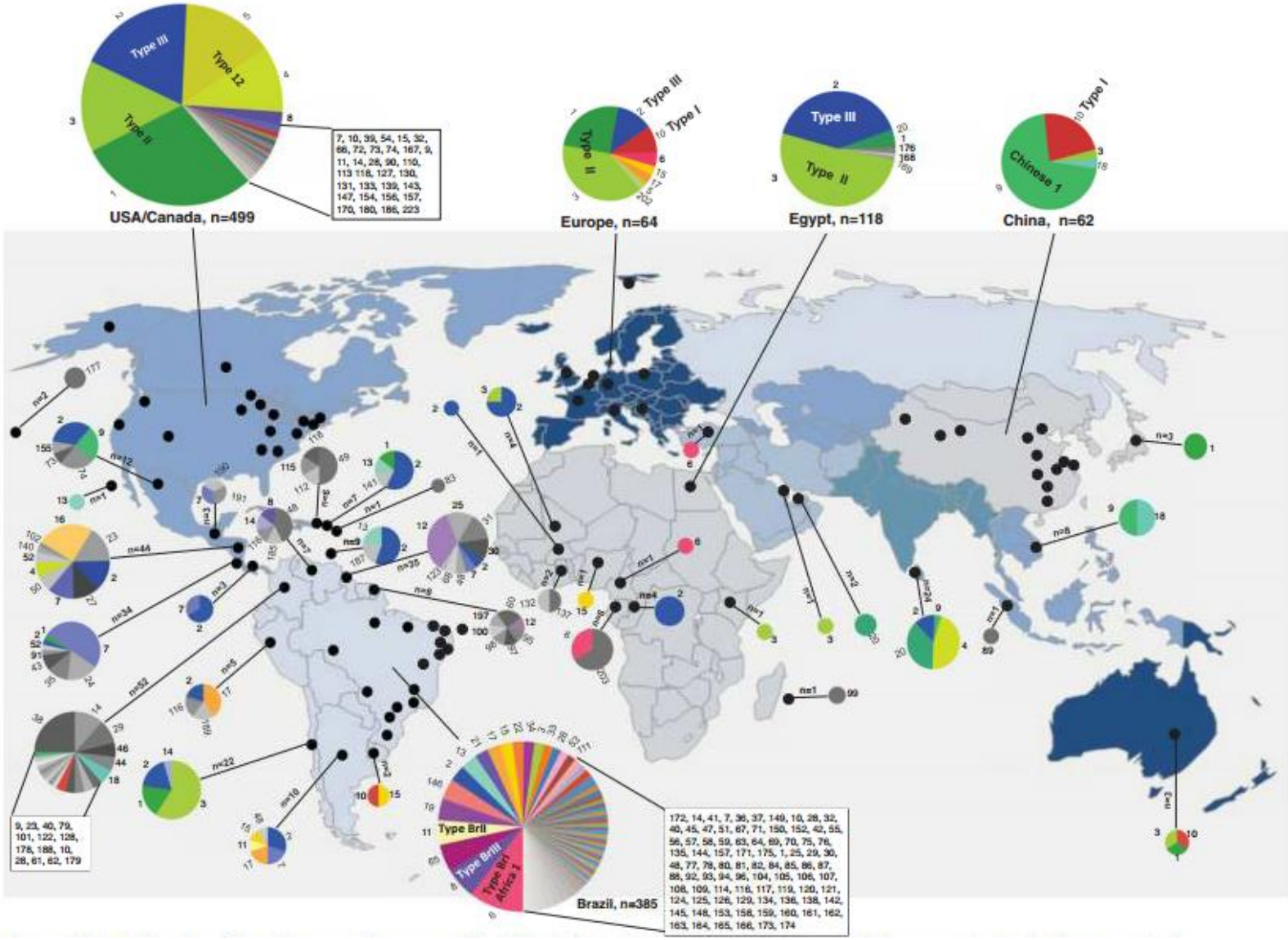

Figure 1.8: Geographical distribution of the *T. gondii* circulating strains by Shwab et al., 2014

Recent studies comparing the circulating strains worldwide and reconstructing the *T. gondii* phylogeography have provided a broader view of the genetic diversity of *T. gondii* in the world, with more than 13 different types (haplogroups) and have suggested a potential common origin of all the modern *T. gondii* strains located somewhere in South America (Shwab et al., 2014; Lorenzi et al., 2016; Bertranpetit et al., 2017). As shown in Figure 1.8, the higher diversity of strains is found in South America while in Europe the vast majority of the strains are type II and mainly archetypal strains can be found (Type I, II and III) (Shwab et al., 2014).

The differences in virulence and pathogenicity between the main lineages have been widely studied in the mouse model:

- Type I: strains from this lineage are highly virulent in mice. Only one tachyzoite injected intraperitoneally (ip) is able to kill mice within 10 days (Lethal Dose (LD) 100%= 1 tachyzoite) (De Craeye, 2012).
- Types II and III: these strains are not considered virulent in mice, at least 1000 tachyzoites are needed to cause death when injected ip (LD >1000 tachyzoites) (De Craeye, 2012).

Remarkably, most of the atypical strains are considered virulent in both mice and humans causing death in mice and severe ocular problems even in immunocompetent humans. However, these strains have not yet been widely studied.

Chapter II:

Public Health, Human and Animal Toxoplasmosis

Although *T. gondii* infection is normally associated with cats and their shedding of oocysts (Torrey and Yolken, 2013), carnivorism is also an important route of transmission for *T. gondii* within intermediate hosts, including humans. Three main routes of transmission are considered to be responsible for almost all the human and animal infections.

2.1 Main routes of transmission

2.1.1 Horizontal transmission, via oocysts

Unsporulated oocysts are the result of the sexual replication in the intestinal tract of the definitive host (Felidae) and are shed in the environment where they become infectious after sporulation. Virtually all warm-blooded animals (intermediate hosts) can get infected when consuming oocyst-contaminated drinking water, vegetables, soil or animal feed (Hide, 2016). Particularly, humans can also get infected while cleaning contaminated cat litter or while gardening (Hide, 2016), see Figure 2.1 for an overview of this transmission route.

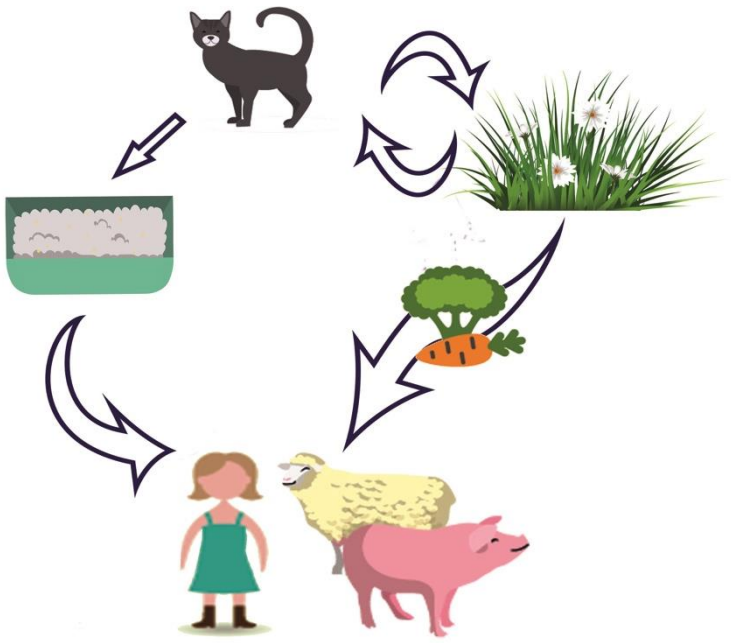

Figure 2.1: Graphical representation of the horizontal transmission via infectious oocysts

2.1.2 Horizontal transmission, via tissue cysts

Once infected, intermediate hosts become carriers of intracellular tissue cysts life-long, actively collaborating to the successfully spread of the disease (Dubey, 2010). Carnivores and omnivores can therefore get infected by ingesting contaminated tissues from a chronically infected animal. The consumption of raw or undercooked meat has been widely proven to be highly effective to initiate infection in intermediate and definitive host (Aspinall et al., 2002; Su et al., 2003; Hide, 2016). The effectivity of this route is actually that high that still today the infection of animals is considered as the reference method for the direct detection of the parasite in tissues (Hide, 2016). Transmission by meat consumption was first described in humans in 1965 by Desmonts (Desmonts et al., 1965) and since then has been considered of pivotal importance (Figure 2.2) in humans. Meat borne transmission of *T. gondii* is estimated to be responsible for at least 30 % of the human *T. gondii* infections (Cook et al., 2000).

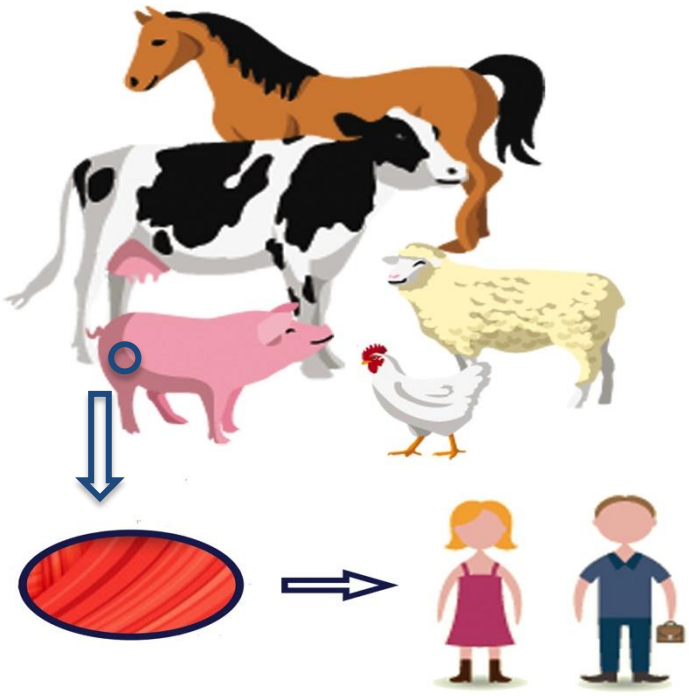

Figure 2.2: Graphical representation of the horizontal transmission via infectious tissue cysts

2.1.3 Vertical transmission

Congenital transmission of *T. gondii* has been extensively documented in several species including, humans, sheep and mice (Hide, 2016). However, not all species are equally susceptible to this route of infection. For example, although different experimental attempts to reproduce congenital transmission were performed in pigs, these were not consistently successful (Basso et al., 2015). In humans, the first report of congenital transmission was described in 1939 (Wolf, 1939). When it occurs, it may be associated with severe clinical signs for the child or even cause abortion (Maldonado and Read, 2017). An overview of the sources of infection for humans that will lead to a vertical transmission are shown in Figure 2.3

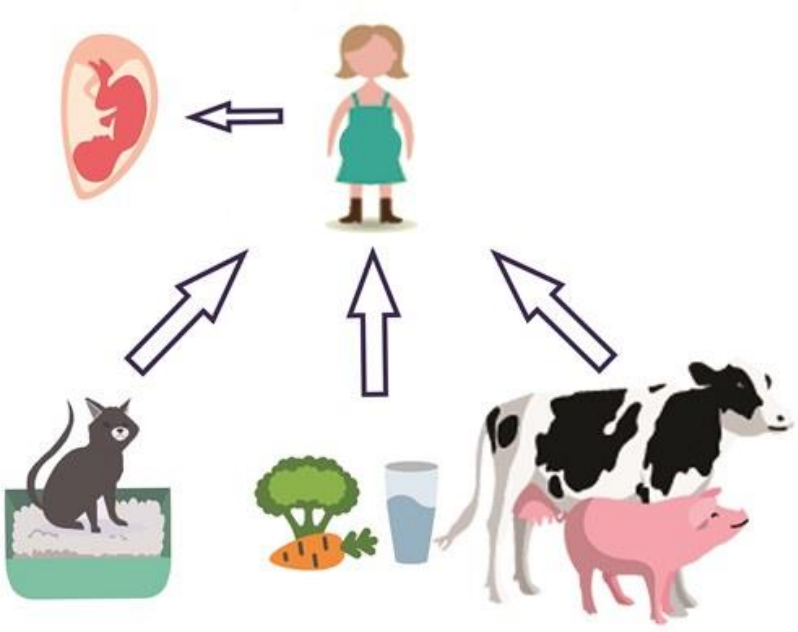

Figure 2.3: Graphical representation of the vertical transmission from the mother to the unborn baby

Despite a high seroprevalence in humans (30-80%) (Opsteegh et al., 2016), relatively few cases of congenital toxoplasmosis are being reported, between 1/1000 and 1/10000 pregnancies according to the country (Dubey, 2010). The low rate of congenital transmission in humans can be explained by the fact that congenital transmission occurs in most cases only when mothers seroconvert during pregnancy (Frenkel, 1974; Maldonado and Read, 2017). Reactivation of chronic infections during pregnancy leading to congenital transmission was demonstrated in only a few individual cases (Ladas et al., 1999; Kodjikian et al., 2004; Valdès et al., 2011).

2.2 Toxoplasmosis, public health and economic impact

Toxoplasma gondii is a zoonotic parasite of global interest. There is an increasing awareness worldwide of its public health and economic importance. Human toxoplasmosis costs in the US are estimated to be more than 3 billion US dollars a year considering only the medical costs (Hoffman et al., 2013). *Toxoplasma gondii* was classified as second of the 15 most important foodborne pathogens in function of economic impact by the United States Department of Agriculture (USDA) (Hoffman et al., 2013), see Figure 2.4. The Center for Disease Control and Prevention (CDC) estimates that foodborne toxoplasmosis is only surpassed by *Salmonella* on the annual numbers of human deaths caused by foodborne hazards. In Belgium, *Toxoplasma* is considered in the top 10 of the most important foodborne pathogens (higher public health impact) (Cardoen et al., 2009).

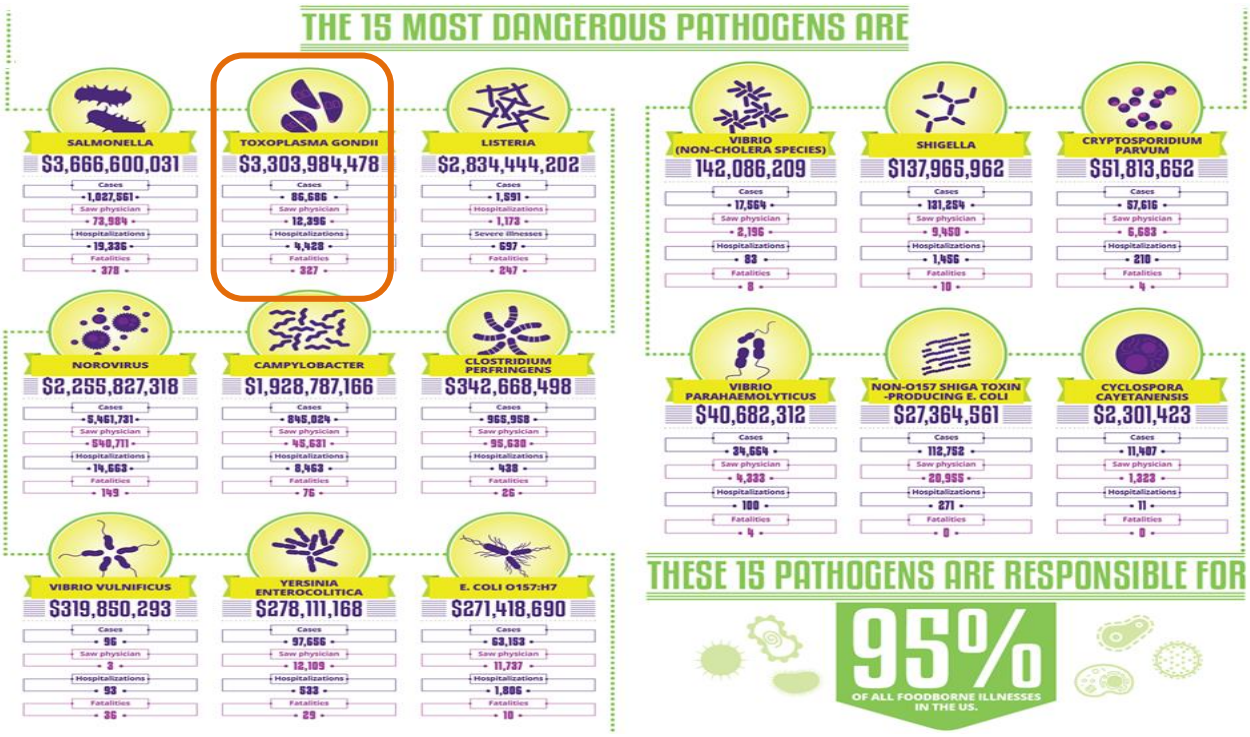

Figure 2.4: Overview of the 15 pathogens responsible for 95% of the foodborne illnesses in US, adapted from USDA, 2013

In addition, *T. gondii* infection in livestock is often associated with reproductive disorders, reduced daily weight gain and weak progeny, with consequently, important economic losses for producers (Buxton et al., 2007; Dubey and Jones, 2008; Dubey, 2010). For example, in Italy, the economic losses associated with lamb mortality and missed lactation in sheep are estimated to be more than 10 million Euro per year (Masala et al., 2003).

Nowadays, the concept of animal health has been strengthened not only to the absence of disease, but also to the important relationship between health and welfare of the animals increasing the number of farms concerned by the wellbeing of their animals and providing them access outdoors. However, the increasingly more ethical and popular animal-friendly production systems (organic farms), where the animals are more exposed to environmental contamination and wildlife, may account for a re-emergence of toxoplasmosis in livestock (Kijlstra et al., 2004; Hill et al., 2010; Dubey et al., 2012). A higher prevalence of *T. gondii* infections in livestock could also lead to an increase of human *T t*oxoplasmosis in the near future.

2.3 Human toxoplasmosis

Human toxoplasmosis has been widely studied since the first decade of the 20th century till today, describing several non-pathognomonic signs and symptoms, differing in severity (Weiss and Dubey, 2009). These range from mild flu like symptoms often wrongly attributed to other causes, to severe encephalitis and even death. Human infections are predominantly asymptomatic in around 80 % of the cases (Robert-Gangneux and Dardé, 2012). However, as explained above, some specific population groups are more susceptible to develop severe toxoplasmosis and a correlation between *T. gondii* genotype and virulence has been described in the literature. Type I strains are considered to be very virulent in human hosts while type II and III strains are considered to have a lower virulence. Additionally, "atypical strains" have been found to be very virulent in humans, especially in people originating from areas where these genotypes are very rare (Europe, North America).

Currently, the estimated seroprevalence in humans ranges between 20 and 80 % of the global population. The important inter-study variations can be explained by the use of different sampling strategies and detection methods (see Table 2.2), but mainly to geographical differences and effects of culinary traditions. An estimated 50-80% of the total European population has been considered to be seropositive for *T. gondii* and has thus been in contact with the parasite (Opsteegh et al., 2016). However, this prevalence does not reflect the current situation in Europe since old studies were included overestimating the actual seroprevalence in Europe. The prevalence is higher in Southern and Central European countries than in countries in the North, mainly reflecting differences in meat consuming habits. For example, Norway and Sweden have a lower estimated prevalence (<15%) than Belgium , where the estimated prevalence is higher than 50 % (Breugelmans et al., 2005).

Nowadays, different strategies developed to better estimate the burden of disease in the field of public health are becoming more popular and have revealed the high importance of long time neglected diseases such as toxoplasmosis, which ranks among the most important. The key feature of these studies is the combination of mortality and morbidity into a single and easy to use metric (Devleesschauwer et al., 2014). For example, a pathogen-food relationship ranking in function of Quality-Adjusted Life Years (QALYs), which is a generic measure of disease burden used to economically evaluate the impact of a disease considering the medical interventions, ranks the combination *T. gondii*/pork in the top 2 above other more known pathogen-food combinations, such as *Salmonella enterica*/poultry and norovirus/complex foods (Batz et al., 2012). Another study in Europe, using the Disability-Adjusted Life Years (DALYs), which is a measure of the overall disease burden, estimated a total of 829,071 DALY's due to foodborne toxoplasmosis (Havelaar et al., 2015). In the Netherlands, *T. gondii* congenital and acquired infections, accounted for 3,570 DALY's in 2011 (Mangen et al., 2014). In Belgium, the estimated disease burden of congenital toxoplasmosis in 2013 was 188 years lost due to disease, poor health or early death (Smit et al., 2017).

Table 2.2: Overview of different *T. gondii* seroprevalence studies in humans

Continent	Country	Estimated seroprevalence	Reference
Europe	Belgium	57 %	Breugelmans et al., 2005
	France	37 %	*Nogareda et al., 2013*
	Netherlands	25 %	*Hof huis et al., 2011*
	Germany	55 %	*Wilkings et al., 2016*
	Spain	30 %	Jaqueti et al., 1991
	Italy	22 %	De Paschale et al., 2008
	Sweden	14 %	Evengård et al., 2001
	Norway	11 %	Jenum et al., 1998
North America	Canada	60 %	Messier et al., 2009
	US	13 %	Jones et al., 2014
South America	Argentina	59 %	Fuente, 1997
	Brazil	92%	Figueiró-Filho et al., 2005
	Colombia	46 %	Rosso et al., 2008
Asia	China	11 %	Liu et al., 2009
	India	24 %	Dhumne et al., 2007
	Indonesia	70 %	Terazawa et al., 2003
	Korea	4 %	Ryu et al., 1996
Oceania	Australia	35 %	Walpole et al., 1991
	New Zealand	33 %	Morris and Croxson, 2004
Africa	Congo	60%	Makuwa et al., 1992
	Egypt	27 %	Azab et al., 1993
	Ethiopia	80 %	Woldemichael et al., 1998

2.3.1 Congenital toxoplasmosis

Toxoplasma gondii vertical transmission from the mother to the foetus is the most known and notorious consequence associated with a *T. gondii* infection. In Europe, approximately 2-8 women on 1000 seroconvert during pregnancy, with an estimated mean of transmission to the foetus of 25 % (EFSA report 2012; Opsteegh et al., 2016; Smit et al., 2017). During 2013, only in Belgium, it was estimated that 69 infants were born with congenital toxoplasmosis (CT) due to a primary infection of

the mother during pregnancy (Smit et al., 2017).

Transmission to the foetus has been widely studied and importantly, a positive correlation was found between gestational age and transmission rate to the unborn, from less than 10 % during the first trimester to higher than 60% during the last months of gestation (Figure 2.5) (Dubey, 2010).

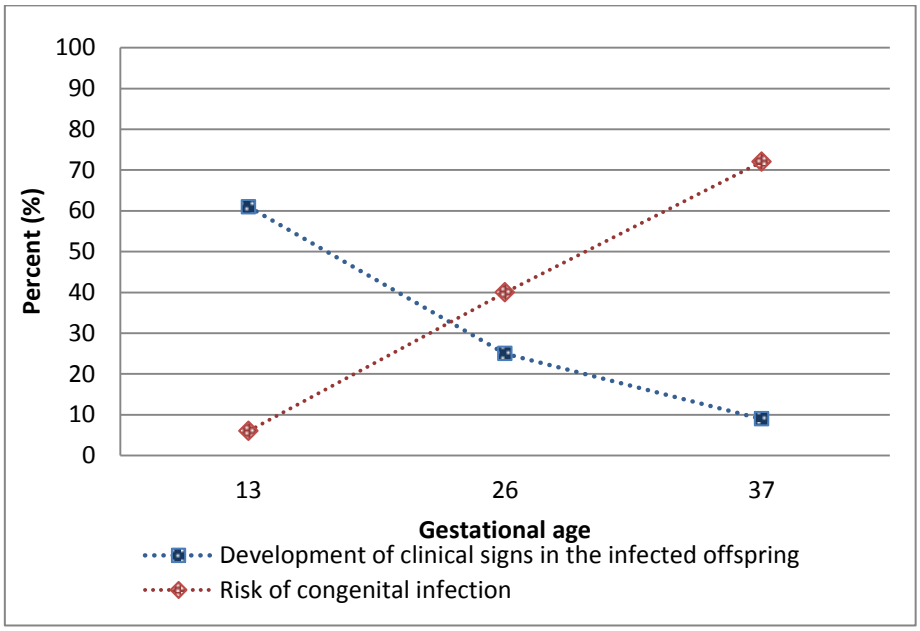

Figure 2.5: Representation of the percentage of congenital transmission and percentage of severity in function of the gestational age, Adapted from Goldstein et al., 2008.

On the contrary, the earlier the transmission occurs during pregnancy the higher the severity and/or malformations on the foetus are, leading to abortion, perinatal death or reduced quality of life in surviving children (Frenkel, 1974; Tenter et al., 2000; Pfaff et al., 2007).

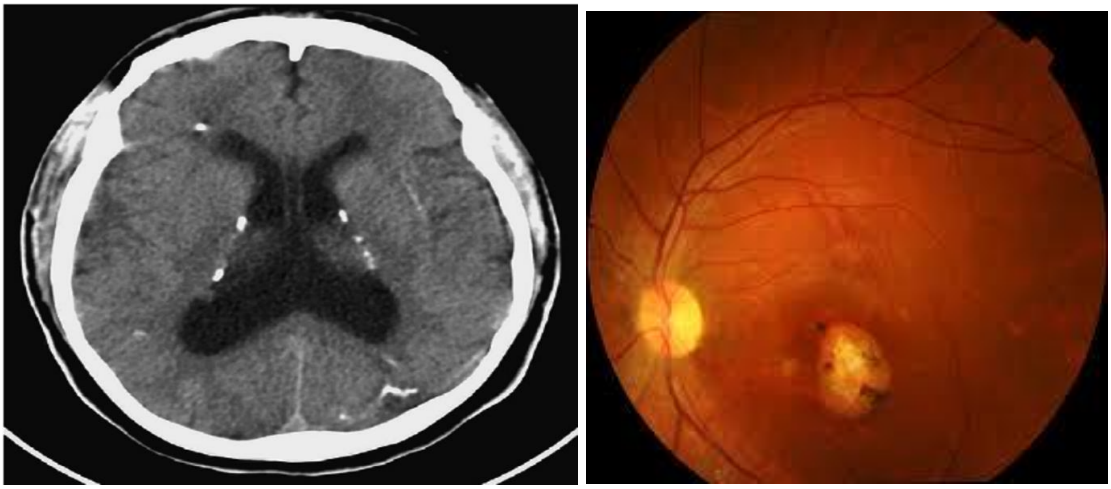

Figure 2.6: Brain calcifications visualised by a brain CT scan (left) and chorioretinitis (right) caused by congenital toxoplasmosis; adapted from Dubey, 2010.

Congenital toxoplasmosis is typically associated with three main clinical signs or symptoms: i) chorioretinitis; ii) intracranial calcifications; iii) hydrocephalus (Figure 2.6) (Dubey, 2010; Maldonado and Read, 2017). In addition to the above mentioned signs, other health problems can also be observed less frequently such as, mental retardation, epilepsy, deafness, blindness, cataract, etc. (Yamamoto et al., 2000; Dubey, 2010). Noteworthy, in some cases the ocular pathologies can only appear several years after birth (Butler et al., 2013).

2.3.2 Immuno-compromised individuals

Toxoplasmosis in immune-compromised people is one of the most severe aspects of this zoonotic disease. Individuals whose immune system is depressed by the acquired immune deficiency syndrome (AIDS) and cancer or transplanted patients undergoing immunosuppressive treatment are susceptible to suffer from neurotoxoplasmosis (NT) due to a deficient T-cell immunoresponse (Mariuz and Luft, 1992; Luft and Remington, 1992; Luft et al., 1993; Robert-Gangneux and Dardé, 2012).

NT ranks high as a cause of death in AIDS patients due to severe encephalitis cursing with necrotic areas and hemorrhage in the brain tissue (Figure 2.7) (Renold et al., 1992; Israelski et al., 1993). Additionally, patients suffering from NT also show other pathologies such as, ocular toxoplasmosis (OT), pneumonitis, pericarditis, etc. (Luft et al., 1993; Robert-Gangneux and Dardé, 2012). In contrast to congenital toxoplasmosis, NT is mainly due to a reactivation of a chronic infection rather than to a primary infection (Luft et al., 1993).

Transplants of infected organs can also be an important cause of neurotoxoplasmosis, especially when transplanting the heart since this is one of the organs with a higher parasitic load in humans (Kittleson and Kobashigawa, 2013).

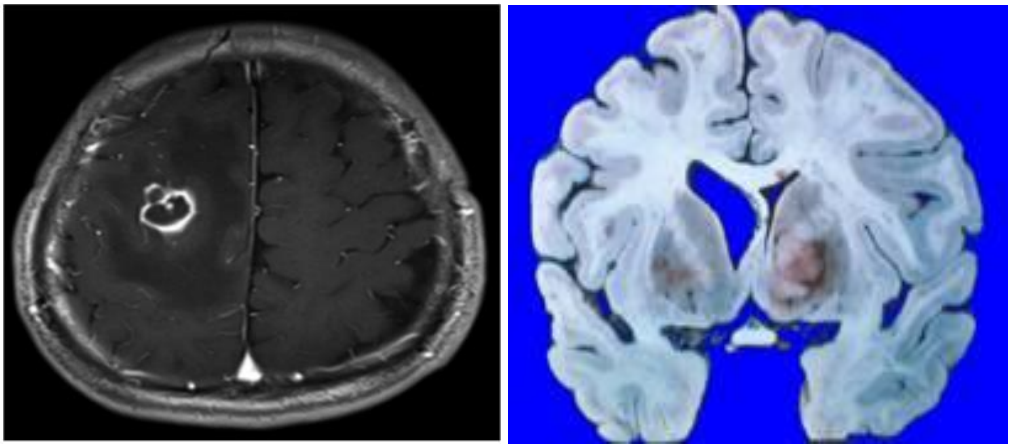

Figure 2.7: MRI of the brain, showing a ring-enhanced mass with mild edema in the right front lobe (left) and a macroscopic picture of a toxoplasmic encephalitis (right)

2.3.3 Immuno-competent individuals

Toxoplasmosis in immunocompetent hosts is generally asymptomatic or will result in mild flu-like symptoms. However, in some cases, ocular toxoplasmosis (OT) may develop in otherwise healthy individuals (Demar et al., 2012; Ozgonul and Besirli, 2017). This manifestation of the disease is strain- and dose-dependent and remarkably, ocular toxoplasmosis in South American countries is mostly associated with infections in North-American and European immigrants by atypical strains, suggesting a host genetic impact on disease severity. Ocular toxoplasmosis can be recurrent with the reactivation or rupture of old tissue cysts (Holland, 2003).

Nowadays, chronic toxoplasmosis is starting to be considered as a risk factor for developing different neurological and psychiatric disorders such as, schizophrenia, Parkinson or depression (Lester, 2010). Moreover, behavioral changes have been described, not only in humans where toxoplasmosis has been associated with reckless driving and traffic accidents (Flegr et al., 2002), but also in rodents where infected animals are less afraid and even attracted to cat odor (Webster, 2007) and in primates which are morbidly attracted to leopard urine (Poirotte et al., 2016).

Once immunity is acquired, immunocompetent individuals are thought to be protected against *T. gondii* infection life-long, although the immunity is not sterile; the sporadic rupture of old tissue cysts is considered to account for a re-stimulation of the immune system (Dubey, 2010). However, acquired immunity is thought to be insufficient to protect against re-infection with a different strain type or by atypical strains (Abdul-Fattah et al., 1992; Valdès et al., 2011).

2.3.4 Prevention and treatment

Prevention is often associated with prophylaxis and vaccination; however, for toxoplasmosis no vaccine is currently available for humans (Buxton et al., 2007). Prevention of toxoplasmosis is mostly focusing on educational programs including hygienic measures and avoiding possible sources of infection. These programs that especially apply for pregnant women and immunosuppressed people (Hill and Dubey, 2002; van der Giessen et al., 2003), have been shown to be cost-saving and effective (Prusa et al., 2017). Within these preemptive measures the most important are:

- Freezing meat and meat products at -20°C for at least 48 h
- Cook the meat at 70°C for at least 1 min
- Avoid consuming raw oysters, clams and mussels
- Washing hands thoroughly and wearing gloves after and during gardening, respectively
- Wash thoroughly fruits and vegetables
- Avoid eating raw vegetables and salads
- Drink mineral bottled water and avoid tap water

Additionally, important efforts are currently done towards identifying different risk factors for *T. gondii* infections at a farm level aiming to achieve *T. gondii* free farms and thus meat. For example, the presence of rodents and cats have been consistently identified as important risk factors in pig farms (Opsteegh et al., 2016).

The first effective treatment for toxoplasmosis was described in 1942 by Sabin and Warren using sulfonamides (Sabin and Warren, 1942). Later on, the curative effect of sulfonamides was enhanced by the use of pyrimethamine (Eyles and Coleman, 1953) and this combined therapy is still today the preferred treatment option for human toxoplasmosis. Both sulfonamides and pyrimethamine are only able to act against tachyzoites, consequently being useful only during the acute phase of infection. Encapsulated bradyzoites are protected against these drugs, which inhibit the dihydrofolic acid synthetase and the dihydrofolic acid reductase, respectively. Concerning CT the most frequently used treatment is spiramycin since this drug accumulates in high concentrations in the placenta without crossing the placental barrier. However, the efficacy of spiramycine for reducing the effects of congenital toxoplasmosis is still to be confirmed (Ajzenberg, 2011).

2.4 *Toxoplasma gondii* infection in animals

As already mentioned, any warm-blooded animal can be infected with *T. gondii* and develop toxoplasmosis; the versatility of *T. gondii* has made it one of the most prevalent zoonotic parasites

worldwide being even found in sea mammals (van de Velde et al., 2016). Within terrestrial animals, free ranging herbivores have a higher prevalence while animals with less environmental contact, eg poultry and pigs raised in intensive farms have a lower prevalence (Dubey, 2010).

Infection with *T. gondii* in animals normally causes no clinical signs or mild non-specific signs such as, fever, anorexia, abdominal pain, etc. However, in some cases serious disease and even death may occur due to necrosis of the intestine and mesenteric lymph nodes. Additionally, focal areas of necrosis may develop in other organs such as heart, eyes, adrenals, etc. (Dubey, 2010).

Additionally, as the interest for *T. gondii* increases, consumption of other types of animals, such as bivalves have been identified as a potential source of infection for man. Even though shellfish cannot develop toxoplasmosis (Paratenic hosts) they do accumulate oocysts from the environment. They may be useful as sentinel animals for investigating environmental *T. gondii* contamination (Palos Ladeiro et al., 2014).

2.4.1 Domestic cats, the Trojan Horse

Cats and other felids play a pivotal role in the life cycle of *T. gondii* since they are the definitive hosts and the only hosts in which the sexual phase occurs. As a result of the sexual replication, millions of infectious and extremely resistant oocysts are shed in the environment (water, crops, soil, etc.), accounting for all the *T. gondii* infections in herbivores and some of those in carnivores. After a primary infection, cats can shed between 25 and 810 million oocysts in the environment during a period of 1-3 weeks (Dubey, 2010). Although most cats have only one oocyst shedding episode in their life, re-infection of a cat by a different *T. gondii* strain may result in a second period of shedding of oocysts (Dubey, 2010).

Most of the warm-blooded animals, including cats are mostly asymptomatic after *T. gondii* infection. However, as in humans, sporadically different non-pathognomonic clinical signs can be observed, ranging from mild clinical signs such as, fever, anorexia, abdominal pain, ocular disease, etc. to more severe signs such as pneumonia or even death (Dubey, 2010). A concomitant infection with the feline immunodeficiency virus (FIV) has been proven to be clinical and epidemiological inconsequential (Dorny et al., 2002).

2.4.2 Livestock

Livestock animals are known reservoirs of the parasite and the consumption of their meat is

estimated to account for at least 30% of the *T. gondii* infections in humans (Cook et al., 2000; Scallan et al., 2011). In this part of the literature study we review the most important features of *T.* gondii infection in the main livestock species.

Pigs

Pigs, similar to other omnivores, can get infected through oocysts contaminated feed and water or by ingestion of chronically infected animals. The seroprevalence in the pig population is dependent of the management system, with low prevalence in intensive, indoor and in/all out management production systems (Belgium intensive farms: 4 % (Jennes, 2017); France intensive farms: 3% (Opsteegh et al., 2016); while for management systems allowing outdoor access the seroprevalence increases importantly (USA free range farms: 25% (Guo et al., 2015), Belgium organic farms: 53% (Jennes, 2017). In Table 2.3 an overview of seroprevalences in pigs in different European countries is presented.

T. gondii infection in pigs occurs normally asymptomatically or with very mild clinical signs such as, anorexia, fever, dyspnea, etc. Congenital transmission in pigs has been described with different consequences varying in severity, ranging from asymptomatic born piglets to abortion of the entire litter. (Dubey, 2010). Following infection of adult pigs the parasite can be found in almost all tissues (see Table 2.4).

Table 2.3: Overview of the *T. gondii* seroprevalence in pigs in different European countries

Country	Estimated prevalence	Reference
Wallonia, Belgium	65.00%	Jennes, 2017
Flanders, Belgium	1.00%	Jennes, 2017
Czech Republic	36.00 %	Bártová and Sedlák, 2011
France	2.90 %	Djokic et al., 2016
Ireland	4.70 %	Halová et al., 2013
Italy	16.30 %	Villari et al., 2009
Portugal	9.80 %	Lopes et al., 2013
Serbia	9.20 %	Klun et al., 2006
Spain	9.70 %	García-Bocanegra et al., 2010
Switzerland	14.00 %	Berger-Schoch et al., 2011

Table 2.4: Overview of the different tissues in which *T. gondii* has been detected in pigs

Tissue	Method	Reference
Heart	MC-qPCR/bioassay	Gisbert Algaba et al., 2017
Brain	MC-qPCR	Gisbert Algaba et al., 2017
M. Gastrocnemius	MC-qPCR/bioassay	Gisbert Algaba et al., 2017
M. Longissimus Dorsi	MC-qPCR/bioassay	Gisbert Algaba et al., 2017
M. Psoas Major	MC-qPCR/bioassay	Gisbert Algaba et al., 2017
M. Diaphragm	MC-qPCR/bioassay	Gisbert Algaba et al., 2017
M. Intercostal	MC-qPCR/bioassay	Gisbert Algaba et al., 2017
Lungs	MC-qPCR	Gisbert Algaba et al., 2017
Tongue	MC-qPCR	Juránková et al., 2013
Liver	MC-qPCR	Juránková et al., 2013
Kidney	MC-qPCR	Juránková et al., 2013

In Europe and North America, even though the overall prevalence in pigs has dropped notably with the introduction of modern and efficient farm management systems and the general hygienic preemptive measures, pork still poses a risk for transmission of toxoplasmosis. Currently, in the United States, it is estimated that 41 % of the cases of foodborne human toxoplasmosis are caused by the consumption of pork, which is considered as the main source of infection for humans (Batz et al., 2012). During the last years, in Europe the increasingly more popular animal friendly production systems (organic farms) may account for a re-emergence of pork as an important source of infection in humans (Kijlstra et al., 2004).

In order to better comprehend the different factors playing a role in *T. gondii* infection in pigs, several studies have been performed aiming to detect the main risk factors. These studies have been recently summarized by Opsteegh et al. (2016). The main on-farm risk factors in pigs are:

- Definitive host-related variables: presence of cats, high population of cats in the area, etc.
- Feed-related variables: feed contamination, dry feeding, etc.
- Housing-related variables: straw bedding, all in all out housing, hygienic measures, etc.
- Cleaning and disinfection-related variables: duration and intensity of the cleaning before housing new animals, inadequate removal of dead animals, etc.
- Confinement level-related variables: outdoor access, access to pasture, etc.
- Rodent-related variables: cats used for rodent control, absence of rodent control, etc.

- Water-related variables: water supply contamination
- Age-related variables: age of the animals, sows, etc.

Chickens

Free-range chickens are known to be extremely susceptible to *T. gondii* infection due to their feeding behavior (picking) (Dubey, 2010). The prevalence in free-range or backyard chickens is considered to be very high even reaching 100% in some studies. On the other hand, animals raised in intensive farms have a very low prevalence (Dubey, 2010). Clinical signs in chickens are rare, however, some of the infected animals can develop clinical signs such as, peripheral neuritis, torticollis, etc… Backyard and free-range chickens are thought to play a role serving as prey and a source of infection for wild felids helping to the spread of the parasite (definitive hosts).

Cattle

In cattle, the correlation between the actual presence of the parasite (tissue cysts) in sero-positive animals is still under major debate (Belluco et al., 2016). Seroprevalence in cattle is considered to be highly variable with values ranging from 1.9 % to 54.5% (Opsteegh et al., 2011). However, direct detection and also isolation of strains with bioassay are successful in very limited cases. The lack of correlation between seroprevalence data and parasite detection in tissues can be explained by the low sensitivity and specificity of most of the available indirect detection methods in cattle (Opsteegh et al., 2016).

Infected animals normally show no clinical signs and although *T. gondii* has been isolated at least twice from aborted bovine fetuses, whether these cows aborted due to toxoplasmosis could not be determined because no histological examination of the fetuses was made (Dubey, 2010).

In contrast to other species, *T. gondii* prevalence by direct detection of the parasite does not increase with age; indeed, calves show higher incidence of infection in tissues than older animals. Also, tissue cysts are not thought to persist long in most of the cattle tissues (Opsteegh et al., 2016). Within the tested tissues the predilection sites for recovering the parasite are, liver, small intestine and skirt steak.

Sheep and goats

Toxoplasma gondii has been identified as one of the main sources of infective abortion in sheep and goats. The actual incidence is difficult to estimate since the disease is usually sporadic (abortion storms) and in only few cases diagnosis is confirmed by examination of the aborted fetuses (Dubey, 2010). Seroprevalence in small ruminants is age-related, reaching 95% in animals older than 6 years (Dubey, 2010). Although endogenous transplacental transmission occurs in persistently infected ewes and does, the majority of infections are due to environmental contamination of water and grass with oocysts (Dubey, 2010).

Chapter III:

Detection Methods

The detection of *Toxoplasma gondii* infection can be achieved by either direct or indirect methods. The first efficient diagnostic tool was developed in 1948 by Sabin and Feldman (Sabin and Feldman, 1948) and consisted of an indirect serological test. Since then, many different indirect methods have been developed for detecting anti-Toxoplasma antibodies in serum samples including, ELISA, MAT, IFA, etc. However, none of those tests has been able to substitute the Sabin Feldman test, which remains the gold standard method for serological detection of *T. gondii* since it is the most sensitive and specific method to detect *T. gondii* infection.

Direct detection of the parasite was first performed by cat and mouse bioassays and histology. With the elucidation of the chemical DNA structure (Watson and Crick, 1953) a new generation of molecular detection methods was developed, such as polymerase chain reaction (PCR) of the B1 gene (Burg et al., 1989) and real time PCR (qPCR) of the 529 repeated element (Homan et al., 2000), among many others. However, still today mouse bioassay is considered the reference method for the direct detection of the parasite since it is the most sensitive method able to demonstrate the presence and the viability of the parasite.

While serology is the method of choice for screening purposes, a positive serological result only demonstrates that a contact with the parasite has occurred and not the actual presence of the parasite in the tissues. Therefore, the combination of both indirect and direct methods is considered the most specific and sensitive approach (Opsteegh et al., 2016).

Here we summarize the most common indirect and direct detection methods for the diagnosis of *Toxoplasma* infection.

3.1 Indirect Methods

Serological tests, although fast and easy to perform in living animals, do not demonstrate the actual presence of the parasite in the tissues. However, they do account for pivotal information on the phase of infection, being able to discriminate between the acute and the chronic phase. This discrimination is possible due to the detection of different antibody isotypes (Ab).

Three immunoglobulin isotypes play a role in the humoral immune response against the parasite (Figure 3.1):

- IgM appears already 4-6 days after a primary infection and for a short period of time and not or almost not after a second infection
- IgG appears between the first and second week after infection, reaches a plateau after 2-3

months and mostly remains present lifelong at residual levels .

- IgA also appears during the first week after infection, disappears after 6-7 months of infection and reappears with every re-infection.

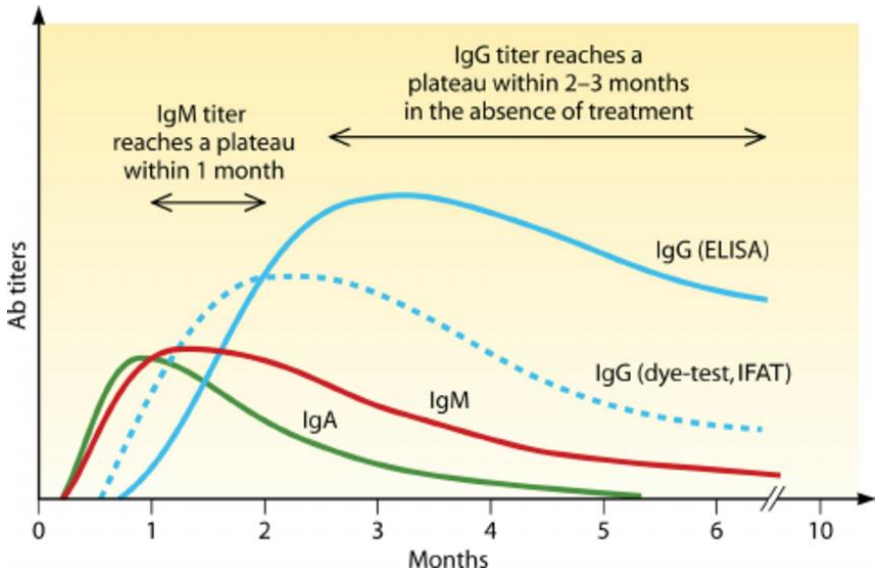

Figure 3.1: Overview of the different antibodies expressed in function of time after infection (Robert-Gangneux and Dardé, 2012)

Therefore, the presence of IgM indicates a relatively recent primary infection, which when testing pregnant women may indicate a seroconversion during pregnancy and thus a risk for transplacental infection of the fetus (Dubey, 2010). Additionally, due to the specific 3-dimensional characteristics of IgM these immunoglobulins are not able to cross the placental barrier. Therefore, the presence of IgM Abs in the newborn indicates actual infection of the baby and not passively acquired maternal Abs.

Based on the different patterns of expression of antibodies the following tests are the most frequently used for serological toxoplasmosis diagnosis.

Sabin Feldman Test

The Sabin Feldman Test or Dye Test is still today considered the gold standard serological method for the diagnosis of *T. gondii* infection. It is a straightforward method which consists of the incubation of a mixture of live tachyzoites and complement of a *T. gondii* seronegative person with a serial dilution of the patient's inactivated serum sample in a methylene blue dye rich medium. Tachyzoites incubated with serum containing anti-Toxoplasma antibodies will lose the capacity of taking the dye as a result of an antibody-complement-mediated lysis reaction while tachyzoites incubated with a negative serum will appear blue under the microscope. This test is able to detect the

presence of different antibodies, IgG, IgA and IgM with high sensitivity and specificity. The main disadvantage of the Dye Test is the requirement of live parasites (tachyzoites obtained from mice infected with the *T. gondii* RH strain).

Agglutination and Modified Agglutination Tests (MAT)

The agglutination assays are easy to use serological methods that are commercially available, which do not require special equipment. Briefly, serial serum dilutions are incubated overnight with formalized tachyzoites. Tachyzoites incubated with a positive serum will precipitate to the bottom of the well of a microtiter plate in the shape of a carpet, while tachyzoites incubated with negative serum will form a dot on the bottom of the well (Figure 3.2).

In order to increase the specificity of the agglutination assays the serum samples can be incubated with mercaptoethanol prior to incubation with the tachyzoites, aiming at denaturizing the less specific IgM, allowing the detection of the highly specific IgG (Dubey, 2010).

Kit Negative Control

Kit Positive Control

Buffer Control (Negative)

Serum Control (positive)

Negative Sample

Negative Sample

Positive Sample

Figure 3.2: Example of the results obtained with MAT on different serum samples

Indirect Enzyme-linked Immunosorbent Assay (ELISA)

The indirect ELISA is a two-step ELISA in which the target antigen(s), coated on a solid surface, bind(s) serum antibodies (Ab). In a next step, the bound antibodies are detected by secondary isotype- and species-specific antibodies labelled with an enzyme. The enzyme will convert a colorless substrate-chromogene complex into a colored reaction product by an oxidation reaction.

The absorbance, measured by a spectrophotometer and expressed as the optical density, is used to determine the relative concentration of the antibodies in the samples, as compared to the known standards or control samples. An overview is shown in Figure 3.3.

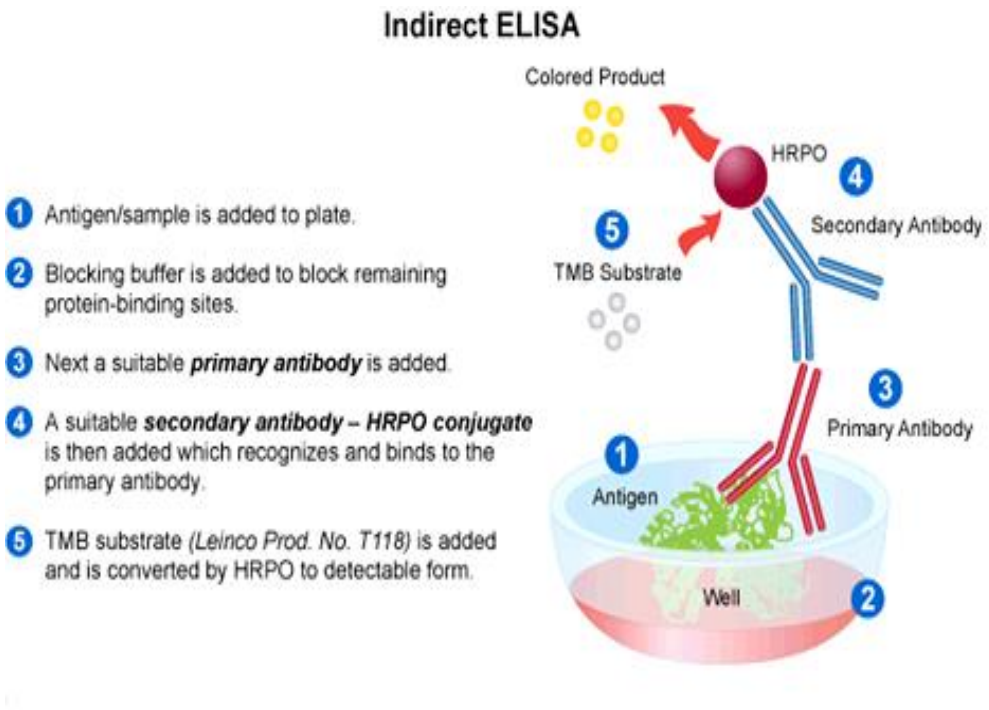

Figure 3.3: Overview of an indirect ELISA, adapted from Leicon technologies

Nowadays, ELISA has become one of the most used techniques for indirect detection of *T. gondii* infections due to the big variety of ready and easy to use kits developed to detect different Abs in many different species, including humans. These are designed either to detect Abs against one single specific native or recombinant antigen (SAG1, GRA7, etc.) or against a mixture of antigens such as, total lysate antigen (TLA) containing almost all the antigens expressed by the parasite. A test using one specific antigen will be more specific but less sensitive since during the infection antibodies will not be developed equally well against different antigens (Basso et al., 2013) and a combination of antigens will render the technique more sensitive but less specific, since there is a higher risk of cross reaction with antibodies developed against other microorganisms giving false positive signals.

Indirect Immunofluorescence Assay (IFA)

In IFA, whole tachyzoites are coated on a glass slide and recognized by the primary serum antibodies. Binding of the serum antibodies is visualized by incubation with secondary isotype- and species-specific antibodies labelled with a fluorescent dye, often fluorescein isothiocyanate (FITC). To decrease background a counterstain with Evans Blue is performed. The fluorescence microscopic

positive samples appear green while negative samples will appear red (Figure 3.4). Once more, depending on the conjugates used, different Ab isotypes can be detected.

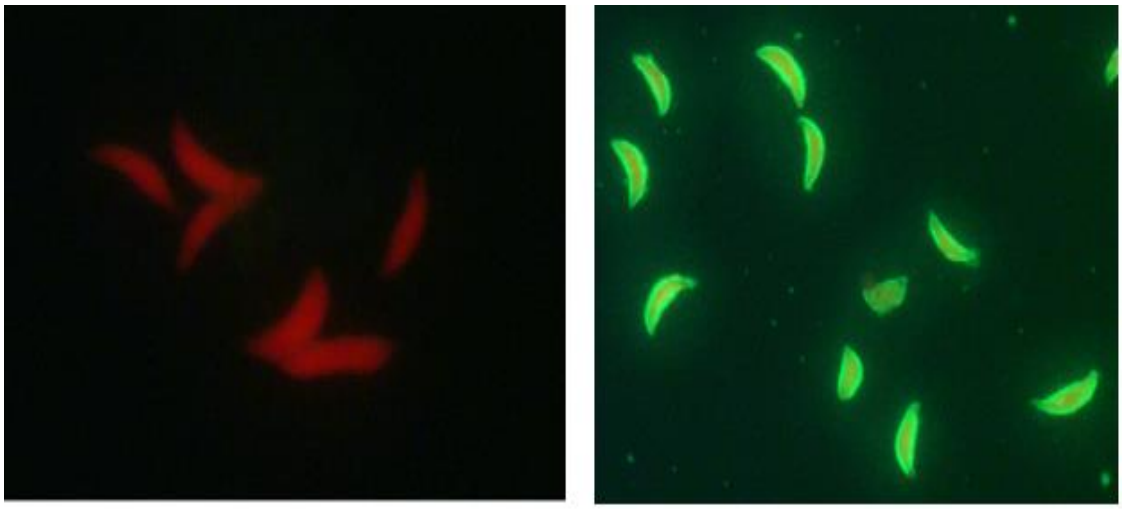

Figure 3.4: Results obtained with a negative serum (left) and a positive serum (right) in IFA. Source: NCR for Toxoplasmosis, WIV-ISP, S. De Craeye

3.2 Direct Methods

The direct detection of the parasite is achieved by using several techniques able to directly identify one of the stages, the parasite's DNA or the infectivity of the parasite.

Bioassay

The infection of animals is still today considered the gold standard for the direct detection of viable parasites. Positive and negative samples are discriminated by the ability of the sample to produce toxoplasmosis in different animals. The most relevant experimental animal species for the detection of *T. gondii* are the mouse and the cat. Cat bioassay is performed by orally feeding cats with suspected tissue samples. This method is able to demonstrate the presence of infectious parasites in large amounts of tissues; in addition, oocysts that are shed by cats following infection can be further used for genotyping of the strain. However, the cat bioassay is ethically questionable, time consuming and expensive. Mouse bioassay is less sensitive than cat bioassay since a smaller amount of sample can be analyzed; however, its sensitivity is still high. The method is less costly than cat bioassay, but also ethically questionable. Nowadays, only a few labs perform bioassays for the diagnosis of toxoplasmosis.

Cell Culture

As an alternative to the use of experimental animals an *in vitro* technique was developed using different cell lines as a growing media (Foulon et al., 1999; Jokelainen, et al., 2012). However, the sensitivity of this technique is lower than that of the bioassays and even of molecular detection methods. Consequently, cell culture is mainly used to maintain strains or multiply strains for antigen preparation.

Microscopy

Direct observation of all the stages of the parasite is microscopically possible:

- Oocysts: *T. gondii* oocysts can be observed after their isolation using sucrose flotation techniques in fecal samples. However, morphological distinction from closely related species such as, *Hammondia hammondi* is not possible and molecular methods are needed to exclude false positives.
- Tachyzoites: This form of the parasite is visible without any specific staining, but they are mostly present only during a short period of time in patient samples (acute phase of infection)
- Tissue cysts: The presence of the parasite in different tissues can be shown by histological sections without any specific staining; however, specific staining conjugates are necessary to differentiate *T. gondii* tissue cysts from other close related parasite species.

The sensitivity of these techniques is also limited, as a low parasite density in blood, tissues or fecal samples, etc. will result in false negative results.

Molecular Methods:

Molecular methods are very sensitive techniques able to detect the parasitic DNA in any type of matrix including all animal and human tissues, soil, vegetables or water. The sensitivity of one of these methods (MC-qPCR) has been recently proven to be even higher than the mouse bioassay. However, the main drawback of all these techniques is the inability to differentiate between dead and thus non- infectious parasites and viable parasites (Opsteegh et al., 2016).

Polymerase Chain Reaction (PCR)-based methods

The appearance and development of several PCR-based methods represented a major breakthrough in scientific history mostly in the field of medical and veterinary diagnosis. These methods allow to amplify concrete specific DNA sequences in order to proof the presence of pathogens in very low

concentrations.

PCR and Real time PCR (qPCR)

The PCR is an easy to use technique, which combines the use of thermostable polymerase enzymes and sequence specific primers for one or more targets. For *T. gondii*, many different target sequences have been widely used from which the most common are the B1 gene and the 529 bp repeated element (Burg et al., 1989; Homan et al., 2000). Although very sensitive and fast, the major disadvantage of this technique is the DNA extraction, which can only be performed in small samples up to 200 mg, resulting in a high number of false negatives and the inability of accurate quantification. As an alternative to classic PCR, real time PCR also called quantitative PCR (qPCR) was developed enabling a precise quantification, but still an insufficient amount of sample can be analyzed. These two techniques have been proven to be very valuable for the diagnosis of human toxoplasmosis, with direct detection of the parasite in different tissues or fluids such as, the placenta amniotic fluid, cord blood, aqueous humor, etc., but insufficiently sensitive to detect the presence of the parasite in the different animal tissues. (Petersen, 2007; Dubey, 2010).

Magnetic Capture- Real Time PCR (MC-qPCR)

To overcome the sensitivity problem, magnetic capture real time PCR (MC-qPCR) was developed combining the use of specific, biotin labelled probes with the capture of those probes with streptavidin-coated paramagnetic beads. The development of the Magnetic Capture (MC-qPCR) allowed the possibility of reliable parasite quantification in contrast with the qualitative results obtained with bioassays and classic PCR, enabling the study of the quantitative distribution of the parasite through the carcasses of different species (Opsteegh et al., 2010; Juránková et al., 2014; Aroussi et al., 2015; Hosein et al., 2016). Furthermore, the method has been recently improved (**Chapter IV**).

Loop-mediated isothermal amplification (LAMP)

As an alternative to PCR and to avoid the use of expensive thermocyclers, LAMP technology was developed. The sensitivity of this technique is comparable to the PCR-based methods but LAMP does not easily allow multiplexing. In addition, the sample size and viability of the parasite continue to be a major drawback (Opsteegh et al., 2016).

Part II: Experimental studies

Situation of the problem and objectives

Nowadays, it is estimated that at least 50% of the European population is *Toxoplasma gondii* seropositive (50%-80%). From these seropositive persons, it is estimated that 1 out of 3 infections is meat borne, identifying the consumption of raw or undercooked meat as one of the main routes of infection for humans. Recent studies ranking disease burden of 14 foodborne pathogens to various food sources, have classified *T. gondii* and pork in the top 2 above other better-known pathogen-food combinations such as, *S. enterica* and poultry or *norovirus* and complex foods. Moreover, in the US, 41% of the foodborne toxoplasmosis cases are considered to be caused by consumption of pork. However, little is known about the parasite burden present in pigs intended for human consumption in Belgium.

The general objective of this study was to study the role of pork and other meat products as a source of *T. gondii* infection in humans

The specific aims of the present PhD thesis were

- To optimize the parasite detection in host tissues (Chapter IV).
- To study the infection kinetics in experimentally infected pigs (Chapter V):
    - Effect of different infectious stages and strains on the parasitic load present in the carcass at slaughter age.
    - Effect of different strains in the presence of the parasite during the acute phase of infection.
    - Anatomical distribution of the parasite in pig carcas
- To study the molecular epidemiology of *T. gondii* in Belgium (Chapter VI):
    - Isolation and genotyping of strains in naturally infected pigs.
    - Comparison of pig strains with those occurring in humans and other animal species in Belgium.

Chapter IV

Optimization of the parasite's detection in the host tissues

Adapted from the publication: A more sensitive, efficient and ISO 17025 validated MC-qPCR method for the detection of archetypal *T. gondii* strains in meat

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Abstract

Toxoplasma gondii is a worldwide prevalent, zoonotic parasite of major importance for public health. Various indirect and direct methods can be used for the diagnosis of toxoplasmosis. Whereas serological tests are useful to prove a contact with the parasite has occurred, the actual presence of the parasite in the tissues of a seropositive animal is not demonstrated. For this, bioassay is still today the reference method. As an alternative, various PCR methods have been developed, but because of the limited amount of sample that can be tested combined with a low tissue cyst density, they have been proven to be insufficiently sensitive. A major improvement of the sensitivity was achieved with a Magnetic Capture based DNA extraction: by combining the hybridization of specific, biotinylated probes with the capture of those probes with streptavidin coated paramagnetic beads, *T. gondii* DNA can selectively be "fished out" from a large volume of meat lysate. Still, several studies showed an insufficient sensitivity compared to the mouse bio-assay. Here we present how we made the method more sensitive (99% LOD of 65.4 tachyzoites per 100 g of meat), economic and reliable (ISO 17025 validated) by adding a non-competitive PCR inhibition control (co-capture of cellular r18S) and by making the release of the target DNA from the streptavidin coated paramagnetic beads UV dependent. The presented results demonstrate the potential of the modified MC-qPCR as a full alternative for the mouse bioassay for the screening of various types of tissues and meat, with the additional advantage of being quantitative.

4.1. Introduction

Toxoplasma gondii is an obligate intracellular and cyst forming protozoon parasite with a complex life cycle. Most warm-blooded animals are intermediate hosts, while only the members of the Felidae are definitive hosts (Tenter et al., 2000). The parasite has three infectious stages: tachyzoites, the free, fast replicating form of the parasite present during the acute phase; bradyzoites, the slow replicating form present in tissue cysts during the chronic phase of the infection and sporozoites, which are the result of the sexual replication in the definitive host and are present in sporulated oocysts (Dubey et al., 1998).

Humans can get infected with *T. gondii* through different routes: by the consumption of raw or undercooked meat from chronically infected animals, by ingesting sporulated oocysts present in water, soil or on vegetables and by vertical transmission through the placenta (Hide, 2016). In humans, the majority of infections are asymptomatic while on rare occasions mild flu-like symptoms are observed (Ho-Yen, 2001). However, an infection of the mother during pregnancy can result in

congenital transmission, which may lead to foetal abnormalities, abortion and reduced quality of life in surviving children (Frenkel, 1974; Maldonado, 2017). Additionally, toxoplasmosis can result in severe disease in immunocompromised people and eye disease in otherwise healthy individuals (Demar, 2012; Ozgonul and Besirli, 2017). In rare cases, mostly in South America, toxoplasmosis is associated with severe disease even in immunocompetent people due to an infection with atypical strains (Pomares, 2011; Sobanski, 2013).

Being zoonotic and worldwide prevalent, there is an increasing awareness of public health importance of *T. gondii* infections. For example, an estimated 50-80% of the European population is seropositive for *T. gondii* and has thus been in contact with this parasite (Opsteegh et al., 2016). In a recent report of the USDA ranking the 20 most important foodborne pathogens, *T. gondii* was classified as second considering the annual number of infections and the associated economic and medical costs (with an estimated yearly cost of more than 3.3 billion US dollars) (Hoffman et al., 2013).

Although *T. gondii* infection is often associated with cats and their shedding of oocysts (Torrey and Yolken, 2013), meat is probably the most important source of infection in developed countries. It has been estimated that 50% of the cases are foodborne (Scallan et al., 2011). A ranking of the disease burden of 14 foodborne pathogens in relation to various food sources estimated that 41 % of the cases of foodborne human toxoplasmosis in the United States are caused by the consumption of pork, which can thus be considered as a main source of infection for humans (Batz et al., 2012). Moreover, a pathogen-food relationship ranking in function of QALYs and costs of illness situates the combination *T. gondii*/pork on the top 2 above other pathogen-food combinations as *Salmonella enterica*/poultry and norovirus/complex foods (Batz et al., 2012). Furthermore, the increasingly more popular animal friendly production systems (organic farms) and the fact that one pig may be consumed by an estimated 200 to 400 individuals may account for a re-emergence of pork as a main source of infection (Fehlhaber, 2003; Kijlstra et al., 2004).

Several indirect and direct methods have been developed for the detection of *T. gondii*. While serology is the method of choice for screening purposes, a positive result only demonstrates that a contact with the parasite has occurred and not the actual presence of the parasite in the tissues. The direct detection of the parasite on the other hand is often laborious, costly and insufficiently sensitive. While bio-assay is, together with cell culture (Jokelainen, 2012), still the only test capable of demonstrating the presence of infectious parasites, it is time consuming, expensive and implies the

use of laboratory animals. Histological and molecular methods have been proven to be insufficiently sensitive for demonstrating the parasite in meat samples because of a non-homogeneous and low tissue cyst density in meat. This, together with the small amounts of sample that can be tested with commercially available kits (up to 50mg with commercially available DNA extraction kits) often result in high numbers of false negatives (da Silva and Langoni, 2001; Hill et al., 2006). Also, although detection of parasites in tissue samples is significantly higher in seropositive pigs, the parasite has been isolated from seronegative animals. This accentuates the need of combining both, indirect and direct methods in order to estimate the role of meat as a source of infection for humans (Opsteegh et al., 2016).

Opsteegh et al. developed a Magnetic Capture qPCR (MC-PCR) that enables analysis of tissue samples of up to 100g (Opsteegh et al., 2010). This method combines a specific DNA extraction and concentrating method (based on the hybridization of specific, biotin labelled probes to a *T. gondii* target sequence with a capture with streptavidin coated paramagnetic beads) with a sensitive and specific qPCR. However, a recent study showed certain disagreements between the MC-qPCR and mouse bio-assay in pig samples (Opsteegh et al., 2016).

We aimed to improve the MC-PCR method to make it even more sensitive, more economic and suitable for ISO validation so that it can be routinely used in a diagnostic lab. We achieved this by adding the co-capture of cellular r18S as a means of keeping track of the extraction and as a non-competitive PCR inhibition control, by making the release of the target DNA UV dependent and more efficient, by reducing incubation times and by reducing the cost through a comparison of reagents. Moreover, once optimized, the new method was subjected to an ISO 17025 validation to objectively confirm its robustness and reliability. We have chosen pork as the main matrix for the validation process due to its perceived importance in the transmission of *T. gondii* to humans.

4.2. Materials and Methods

4.2.1 Toxoplasma gondii strains

In order to study the main *T. gondii* lineages, different strains were used: *T. gondii* RH strain (Type I, high virulence in mice), *T. gondii* IPB-LR (type II, low virulence in mice) and *T. gondii* IPB-G (Type II/I, a hybrid rare strain virulent in mice). The strains were genotyped by the CBR (Limoges, France) as described by Ajzenberg, et al. (2010).

4.2.1.1 Preparation of a Quantified Tachyzoite Suspension for Spiking Experiments

A purified *T. gondii* RH strain tachyzoite suspension was quantified using a Bürker counting chamber and a phase contrast microscope (amplification 400X) (Nikon Eclipse Ni, Nikon, Brussels, Belgium) and diluted accordingly to obtain a final concentration of 10^7 tachyzoites per ml in PBS supplemented with 0.01% bovine foetal calf serum (BFCS). Starting from this stock suspension, the following dilutions were made: 10^6, 10^5, 10^4, 5000, 10^3, 500, 250, 125, 10^2, 50, 25 and 10 tachyzoites per ml and stored at -20°C till further use.

4.2.1.2 Preparation of Tissue Cysts

Seventy-five Swiss white outbred mice (Janvier Labs, Rennes, France) were intraperitoneally inoculated with the *T. gondii* IPB-LR (type II) or IPB-G (Type II and I) strains. The mice were observed daily and a humane-endpoint was defined to limit the suffering of the mice in case of disease (Ethics committee licence *n*: 20140704-02). After 6 weeks post infection, the mice were euthanized by decapitation and their brains were collected and homogenized in the presence of PBS supplemented with penicillin and streptomycin using a potter homogenator. The concentration of tissue cysts was determined by counting three times a volume of 8 µl with a phase-contrast microscope (Nikon Eclipse Ni, Nikon, Brussels, Belgium).

4.2.1.3 Isolation and Purification of T. gondii Tissue Cysts for Use in Spiking Experiments

Toxoplasma gondii tissue cysts were separated from the brain homogenate by centrifugation on a Percoll gradient. The protocol was kindly provided by the French Biological Resource Centre (BRC) Toxoplasma, and was based on the publication of Blewett et al. (1983). Briefly, the homogenized brains were transferred to a 30 ml conical glass centrifuge tube and two different concentrations of Percoll (Ge Healthcare, Diegem, Belgium) were carefully applied on the bottom of the glass tube (first, a 30% Percoll solution and then a 90% Percoll solution). After centrifugation at 1750 g for 15 min at room temperature (RT), the brain cysts were concentrated in the 30% Percoll layer. This layer was then collected and the Percoll was washed away by dilution in PBS, followed by centrifugation at 1750 g. The obtained brain cysts pellet was then resuspended in a few ml of PBS and was then transferred to a small Petri dish and observed with a phase contrast microscope (Nikon Eclipse Ni, Nikon, Brussels, Belgium). The required amount of brain cysts for each spike were then fished out with a 20µl micropipette and added to meat samples.

4.2.2 Capture Probe design and UV mediated elution

We aimed to match the efficiency of the magnetic capture described by (Opsteegh et al., 2010) with the sensitive ISO 15189 validated qPCR already in use in the laboratory. This qPCR method has been published before and amplifies an amplicon of 106bp inside the 529bp repeated element (GenBank ascension number: AF146527) (De Craeye et al., 2011). For this, the reverse capture probe (ToxCap RB) had to be redesigned further away on the target sequence. The sequence of the new capture probe was evaluated for secondary structures, melting temperature and dimer formations using several bioinformatics analyses: To predict secondary folding structures and hybridization of linear ssDNA the Mfold web server (online: http://unafold.rna.albany.edu) was utilized. Further basic oligo analysis was performed using the OligoAnalyzer 3.1 on the Integrated DNA Technologies website (IDT, online: http://eu.idtdna.com/calc/analyzer). To confirm the results obtained with the bioinformatics analysis different experiments were performed (data not shown).

As an ISO 17025 validation was aimed for the implementation of the modified MC- qPCR for diagnostic purposes, a non-competitive internal amplification control (NCIAC) had to be designed to make the whole course of the process traceable, starting from the lysis step till the qPCR result. The cellular r18S gene was chosen for this purpose as it was already implemented in the ISO 15189 validated and previously published qPCR (De Craeye et al., 2011). Here, both primers and the dual labelled probe were selected on a very conserved region of the r18S gene and are able to amplify and detect an amplicon of 138bp within the DNA of all livestock species and was also tested successfully on human DNA and other animal species. To further extend this "universality", two new capture probes (r18SCapFw and r18SCapRV) were designed further away of the primers VF1 and VR1. Using the r18S co-capture as an NCIAC enables not only to exclude false negative results due to PCR inhibition; a suboptimal qPCR result will also show something went wrong during the extraction and DNA capture process.

Whereas the very high binding affinity of the streptavidin-biotin interaction is especially suitable for the magnetic capture method, it also hampers the subsequent release of the captured DNA. The necessity to break the streptavidin-biotin to elute the DNA was avoided by designing the capture probes with a UV cleavable spacer between the biotin and the oligo molecules (5' PC Biotin).
An overview of all the used oligos is given in Table 4.1. All the oligo's were synthetized by Integrated DNA Technologies (IDT, Leuven, Belgium).

Table 4.1: Overview of the used oligonucleotides and their modifications.

DNA Oligonucleotide			Sequence and modifications	Tm (°C)	Ref
Capture Probes (Magnetic Capture)	T-CP	ToxCap Fw	5'-PCBio/CTTGGAGCCACAGAAGGGACAGAAGTCGAAGGGGACT ACAG ACGCGATGCCGCTCCTCCAGCCGTCTTGG- 3'	82.9	Opsteegh,2010
		ToxCap RB	5'-PCBio/CTCCTCCTCCCTTCGTCCAAGCCTCCGACTCTGTCTCCCT CGCCCTCTTCTCCACTCTTCAATTCTCTCC - 3'	81.7	This study
	U-CP	ToxCap UFw	5'-PCBio/CUUGGAGCCACAGAAGGGACAGAAGUCGAAGGGGAC UACAGACGCGAUGCCGCUCCUCCAGCCGUCUUGG 3'	82.9	This study
		ToxCap URB	5'-PCBio/CUCCUCCUCCCUUCGUCCAAGCCUCCGACUCUGUCUC CCUCGCCCUCUUCUCCACUCUUCAAUUCUCUCC - 3'	81.7	This study
	NCIAC	r18SCapFw	5'-PCBio/CGTGATGGGGATCGGGGATTGCAATTATTCCCCATGAA CGAGGAATTCCCAGTAAGTGCGGGTCATAAGCTTGCG -3'	79.5	This study
		r18SCapRv	5'-PCBio/CGCTCTCGCCAACGTTAATGATCCTTCCGCAGGTTCACC TACGGAAACCTTGTTACGACTTTTACTTCCTCT- 3'	77.8	This study
qPCR	*T. gondii*	T2	5'-CGGAGAGGGAGAAGATGTT- 3'	60.1	De Craeye, 2011
		T3	5'-GCCATCACCACGAGGAAA- 3'	59.9	De Craeye, 2011
		TP	5'-6FAM/CTTGGCTGC/ZEN/TTTTCCTGGAGGG/3IABkFQ- 3'	66.4	De Craeye, 2011
	NCIAC	VF1	5'-GATTAAGTCCCTGCCCTTT- 3'	58.0	De Craeye, 2011
		VR1	5'-GATAGTCAAGTTCGACCGTCTT- 3'	60.8	De Craeye, 2011
		VP	5'-TexRed/CACACCGCCCGTCGCTACTACC/3IAbRQSp- 3'	70.1	De Craeye, 2011

Tm: melting temperature; Ref: reference; T-CP: thymine captures; U-CP: uracil modified captures; NCIAC: not competitive inhibition amplification control. Modifications: The capture probes were modified with a photo cleavable biotin (PCBio) at the 5'end. The detection probes have the following reporter/quencher combinations: FAM/ZEN+3IABkFQ to detect *Toxoplasma gondii* and TexRed/3IAbRQSp for the NCIAC.

4.2.3 MC-qPCR

Prior to the optimization of the modified MC-qPCR method a wide-ranging market study was performed in order to choose the best quality-prize reagents and different concentrations of the MC-qPCR reagents were tested. Moreover, different temperatures and incubation times were assessed for the hybridization of probes. In the next point, the final protocol of the modified MC-qPCR method is described.

4.2.3.1 Sample Preparation

4.2.3.1.1 Homogenization and Lysis

A meat sample was cleaned by removing fats and connective tissues and was then cut into small pieces of approximately 1 cm^3 using sterile knives and tweezers. Hundred grams of the cut meat were then transferred to a filtered pedal homogenator bag (Bagpage XR, Interscience, Paris, France) with 2.5 ml of lysis buffer (100 mM Tris HCL, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 40mg/l proteinase k (30 mAnson-U/mg; Amresco, Ohio, USA), pH = 8.0 ± 0.2) per gram of tissue (250ml) as described by (Opsteegh et al., 2010).

The cut meat sample and the lysis buffer contained inside the bag was homogenized in a pedal homogenator (Labconsult, Brussels, Belgium) for 2 min at maximum speed and incubated at 55°C overnight. The following day, the sample was homogenized for one additional minute with the pedal homogenator and incubated 45 more minutes or until the foam disappeared. The lysate was then recovered through the filter of the bag and transferred to a 500 ml centrifuge bottle and centrifuged at 3500 g at 20 °C for 45 min to remove remaining cell debris. Fifty millilitres of the cleared lysate was then transferred to a 50ml polypropylene tube for further use and as a backup.

4.2.3.1.2 Removal of the Free Biotin

To avoid interference of naturally present free biotin in the meat lysate, it was removed using streptavidin coated agarose beads. Briefly, 12 ml of the cleared meat lysate was transferred to a 15 ml polypropylene centrifuge tube and incubated at 100°C for 10 min to inactivate the proteinase K. After inactivation, the extract was cooled down to room temperature and centrifuged at 3900 g to settle the eventual formed flocculation. The supernatant was then transferred to a new 15 ml tube. Meanwhile, 50 µl of streptavidin agarose beads (binding capacity >330 nmol/ml; solulink, San Diego, USA) were washed three times in PBS. The washed beads were then added to the lysate and the whole was incubated for 45 min at room temperature under gentle rotation. The beads were then removed by a centrifugation step at 3500 g for 15 min and 10ml of the biotin-free supernatant was transferred to a new 15 ml centrifuge tube for DNA extraction (magnetic capture).

4.2.3.2 Magnetic Capture of the Target DNA

4.2.3.2.1 Hybridization with the Biotinylated Capture Probes

The specific biotinylated capture probes were added to 10ml of the cleared biotin free lysate to capture cellular r18S and *T. gondii* (AF146527) DNA at a final concentration of 20 attomols and 1 picomol per ml, respectively. See Table 4.1 for an overview of the used capture probes.

Subsequently, the sample was first incubated at 95°C for 15 min followed by incubation at RT for 15 min in order to hybridize the probes with the complementary target DNA sequences.

4.2.3.2.2 DNA Capture Using Streptavidin Coated Paramagnetic Beads

During the hybridization step, the streptavidin coated paramagnetic beads with a binding affinity > 2.5 nmol/mg (Solulink, San Diego, USA) were prepared as follows: 200µg of beads (or 20 µg/ml of biotin-free lysate) were transferred to a 1.5ml microtube and washed by adding 1ml of binding and washing buffer (B&W buffer, 50 mM Tris HCl, 150 mM NaCl, 0.005 % tween 20, pH 8.0 ± 0.2). After mixing, the buffer was removed by placing the tube in a magnetic rack (Magnarack, Invitrogen, Massachusetts, USA) to retain the beads against the magnet. The supernatant was removed and this washing step was repeated twice. Finally, the beads were resuspended in 100µl of B&W.

Once the hybridization step finished, the 200 µg of washed paramagnetic beads were added to each sample and the whole was incubated at room temperature while rotating for 60 min in order to capture the biotinylated probes hybridized with the target DNA. The tubes were then placed on a magnetic rack (Dynamag 15, life technologies, Massachusetts, USA) until the streptavidin coated paramagnetic beads were collected along the tube wall. The supernatant was then discarded and the beads were resuspended in 1ml of B&W buffer and carefully transferred to a 2ml microtube. The beads were then washed with 1ml of B&W buffer to remove the remaining lysate. Finally, the beads were resuspended in 100µl of TE buffer pH 8.0 (Applichem, Darmstadt, Germany).

4.2.3.2.3 Elution

Elution of the captured DNA was achieved by exposing the 2ml microtube containing the streptavidin coated paramagnetic beads in 100µl TE buffer to UV light (300-350nm) during 5 min. For this, a midrange trans illuminator can be used. After each minute the samples were mixed and vortex vigorously. To make the UV elution more convenient and to avoid possible health hazards related to the use of agarose gel trans illuminators (still often contaminated by the use of Ethidium Bromide), we designed an UV elution chamber fitted with a broad spectrum UV lamp with a wave length between 305 and 315 nm (PL-S 9W/12/2P, Philips, Brussels, Belgium) in which 1.5ml microtubes fit (distance to the lamp 2cm), hereon we will refer to the lamp as UVb. After exposure, the 2 ml microtube was placed on a magnetic rack to retain the beads and the supernatant containing the released DNA was transferred to a new 1.5 ml microtube and conserved at -20°C until further testing.

4.2.3.3 Detection of the Target DNA (qPCR)

The purified DNA extract was analysed as described before (De Craeye et al., 2011). Briefly, 10µl of the DNA extract was tested in a final reaction volume of 25µl containing 12.5µl of ExTaq 2X probe mix (Takara, Saint-Germain-en-Laye, France), 400nM of primers T2 and T3, 200nM of primers VF1 and VR1, 66nM of probe TP and 40nM of probe VP. All stock solutions of the primers and probes were made in TE buffer pH8.0 (Applichem, Darmstadt, Germany). To check for inhibition and possible competition between the PCR targets each DNA sample was tested twice in each PCR run: once in duplex (D), testing for the presence of *T. gondii* DNA and cellular r18S; and once in simplex (S) with only the primers and probes to amplify the *T. gondii* target (without VF1, VR1 and VP). The real-time PCR was performed on a BioRad CFX 96 thermocycler (Hercules, California, USA) with the following 2-step cycling program: an initial DNA denaturation and Taq polymerase activation of 3 min. at 95°C, followed by 41 cycles of 15s at 95°C and annealing/extension of 20s at 60°C. In each PCR run non-template controls were included. The qPCR results were analysed using the BioRad CFX manager software to obtain the crossing point (Cp) values.

4.2.2.4 ISO 17025 Validation

To investigate the reliability of the MC-qPCR, a method validation according to the ISO 17025 standard was performed. For this, the following parameters were assessed: repeatability, intra-reproducibility, inter-reproducibility (2° and 3° line control tests), cross-contamination, selectivity, limit of detection (LOD), cross reactivity, specificity and sensitivity. The specificity and sensitivity were assessed on tissues collected from experimentally infected pigs tested with both MC-qPCR and bio-assay (gold standard method).

4.2.5 Experimental Infections in Pigs

To study the specificity and sensitivity of the method, ten 3-week old *T. gondii* seronegative piglets (confirmed by 2 different serological methods: the modified agglutination test (ToxoScreen DA, Biomérieux, Capronne, France) and an in house immunofluorescence test (based on Toxo-Spot IF, Biomérieux, Capronne, France) (Verhelst et al., 2015) were purchased and divided into groups according to the following experimental setup: group 1, two negative control animals; group 2, four animals infected with 6000 tissue cysts of the *T. gondii* IPB-G strain (oral inoculation); group 3, four animals infected with 6000 tissue cysts of the *T. gondii* IPB-LR strain (oral inoculation). For this an inoculum prepared from brain homogenate from previously infected mice was prepared and quantified by counting the number of tissue cysts with a phase contrast microscope (dark field setting, 100X amplification).

The piglets were separated in different stables according to groups, observed daily and a humane-endpoint was defined to limit the suffering of the animals in case of disease and also serologically monitored weekly to confirm an established infection in the positive animals and to monitor the absence of it in the negative animals. Four months post infection (p.i.), the pigs were euthanized using Sodium pentobarbital (Iv) and the following tissues were collected: heart (Ha), M. gastrocnemius (Ga), M. psoas major (PM), M. longissimus dorsi (LD), diaphragm (DI), M. intercostal (IC) and brain (BR).

The collected tissues samples (except the brain tissue) were first cleaned by removing fats and connective tissues and then cut into small pieces ($1cm^3$). The whole cut sample was then mingled and 100g were kept for bioassay and 100g for MC-qPCR. Brain tissue (50g) was tested only by MC-qPCR.

4.2.6 Bioassay

Hundred grams of cut tissue were homogenized in a blender with 150 ml sterile saline (0.9 % NaCl) supplemented with 0.25% trypsin (Gibco) and gentamycin (0.4mg/ml). The homogenate was then transferred to a 2l flask and incubated in a water bath at 37°C for 1h 45 min under continuous stirring (magnetic stirrer). The digest was then filtered through a gauze, transferred to a 500 ml centrifuge bottle and centrifuged at 2100 g at RT for 20 min.

The supernatant is then removed and the pellet resuspended in 100 ml of saline with gentamycin and centrifuged at 2100 g at room temperature for 15 min. This step was repeated once more in order to remove most of the trypsin present in the pellet. The final pellet was then resuspended in 10 ml of PBS supplemented with gentamycin and IP injected into 5 Swiss white female mice, 1ml each (Ethics committee licence *n*: 20140704-02).

The mice were observed daily and a humane-endpoint was defined to limit the suffering of the mice in case of disease. In this case, the mice were euthanized and the lungs and, if present, ascites from the peritoneal cavity were collected and tested in PCR as described before (Verhelst et al., 2015). Six weeks after the inoculation, the mice were euthanized and their blood recovered to prepare serum. The sera were tested by an indirect immunofluorence assay (IFA) as described before (Verhelst et al., 2015).

4.2.7 Statistical Analysis

For determining the LOD, a generalized linear model was fit between detection rates and initial concentrations to obtain the relation between concentration and detection rate using a PROBIT link. The concentration that corresponds with 95, 96, 97, 98 and 99% detection rates was calculated and confidence intervals around this concentration were obtained by means of a 1000-fold bootstrap.

The Cohen's kappa coefficient measures inter-rater agreement for qualitative (categorical) items, this statistical analysis was used to compare the results obtained with the two direct methods used on this study (bioassay and MC-qPCR). The data analysis was performed with the Richard Lowry 1998-2016 software available on the VassarStats website for Statistical Computation (http://vassarstats.net/kappa.html).

4.3 Results

4.3.1 Optimization of the MC-qPCR

4.3.1.1 Removal of Free Biotin

Biotin naturally present in the tissue lysate will reduce the sensitivity of the detection as it will preferably bind to the streptavidin coated magnetic beads. It is therefore essential to insert a biotin removal step before adding the biotinylated capture probes as described by (Opsteegh et al., 2010). The biotin removal was achieved by using streptavidin coated agarose beads.

To investigate the effect and the amount of streptavidin agarose beads needed, the magnetic capture was tested under different conditions: on a reference sample (performed with 50µl of beads), a capture "blocked" sample (using beads exposed to an excess of biotin prior to the capture procedure) and a series of samples supplemented with an increasing amount of biotin (0, 1, 3, 4, 6 and 10 nm). All obtained samples were then tested in qPCR and the obtained Cp values were compared.

The Cp value of the reference sample can be considered as an optimal capture, as no more free biotin should have been present during the capture process, while the capture "blocked" sample shows what happens when the paramagnetic beads have been exposed to an excess of biotin, thus being restricted in their capacity to capture the biotinylated probes. A clear negative effect, as measured by higher Cp values, due to the presence of free biotin could be measured in the qPCR (supplementary table S4.1).

Supplementary Table S4.1. Magnetic capture real time PCR (MC-qPCR): effect of free biotin in the capture and use of Streptavidin agarose beads to detect *Toxoplasma gondii*

Tachyzoites[a]	Supplemented Biotin	Streptavidin beads	Magnetic beads[b]	*T. gondii*		
				rep1	rep 2	Average
10*E4	0 nmol	50 µl	ok	27.44	27.39	27.42
10*E4	10 nmol	50 µl	ok	27.42	27.43	27.43
10*E4	1 nmol	10 µl	ok	29.18	29.05	29.12
10*E4	3 nmol	10 µl	ok	29.59	29.49	29.54
10*E4	4 nmol	10 µl	ok	29.90	29.98	29.94
10*E4	6 nmol	10 µl	ok	31.10	30.86	30.98
10*E4	10 nmol	10 µl	ok	31.69	31.94	31.82
10*E4	0 nmol	50 µl	Blocked	30.16	30.23	30.20

[a] Concentration of tachyzoites spiked to the sample

[b] Magnetic beads previously incubated with free biotin to block the binding sites (blocked) or not blocked (ok)

nmol, nanomol; rep 1, replicate 1; rep 2, replicate 2.

This confirmed that indeed the presence of free biotin is detrimental for the sensitivity of the method. Therefore, the use of 50 µl of Streptavidin agarose beads per sample was selected as a precautionary measure taking into account the unknown variability in biotin concentration due to the matrix effect and even within animal-level factors.

4.3.1.2 Optimization of the Capture Process

4.3.1.2.1 r18S Co-capture

The co-capture of cellular r18S was used as a non-competitive internal amplification control (NCIAC). Therefore, different concentrations of the specific biotinylated capture probes (from $2x10^5$ to 20 attomols/ml of biotin-free extract) were tested on samples with and without the addition of *T. gondii* DNA. The goal was to have a NCIAC qPCR result, which does not handicap the capture and amplification of the *T. gondii* target while obtaining stable, highly reproducible inter-sample Cp values.

Indeed, a decrease of the concentration of the r18S capture probes also reduced the recovery of cellular r18S DNA, resulting in an increase of the Cp value. After analysis of the data, 20 attomols per ml of biotin-free extract was selected as the optimal concentration for further experiments (data not shown).

4.3.1.2.2 Toxoplasma gondii DNA Capture

In order to maximize the robustness of the *T. gondii* DNA capture process, various parameters related with the capture probes were investigated. First, to match the magnetic capture with the previously published and already ISO 15189 validated qPCR at the laboratory (De Craeye et al., 2011), a new reverse probe was selected (see table 4.1).

Then, as the probes were designed with an UV cleavable spacer, the effects of the presence of the capture probes in the qPCR reaction were investigated. For this, two experiments were designed. (Here, the DNA extracts resulting from the magnetic capture were only tested for the presence of *T. gondii* DNA, thus without the NCIAC): In the first experiment, qPCR reactions were performed using the capture probes as primers (no other primers added). This experiment confirmed the capture probes are used as primers in the qPCR reaction. Agarose gel electrophoresis confirmed the presence of the expected extended amplicon of the *T. gondii* DNA target. Secondly, to exclude any interference of the presence of the capture probes on the efficiency of the qPCR detection, a series of MC-qPCR tests were performed using capture probes in which the Thymine was replaced by Uracil bases. By including an initial UNG degradation step (Thermo Fisher Scientific) just before the start of the qPCR cycling protocol (37°C for 10 min., following the manufacturer's instructions), those Uracil containing captures were destroyed and amplification products were solely synthetized using the PCR primers. Our results indicated that indeed the captures were used in the qPCR reaction, but without interference. The curves showed the same efficiency in all the experiments (data not shown).

As the concentration of the capture probes in the magnetic capture is of paramount importance (a maximum of target DNA has to be hybridized, without having an excess of probes competing for binding to the beads) a series of experiments with two different concentrations were tested (1 pmol and 2 pmol per ml of biotin-free lysate) on lysates spiked with different tachyzoite concentrations (10^5, 10^2 and 10 tachyzoites/12ml of lysate). The obtained qPCR results for both probe concentrations showed little or no difference (data not shown). Hence, the concentration of 1 pmol per ml of biotin-free lysate was selected for further experiments.

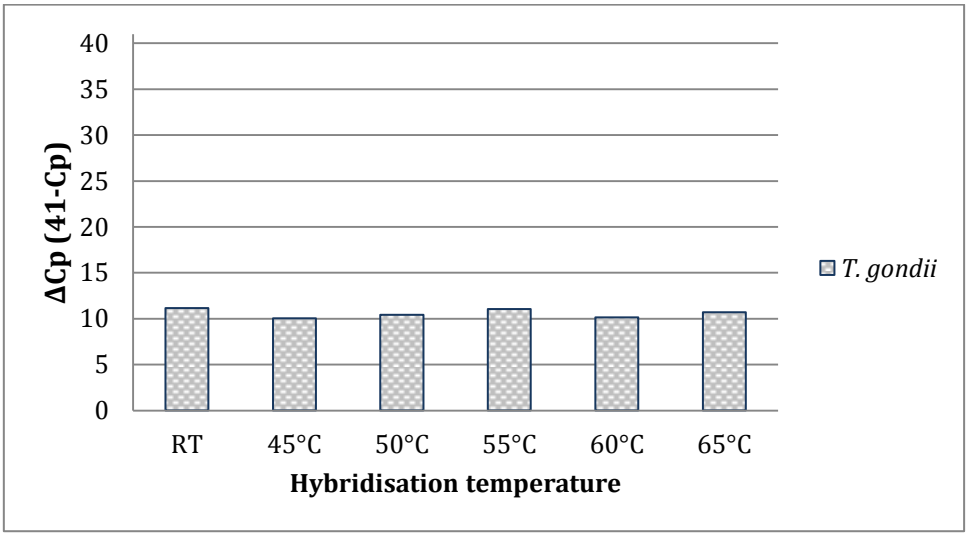
Once the optimal concentration of all the capture probes was known all the capture probes were combined into a single 1000 times concentrated Capture Mix (CM). Aliquots were stored at -20°C. No difference was seen in the qPCR results using 10µl of CM per 10ml of meat lysate or by adding each probe separately (supplementary Table S4.2). The use of the CM was selected for the further optimization and validation of the method.

Supplementary Table S4.2. Magnetic capture real time PCR (MC-qPCR): combination of all the capture probes in one mix

		Capture Mix	Separated captures
Toxoplasma gondii	**rep 1**	23.33 Cp	23.33 Cp
	rep2	23.34 Cp	23.34 Cp
	Average	23.33 Cp	23.33 Cp
r18S	**rep 1**	18.27 Cp	18.55 Cp
	rep2	18.30 Cp	18.61 Cp
	Average	18.28 Cp	18.58 Cp

rep 1, replicate 1; rep 2, replicate 2; Cp, crossing point.

Finally, as the hybridization of the capture probes to the target DNA is a crucial step, different hybridization temperatures (45°C, 50°C, 55°C, 60°C and 65°C) after the initial denaturation at 95°C were tested. As no substantial difference was observed within the tested temperature range, we also tested the hybridisation process by incubating at RT for 15 min. Here, equal results were obtained (supplementary Fig. S4.1) showing that the hybridization is completed during the cooling down of the samples. We therefore selected 15 min. of hybridization time at RT in the final protocol, making the use of a hybridisation oven redundant.



Supplementary Fig. S4.1. Optimization of the Magnetic Capture real time PCR and effects of different temperatures of hybridization on the probes with the target DNA.

4.3.1.3 Streptavidin Coated Paramagnetic Beads

Although expensive, the streptavidin coated paramagnetic beads are an essential reagent for the concentration and purification of the target DNA. In the aim of reducing the costs of the MC-qPCR

method, we tested different concentrations of streptavidin paramagnetic beads (80, 40, 20, 10 and 5 µg/ml of biotin-free lysate) to determine the lowest concentration that could be used without diminishing the sensitivity. Similar results were obtained for all tested concentrations (supplementary Table S4.3). The addition of more paramagnetic beads does thus not correspond with a higher recovery of target DNA. Therefore, 20µg (20 µl) of streptavidin magnetic beads per ml of biotin-free extract was selected as the amount to be used in all further experiments.

Supplementary Table S4.3. Magnetic capture real time PCR (MC-qPCR): titration of Streptavidin coated paramagnetic beads (M. beads)

		Concentration of M. beads (µg/ml)				
		40	**20**	**10**	**5**	**0**
Toxoplasma gondii	**rep 1**	34.28 Cp	34.1 Cp	35.7 Cp	35.49 Cp	-
	rep 2	34.25 Cp	33.67 Cp	34.33 Cp	34.51 Cp	-
	Average	34.27 Cp	33.89 Cp	35.02 Cp	35.00 Cp	-

rep 1, replicate 1; rep 2, replicate 2

4.3.1.4 UV elution

To prove the UVb elution is more efficient and convenient compared to elution based on thermal denaturation, the methods were compared. For this, streptavidin coated paramagnetic beads that had been used to capture DNA from the same lysate were subjected to thermal elution (incubation in boiling water for 10min) or to UVb irradiation and the respective qPCR results were compared. When performing thermic release of the targets, inhibition was observed in the subsequent qPCR decreasing the sensitivity of the method. On the other hand, using UVb elution, no inhibition was observed and thus higher sensitivity was obtained (Table 4.2).

Table 4.2. Comparison of the two elution methods used with magnetic capture real time PCR (MC-qPCR): UVb-mediated and thermic elution.

Method	**UVb**						**Thermic[b]**					
Sample	Negative			Positive			Negative			Positive		
Eluate dil[a]	1:1	1:10	1:100	1:1	1:10	1:100	1:1	1:10	1:100	1:1	1:10	1:100
T. gondii	N/A	np	np	33.41	np	np	N/A[c]	N/A	N/A	Inh[c]	Inh[c]	39.37
r18S	20.31	np	np	20.22	np	np	Inh[c]	23.43	30.48	30.07[c]	23.87	27.48

N/A, target not amplified; np, not performed.

[a] Dilution (dil) at which the obtained eluate was tested with qPCR.

[b] Incubation at 100°C for 10 min.

[c] Those qPCRs showed inhibition either due to the absence of an amplification signal for the non-competitive internal amplification control (NCIAC), or due to a poor observed PCR efficiency.

4.3.1.4.1 UVb Exposure Time

To determine the optimal time of UVb exposure, the following experimental setup was designed: a positive meat sample (spiked with high numbers of tissue cysts) was processed following the MC-qPCR procedure till the washing step. After the last washing, the beads were resuspended in 100µl elution buffer and exposed to UVb for one minute. The tube was then placed on the magnetic rack and the eluate recovered. This elution step was repeated 5 more times, so six consecutive eluate fractions were obtained and each was tested separately in qPCR. The experiment was performed twice in order to estimate variability.

In order to estimate the contribution that every additional minute of UVb exposure would have on the final Cp result (Cp_{final}), the following formula was applied:

$$\boldsymbol{Cp_{final} = Cp_{ref} - \sum_{i}^{n} \log_2 \left[Au_1 \left(1 + \frac{1}{2^{\Delta Cp_i}} \right) \right]}$$

With:

Cp_{final} : the final Cp value obtained after n minutes of UVb exposure.

Cp_{ref}: the Cp obtained after the first minute of UVb exposure.

i : represents each minute of UVb exposure, of a total of n minutes.

$\Delta Cp_i = Cp_i - Cp_{ref}$: the Cp obtained after i minutes of exposure minus Cp_{ref}.

Au1: represents an arbitrary amount of DNA released from the beads. As we cannot know at this point how much DNA is actually captured, nor the amount of DNA eluated, we attributed to the latter an arbitrary value. In the hypothesis most of the DNA is released after the first minute of UVb exposure, the arbitrary value at that time point was set to be 1.

As hypothesized, the results show that most of the DNA is eluted after the first minute of UVb exposure, as the eluate at this time point has the lowest Cp values for both r18S and *T. gondii* (Table 4.3). Considering the contributions of each additional minute of UVb elution, after 5 minutes there is no more significant contribution to the final Cp values. Five minutes of UVb exposure was thus selected in the final protocol.

Table 4.3. Results and data analysis of the optimization of the elution time using magnetic capture real time PCR (MC-qPCR)

	S1					S2				
Time (i)	**Cp**	**ΔCp_i (Cp_i-Cp_{ref})**	**AU**	**Estimated cont. to Cp**	**Cp_{final}**	**Cp**	**ΔCp_i (Cp_i-Cp_{ref})**	**AU**	**Estimated cont. to Cp**	**Cp_{final}**
1	33.81	Cp_{ref}	1	33.81	33.81	34.46	Cp_{ref}	1	34.46	34.46
2	34.37	0.56	1.68	-0.74	33.06	34.08	-0.38	2.3	-1.2	33.26
3	35.82	2.01	1.25	-0.32	32.74	35.83	1.37	1.4	-0.47	32.79
4	37.08	3.27	1.1	-0.14	32.60	36.49	2.03	1.3	-0.316	32.47
5	37.41	3.6	1.08	-0.11	32.49	36.89	2.43	1.2	-0.25	32.22
6	38.04	4.23	1.05	-0.07	32.41	38.3	3.84	1.1	-0.098	32.12

Cp_{final}, the final Cp value obtained after n min of UV exposure; I, each min of UV exposure for a total of n min; Cp_{ref}, the Cp obtained after 1 min of UV exposure; $\Delta Cp_i = Cp_i - Cp_{ref}$, the Cp obtained after i min of exposure minus Cp_{ref}; Au, an arbitrary amount of DNA released from the beads, here set to be equal to 1 for the reference (the first minute of exposure).

4.3.2 ISO 17025 validation

To test the robustness of the MC-qPCR method and to be able to apply it for diagnostic purposes, an extensive ISO 17025 validation was performed. An overview of the results obtained during the validation is shown in Table 4.4.

Table 4.4. Overview of the results obtained by the magnetic capture real time PCR (MC-qPCR) during the ISO 17025 validation.

Parameter	**Results**						**Conclusion**
Repeatability	1.13% CV						**OK**
Inter- and Intra-reproducibility	**Intra-rep** 4.75% CV	**2° line** OK	**3° line** OK				**OK**
Cross-contamination	No cross contamination measured when alternating positives and negatives						**OK**
Selectivity	BLAST						**OK**
LOD	**Cyst** 1 /100g of meat		**Tachyzoites** 65.4 /100g of meat				**OK**
Cross-reactivity	***T. gondii*** Pos	***N. caninum*** Neg	***S. auch.*** Neg	***S. meis.*** Neg	***S. cruzi*** Neg	**NMC** Neg	**OK**
Specificity	100 %						**OK**
Sensitivity	94.12 %						**OK**
Matrix effect	**Heart** 33.64[a]	**Lungs** 34.59 [a]	**Liver** 33.28 [a]	**Brain** 34.89 [a]	**Muscle** 33.40 [a]		**OK**
	Chicken 33.22 [a]	**Lamb** 32.60 [a]	**Horse** 33.11 [a]	**Pork** 33.34 [a]	**Beef** 32.62 [a]		

CV, coefficient of variation; OK, satisfactory results; LOD, limit of detection; Pos, positive; Neg, negative; *T. gondii*, *Toxoplasma gondii*; *S. Auch*, *Sarcocystis aucheniae*; *S. meis*, *Sarcocystis meischeriana*; *S. cruzi*, *Sarcocystis cruzi*; NMC, negative magnetic capture.
[a]Crossing point

4.3.2.1 Matrix effect

As the performance of a DNA extraction method can vary greatly depending on the matrix, the effect of the latter on the MC-qPCR method was assessed. Hundred grams of various types of tissues (brain, muscle, heart, lung, liver) were obtained from seronegative pigs, spiked with 5000 tachyzoites and tested with the MC-qPCR method. Also, meat samples from different species (pork loin, chicken breast, leg of lamb, beef and horse steak) were tested at the same concentration of tachyzoites and no matrix effect was observed (Table 4.4).

4.3.2.2 Repeatability and Cross-contamination

To study the repeatability of the method, 5 samples (100g of meat) were spiked with the same number of tachyzoites (10^5 tachyzoites) and processed in parallel and in alternation order with negative control samples (NMC) to investigate the possibility for cross-contamination.

The results show that the method is highly reproducible with a Coefficient of Variation (CV) of 1.13% (Table 4.4). A CV lower than 3% is accepted for a qPCR detection method according to the consulted literature (Estes and Sevall, 2003; Moens et al., 2009). Also, no false positive results were obtained demonstrating that the method as performed (with all pre-emptive measures, from the sample reception till the qPCR result) was indeed free of cross contamination.

4.3.2.3 Intra- and Inter Reproducibility

Different persons working in the same lab should obtain the same the results, anytime. To test this, two positive samples (spiked with 10^5 tachyzoites) and two negative samples were processed in parallel at two different time points by two different operators. Both operators obtained comparable results with a CV < 5% (a CV < 10% is the acceptance inter-assay criteria according to the consulted literature) (Estes and Sevall, 2003; Moens et al., 2009).

To test inter-reproducibility, a second line control was performed: 2 positive and 2 negative samples were prepared and tested blindly by 2 different operators, both operators reported the same results. Moreover, a third line control has been organized with the RIVM (Centre for Infectious Disease Control, National Institute for Public Health and the Environment, 16 Bilthoven, the Netherlands) and the results obtained by WIV-ISP had full agreement with the ones obtained by RIVM (Table 4.4).

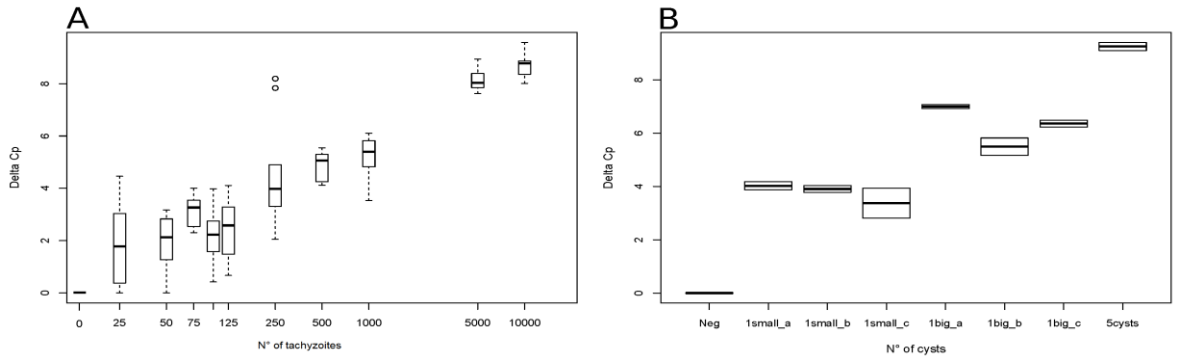
4.3.2.4 Cross-reactivity

The selectivity and specificity of the method was assessed by analysing the biotinylated capture probes, the primers and the PCR detection probes using the NCBI BLAST tool in order to find correspondences with other closely related coccidian species. The blast results showed that the used oligos should be sufficiently selective for the detection of *T. gondii*, avoiding cross reaction with other close related species (Data not shown).

To confirm the BLAST results and to test for possible cross-reactivity with closely related coccidian parasites, samples were spiked with DNA from *Neospora caninum, Sarcocystis cruzi, Sarcocystis aucheniae and Sarcocystis meischeriana*. *Neospora caninum* DNA was kindly provided by Dr. Frank Katzer from Moredun Research Institute, Edinburgh, UK; and the *Sarcocystis* spp. DNA from Dr. Gastón Moré from the National University of La Plata, Argentina. These samples were processed in parallel with a positive control of extraction for *T. gondii* (PMC) and a negative control of extraction (NMC). Only the *T. gondii* spiked sample showed an amplification in the qPCR, confirming the absence of cross-reaction with the tested closely related coccidian (Table 4.4).

4.3.2.5 Limit of Detection

To precisely determine the limit of detection (LOD) in terms of number of parasites present in 100g meat, samples spiked with different concentrations of tachyzoites (10 000, 5000, 1000, 500, 250, 125, 100, 75, 50 and 25) were tested. The LOD was determined using PROBIT analysis on the obtained results. The lowest concentrations of tachyzoites at which 99% of the positive samples will test correctly was estimated to be 65.4 per 100g of meat (95% CI: 31.3-94.4) (Figure 4.1).



C

LOD upper quantile	Lower limit confidence interval	Estimated LOD	Upper limit confidence interval
0.95	26.7	48.5	67.5
0.96	27.6	51.4	71.6
0.97	28.6	54.9	77.3
0.98	29.7	59.2	84.4
0.99	31.3	65.4	94.4

Fig. 4.1. Box and whisker plots for Delta crossing point (Cp) values for different concentrations of *Toxoplasma gondii* tachyzoites (A) and tissue cysts (B). Upper quantiles for limit of detection (LODs) are

given with their estimated values and corresponding confidence intervals in (C) for the different tachyzoites concentrations.

The LOD results show that, as in meat *T. gondii* is present in the form of bradyzoites encapsulated by hundreds in resistant tissues cysts, as little as one cyst per 100g of sample should always be detected. In order to confirm this, negative samples were spiked either with 1 small cyst (3 replicates), 1 big cyst (3 replicates) or 5 cysts (1 sample). All the samples were tested positive, confirming the LOD of the method as 1 cyst per 100g of meat (Figure 4.1 and Table 4.4).

4.3.2.6 Specificity and sensitivity

The specificity and sensitivity was assessed on tissues collected from pigs experimentally infected with either the *T. gondii* IPB-LR or *T. gondii* IPB-G strains. The collected tissues (heart (Ha), M. gastrocnemius (Ga), M. psoas major (PM), M. longissimus dorsi (LD), diaphragm (DI), M. intercostales (IC) and brain (BR)), were processed, homogenized and divided in two (except for the brain tissue): one part was then analysed with mouse bio-assay (considered as the reference method) and the other by MC-qPCR.

The results obtained in both tests were then compared to calculate the specificity and sensitivity. The following criteria were applied in the analysis: 1. Samples from uninfected control pigs should always be negative. Here, any positive result in either method was thus considered as false positive. 2. Samples from experimentally inoculated, seroconverted pigs can test either positive or negative. A sample was thus considered as true negative when testing negative in both methods, bioassay and MC-qPCR, whereas a sample was considered as a true a positive when it tested positive in either of the methods. Consequently, when for a given sample from an experimentally infected and seroconverted pig the MC-qPCR result was positive, while the bioassay result was negative, the latter was considered as a false negative, whereas inversely the MC-qPCR result was negative, while the bioassay result was positive, the former was considered as a false negative.

The obtained results are summarized in Tables 4.4 and 4.5. Data analysis of the results yielded a 100% specificity for both methods (MC-qPCR and Bioassay) and a sensitivity of (94.12% with a 95% CI of 80.32 - 99.28) for MC-qPCR, higher compared to bioassay (86.49% with a 95% CI of 71.23 - 95.46). Moreover, the Cohen's Kappa ratio for both methods is 0.74 which implies substantial agreement between the methods according to (Landis, 1977).

Table 4.5. Overview of mouse bioassay and the magnetic capture real time PCR (MC-qPCR) results obtained for muscle (100 g) and brain (50 g) samples from experimentally infected pigs.

			PM	Ga	D	Ic	LD	Ha	Br
***Toxoplasma gondii* IPB-LR strain**	*n*=3	Bioassay	3/3[a]	2/3	2/3	2/3	3/3	3/3	np
		MC-qPCR	3/3	2/3	2/3	3/3	3/3	3/3	3/3
		g/test	±100 g	±100 g	±100 g	±100 g	±100 g	±100 g	±50 g
***Toxoplasma gondii* IPB-G strain**	*n*=4	Bioassay	4/4	1/4	3/4	3/4	3/4	4/4	np
		MC-qPCR	3/4	2/4	3/4	4/4	4/4	4/4	4/4
		g/test	±100 g	±100 g	±100 g	±100 g	±100 g	±100 g	±50 g
Detected Bioassay			7/7	3/7	5/7	5/7	6/7	7/7	np
Detected MC-qPCR			6/7	4/7	5/7	7/7	7/7	7/7	7/7
Negative	*n*=2	Bioassay	0/2	0/2	0/2	0/2	0/2	0/2	np
		MC-qPCR	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		g/test	±100 g	±100 g	±100 g	±100 g	±100 g	±100 g	±50 g
Detected both methods			0/4	0/4	0/4	0/4	0/4	0/4	0/4

[a] number of positive samples in function of the total number of samples.

PM, psoas major; Ga, gastrocnemius; D, diaphragm; Ic, intercostal; LD, longissimus dorsi; Ha, heart; Br, brain; np, not performed; *n*, number of pigs.

4.4 Discussion

Although the introduction of PCR in the detection of *T. gondii* infection represented a step forward, it has been often proven insufficiently sensitive to detect *T. gondii* in meat (da Silva and Langoni, 2001; Garcia et al., 2006). This lack in sensitivity is mainly due to a combination of the used DNA extraction methods (often commercially available kits) that are designed for small amounts of samples, with a (sometimes) very low *T. gondii* tissue cysts density in meat. It is thus mostly the preceding extraction that handicaps the otherwise very sensitive PCR methods. Apart from the possibility to isolate the infecting strain, this is the main reason why still today mouse bioassay is considered the most sensitive method after the cat bioassay only performed in short number of labs in the world (Dubey et al., 1998; Djokic et al., 2016).

However, as the parasites must be viable to be detected by bioassay, bioassay can give false negative results when incorrectly conserved or stale samples are tested. Today, bioassay is more and more an ethically questionable method and implies a long experimental practice, the need of animal facilities conform to regulations and the huge associated costs, making it uneasy to implement the method for screening (food safety) purposes. There is thus a need for the development of an at least even sensitive and more easily implementable alternative.

The previously published MC-qPCR method by Opsteegh et al, 2010, by hugely scaling up the

amount of sample from which DNA can selectively been extracted and by concentrating it into a volume testable in PCR, already represented a major step towards a sensitive and quantitative alternative to detect *T. gondii* in meat. For this, 100g of meat is completely lysed in order to release all the DNA. Then, by using selective biotinylated probes and streptavidin coated paramagnetic beads, the *T. gondii* DNA eventually present is concentrated into a very small volume and then tested in real-time PCR (Opsteegh et al., 2010).

Still, as has been proven in the latest EFSA report (Opsteegh et al., 2016), some discordances continue to exist between the (Opsteegh et al., 2010) method and the mouse bioassay and even with a qPCR on trypsin digested tissues. Major differences in sensitivity were for example found in the described experimental studies in pigs: whereas 25.6 and 43.4% of the samples tested positive by mouse bioassay and qPCR respectively, only 16.9% were found positive by MC-qPCR. This can certainly in part be explained by the fact that the bioassay and the qPCR were performed on trypsin-digested heart samples while frozen diaphragms were used for the MC-qPCR (EFSA, 2016). Nevertheless, some technical improvements to make the MC-qPCR more sensitive and robust were thus necessary.

In this paper we present a modified and improved MC-qPCR for detecting *T. gondii* DNA in meat: we added a co-capture of the host's cellular r18S as NCIAC, we made the DNA elution more efficient (UVb mediated release of the DNA from the paramagnetic beads), we reduced incubation times where possible, and we reduced the reagent costs by performing an extensive market screening and testing (e.g. the use of different paramagnetic beads, a very expensive reagent). Then, after several rounds of optimization steps, the whole process was fully validated according to ISO 17025 standards permitting its use as an accredited method.

The robustness of the modified MC-qPCR method is clearly reflected in the results of the various parameters assessed during the validation process: the repeatability, intra- and inter reproducibility, cross-contamination, selectivity, the absence of cross reactivity and the broad range of matrices that can be tested. Furthermore, with a 99% LOD of 65.4 tachyzoites per 100g meat, a better detection limit was achieved compared to the original method (95% LOD of 227 tachyzoites per g sample). We also confirmed the detection limit of 1 tissue cyst per 100g sample by performing spiking experiments using purified tissue cysts.

The specificity and sensitivity were investigated by comparing our modified MC-qPCR with the

mouse bioassay on tissues of experimentally infected pigs. Here, to obtain the best homogeneity, the whole organ or muscle tested from each animal was first cut into small pieces, mingled and then divided for each test. The modified MC-qPCR was able to detect a higher number of infected tissues (94.12%) compared to the bioassay (86.49%). These results are by far better compared to previously published results where the bioassay using mice remained the most sensitive method (Gamble et al., 2005; Garcia et al., 2006; Gardner et al., 2010). Also, we obtained a calculated Cohen's Kappa coefficient between the modified MC-qPCR and the bioassay of 0.74 ($P< 0.005$) indicating substantial agreement while in the recent EFSA report a Cohen's kappa coefficient of only 0.36 was obtained comparing different matrixes (heart for mouse bioassay and diaphragm for MC-qPCR (Landis and Koch, 1977; Opsteegh et al., 2016).

Although the combination *T. gondii*/pork meat has been ranked as second of pathogen-food combinations in function of QALYs and the costs of illness (Batz et al., 2012), toxoplasmosis in humans has been associated with the consumption of other livestock species in general. Moreover, a quantitative risk assessment performed in the Netherlands estimates that 68 % of the meat-borne *T. gondii* infections could actually be due to the consumption of beef and only 11% due to pork meat (Opsteegh et al., 2011). Furthermore, the predilection tissues for the detection of the parasite can vary from one livestock species to another: while heart has been described as the predilection tissue for sheep and pork, a recent publication suggests liver as a better tissue to demonstrate the presence of *T. gondii* in cattle (Opsteegh et al., 2016). In the current study, we have tested the most common livestock species and various matrixes confirming the wide-ranging applicability of the method.

Our results demonstrate the potential of the modified MC-qPCR as a full alternative for the mouse bioassay for archetypal *T. gondii* strains (Type I, Type II, Type III), with the additional advantage of being quantitative, thus permitting the estimation of the parasitic load in various types of tissues. When the infecting *T. gondii* strain needs to be isolated for further studies, the viability of detected *T. gondii* needs to be demonstrated to better estimate the risk for the consumers. Also, the bioassay might still be necessary when working in areas such as, South America where atypical *T. gondii* strains prevail. Unfortunately, atypical strains such as MAS, TgCatBr9, VAND, RUB, TgCatBr5, present in South American countries, could not be experimentally evaluated during this study and special attention should be paid when working with samples originating from this areas since the high levels of polymorphism at the target locus could render the method less sensitive.

Here, in the spirit of reducing the number of experimental animals needed, a first screening with the

MC-qPCR could be performed and only positive samples would then be tested in bioassay.

Based on the results of the validation and the comparison with the mouse bioassay, we can state that our enhancements to the MC-qPCR significantly improved this method, making it more robust, reliable and sensitive at a lesser cost.

Acknowledgements

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Chapter V

Toxoplasma gondii infection kinetics of experimentally infected pigs

Adapted from the accepted publication: Pork as a source of transmission of *Toxoplasma gondii* to humans: a parasite burden study in pig tissues after infection with different strains of *Toxoplasma gondii* in function of time and different parasite stages

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Abstract

Toxoplasma gondii is an ubiquitous apicomplexan parasite which can infect any warm-blooded animal including humans. Humans and carnivores/omnivores can also get infected by consumption of raw or undercooked meat containing cysts. This route of transmission is considered to account for at least 30% of human toxoplasmosis cases. To better assess the role of pork as a source of infection for humans, the parasite burden resulting from experimental infection with different parasite stages and different strains of *T. gondii* during the acute and chronic phases was studied. The parasite burden in different tissues was measured with a ISO 17025 validated Magnetic Capture-qPCR. A high burden of infection was found in heart and lungs during the acute phase of infection and heart and brain were identified as the most parasitized tissues during the chronic phase of infection, independently of the parasite stage and the strain used. Remarkably, a higher parasite burden was measured in different tissues following infection with oocysts of a type II strain when compared with a tissue cyst infection with either a type II or a type I/II strain. However, these results can have been affected by the use of different strains and euthanasia time points. The parasite burden resulting from a tissue cyst infection was not significantly different between the two strains.

5.1 Introduction

Toxoplasma gondii is an obligate intracellular cyst-forming coccidian parasite. Being worldwide prevalent and zoonotic, *T. gondii* is considered one of the most successful parasites (Halonen and Weiss, 2013). It has a complex life cycle: it can infect virtually any warm-blooded animal (intermediate host), while the sexual replication only takes place in the gastrointestinal tract of the definitive hosts, which are domestic and wild Felidae (Tenter et al., 2000).

The life cycle in the intermediate host, including man, is characterised by an acute phase involving fast asexual intracellular replication of tachyzoites in almost all tissues, followed by a chronic phase involving the development of tissue cysts containing bradyzoites, mainly in the central nervous system and in skeletal muscles, which may persist lifelong (Dubey, 2010). Different infection routes have been demonstrated: 1. Consumption of meat from a chronically infected animal; 2. Ingestion of sporulated oocysts (resulting from the sexual replication in felids) via contaminated water, soil or vegetables; 3. Vertical transmission through the placenta (Opsteegh, 2016).

The consumption of raw or undercooked *T. gondii* infected meat is considered a main route of transmission for humans (Cook et al., 2000): in developed countries 50% of the infections are

estimated to be meatborne (Scallan et al., 2011) and pork is considered to account for 41 % of foodborne human toxoplasmosis cases in the USA (Batz et al., 2012).

The presence of anti-toxoplasma antibodies and direct detection of the parasite in different tissues have been widely used in different animal species for the demonstration of a *T. gondii* infection (Tenter et al., 2000; Dubey, 2010). The development of a Magnetic Capture qPCR (MC-qPCR) enables reliable parasite quantification in contrast to the qualitative results obtained with bioassays and classic PCR. As such, the MC-qPCR allows the study of the quantitative distribution of the parasite through the carcasses in different animal species (Opsteegh et al., 2010; Juránková et al., 2013; Hosein et al., 2016; Aroussi et al., 2015), and the assessment of the risk of infection for the consumer of the different animal tissues. In pigs, the parasite burden has been studied following experimental infection with either oocysts (Opsteegh et al., 2010; Juránková et al., 2013) or tissue cysts (Verhelst et al., 2011; Jennes et al., 2017), but the potential effect of different parasite stages on the parasite burden is not known. In the definitive host, ingestion of oocysts has been demonstrated to be less efficient than ingestion of tissue cysts to induce infection. Hence, the infectious stage inducing infection in pigs may have an impact on the parasite burden of the tissues. In addition, the parasitic load has been shown to be strain dependant with a more pronounced clearance of the parasite in some tissues when using a hybrid Type I/II strain (*T. gondii* ISP-Gangji) than using a classical type II strain (Verhelst et al., 2011; Jennes et al., 2017).

The aim of this study is to use the recently upgraded and ISO 17025 validated MC-qPCR (Gisbert Algaba et al, 2017) to study the potential effect of different parasite stages (oocysts and tissue cysts) and different strains (Type II and Type I/II strains) on the parasite burden in pigs during acute and chronic infections. By comparing the parasitic loads of different tissues under different conditions, the role of pork as one of the main sources of infection for humans can be better assessed.

5.2. Materials and Methods

5.2.1 *T. gondii strains*

In order to study the most prevalent *T. gondii* genotype in Europe (Type II), the following strains were used: *T. gondii* IPB-LR (tissue cysts) (type II, low virulence in mice), *T. gondii* IPB-Gangji (tissue cysts) (Type II/I, a rare hybrid strain, virulent in mice) and *T. gondii Tg-SweF2* (oocysts) (type II, low virulence in mice). The strains were genotyped by the Center of Biological Resources (Limoges, France) as described by Ajzenberg, et al. (2010).

5.2.1.1 Preparation of Tissue Cysts

Swiss white outbred mice (Janvier Labs, Rennes, France) were intraperitoneally inoculated with the *T. gondii* IPB-LR (type II) or IPB-G (Type II /I) strains. After 6 weeks of incubation, the mice were euthanized and their brains were collected and homogenized in PBS supplemented with penicillin and streptomycin using a potter homogenator. The concentration of tissue cysts was determined by counting three times a volume of 8µl with a phase-contrast microscope.

5.2.1.2 Isolation, purification and preparation of oocysts

In order to perform an experimental infection with oocysts, feces from a cat naturally infected with the *T. gondii Tg-SweF2 strain* were obtained from the National Veterinary Institute of Sweden (SVA, Uppsala, Sweden). The oocysts were first isolated with sucrose flotation followed by a Caesium Chlorine gradient as described by Staggs et al. (2009). Briefly, the feces were homogenized with MilliQ water and 0.2% Tween®20, the suspension filtered through gauze and centrifuged. Subsequently, the pellet was resuspended in a sucrose solution (d:1.15 g/ml) and centrifuged at 800 g for 10 min. The supernatant containing the oocysts was carefully transferred to a new 50 ml Polypropylene tube and the sucrose washed away. Subsequently, the oocysts were aerated in 2% H_2SO_4 and at 22°C for 7 days to allow sporulation. Once sporulated, a Caesium Chlorine gradient was applied and the oocyst suspension was stored in 2% H_2SO_4 at 4°C till further use.

The purified oocyst suspension was quantified using a Bürker counting chamber and a phase contrast microscope and diluted accordingly to obtain a final concentration of 10^5 sporulated oocysts per 5 ml in PBS, supplemented with penicillin/streptomycin.

5.2.2 *Experimental Infections in Pigs*

5.2.2.1 Acute phase

To study parasite distribution and load in the acute phase, 36 three-week old *T. gondii* seronegative piglets (confirmed by the modified agglutination test (MAT, ToxoScreen DA, Biomérieux, Capronne, France) and an in house immunofluorescence test (IFT, based on Toxo-Spot IF, Biomérieux, Capronne, France)) (Verhelst et al., 2015)) were divided into three groups according to the following experimental setup: group A1, 15 animals orally infected with 6000 tissue cysts of the *T. gondii* IPB-LR strain; group A2, 15 animals orally infected with 6000 tissue cysts of the *T. gondii* IPB-G strain; group A3, six negative control animals.

Three animals from each experimental group (groups 1 and 2) were killed after 2, 4, 8, 14 and 28

days post infection. The piglets were serologically monitored by IFA as described by (Verhelst et al.,2015) and the heart (Ha) and lungs (Lu) were collected and tested with MC-qPCR.

5.2.2.2 Chronic phase

To study the chronic phase, 13 three-week-old *T. gondii* seronegative piglets (confirmed by two different serological methods: MAT and IFT (Verhelst et al., 2015)) were divided into four groups according to the following experimental setup: group C1, two negative control animals; group C2, three animals orally infected with 6000 tissue cysts of the *T. gondii* IPB-LR strain; group C3, four animals orally infected with 6000 tissue cysts of the *T. gondii* IPB-G strain; group C4, three animals orally infected with 10^5 oocysts of the *T. gondii* Tg-SweF2 strain (Table 5.1). Hundred thousand oocysts (10^5 * 8 sporozoites) and 6000 tissue cysts (6000 * >100 bradyzoites) were inoculated in order to infect pigs with a similar number of parasites.

Table 5.1: Overview of the different experimental animal groups

phase	Group	N° of animals	Challenge	Infectious form	Strain type
Acute phase	A1	15	6000 tissue cysts	Bradyzoites	LR (Type II)
	A2	15	6000 tissue cysts	Bradyzoites	Gangji (Type II/I)
	A3	6	na	na	Control group
Chronic phase	C1	2	na	na	Control group
	C2	3	6000 tissue cysts	Bradyzoites	LR (Type II)
	C3	4	6000 tissue cysts	Bradyzoites	Gangji (Type II/I)
	C4	3	10^5 oocysts	Sporozoites	Swe2 (TypeII)

na: not applicable.

The piglets were serologically monitored weekly from day 0 till euthanasia to confirm an established infection in the positive animals and to monitor the absence of infection in group C1. The pigs were euthanized 90 days post infection (dpi) in group C4 and between 130 and 182 dpi in groups C2 and C3. The following tissues were collected: heart (Ha), lungs (Lu), m. gastrocnemius (Ga), m. psoas major (PM), m. longissimus dorsi (LD), diaphragm (DI), m. intercostales (IC) and brain (BR).

The collected tissue samples (except the brain tissue) were first cleaned by removing fats and connective tissues and then cut into small pieces of $1cm^3$.

5.2.4 MC-qPCR

In order to determine the parasitic load, the MC-qPCR was performed as described by (Gisbert Algaba et al., 2017). In brief, the meat samples were homogenized in the presence of lysis buffer (100 mM Tris HCL, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 40mg/l proteinase k (30 mAnson-U/mg; Amresco, Ohio, USA), pH = 8.0 ± 0.2) with a pedal homogenator (Labconsult, Brussels, Belgium) and lysed overnight at 55°C. Fats and cell debris were then removed by centrifugation and the free biotin possibly present in the crude extract was removed by adding streptavidin coated agarose beads (binding capacity >330 nmol/ml; solulink, San Diego, USA).

The specific biotin labelled probes against the *T. gondii 529bp RE* (accession number: AF146527) and cellular r18S DNA were added to the biotin-free lysates and the samples were first denatured at 95 °C followed by an incubation at room temperature in order to hybridize the probes with the complementary target sequences. After hybridization, the biotin labelled probes bound to the target DNA were captured using streptavidin magnetic beads (binding capacity > 2.5 nmol/mg (Solulink, San Diego, USA) and after washing and re-suspension in elution buffer, the target DNA was released from the beads by exposure to UV light.

Finally, the qPCR was performed on the final DNA extract to detect the presence of *T. gondii* DNA and the results analysed using the BioRad CFX manager software to obtain the crossing point (Cp) values.

5.2.5 Statistical Analysis

All the samples with an exponential-amplification curve crossing the threshold (Cp) were considered positive for *T. gondii*, samples with no amplification curve for the *T. gondii* target but amplification of the NCIAC (Not Competitive Internal Amplification Control) were considered negative. As described in Gisbert Algaba et al. (2017), the limit of detection of the method is 65.4 parasites per 100 g of tissue. For each round of samples a positive control with a known number of parasites (calibrator) was performed to correct for possible deviations due to manipulation errors. The number of parasites (n° p) was calculated according to the following formula:

$$log_{10}(n° \, p) = \frac{Cp_{value} - 44.75}{-3.0788}$$

The formula resulted from a standard curve established with known concentrations of parasites ranging from 100 to 10^5 spiked in 100g of tissue.

A linear model was fit to look for the differences between the groups to be compared after a log10-

transformation of the parasitic load. Restricted maximum-likehood estimates were calculated taking into account the non-detects, which were modelled as '<log(LOQ)'. Model estimates and their variance-covariance matrix were used to set up contrasts to compare infection types per tissue, tissues per parasite stage types and days. P-values for each contrast were corrected according to Sidak in order to set the global type I error rate at 0.05. The normal distribution of the residuals was assessed by means of a normal quantile plot. All data was analysed using S-Plus 8 for Linux (doi:10.17632/bk47m9hksm.2).

5.3. Results

5.3.1 Acute phase

All the animals in groups A1 and A2 seroconverted and showed some mild clinical signs such as, fever, apathy and anorexia during the first week after infection.

Regarding the comparison between the two strains used (LR and Ganji) at the level of parasitic loads during the acute phase of infection, statistical analyses could not be performed due the number of negative values. The results of the acute phase are presented in Figures 5.1 and 5.2, for the lungs and hearts, respectively.

5.3.1.1 Lungs

During the acute phase of infection, at day 2 post-infection (dpi) *T. gondii* was already present in the lungs of 2 out of 3 animals of both infected groups (A1 and A2). At 8 dpi the concentration of parasites reached a maximum and the parasite was found in all the tested animals for both groups (A1 and A2) while the parasite burden decreased slowly in the following days (14 and 28) (Figure 5.1).

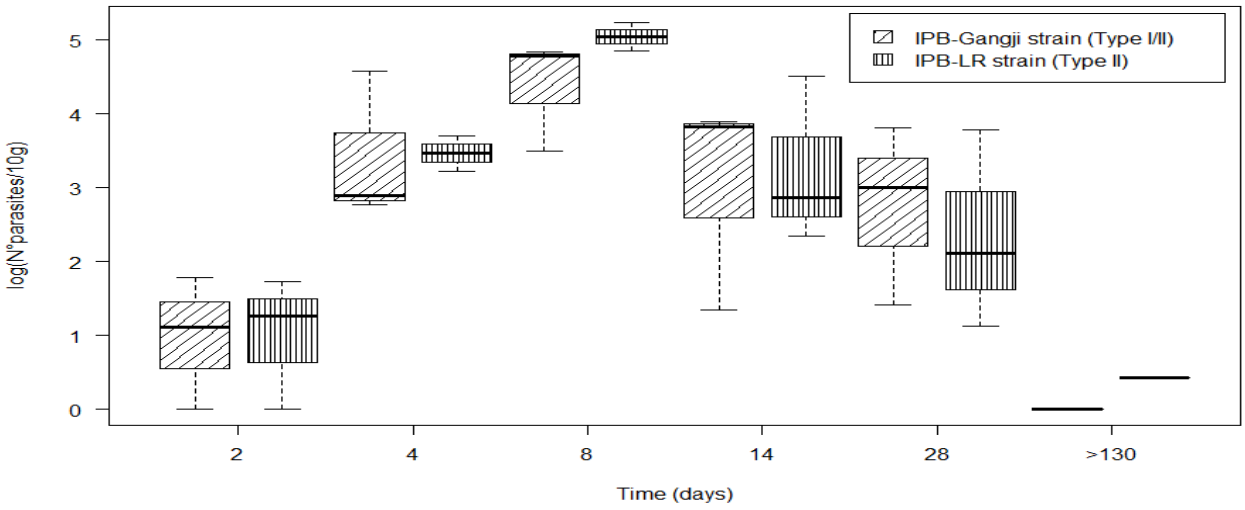

Fig. 5.1. Box and whisker plots for log (N° parasites/10g) in function of days after infection for lungs. A vertical pattern of the plots is used for the results of animals infected with the IPB-LR *T. gondii* strain (Type II) and a diagonal pattern of the plots is used for the results of animals infected with the IPB-Gangji *T. gondii* strain (Type I/II).

5.3.1.2 Heart

In the heart that has been previously described as the predilection tissue of the parasite in pigs (Opsteegh et al., 2010), the parasite was not found in any of the animals of the experimental groups (A1 and A2) at 2 dpi and only in 1 animal of the A1 group (A1:1/3) and 2 of the A2 group (A2: 2/3) at 4 dpi. In contrast to what was observed in the lungs, in hearts the median parasite burden remained stable after 8 dpi (P> 0.1) (Figure 5.2).

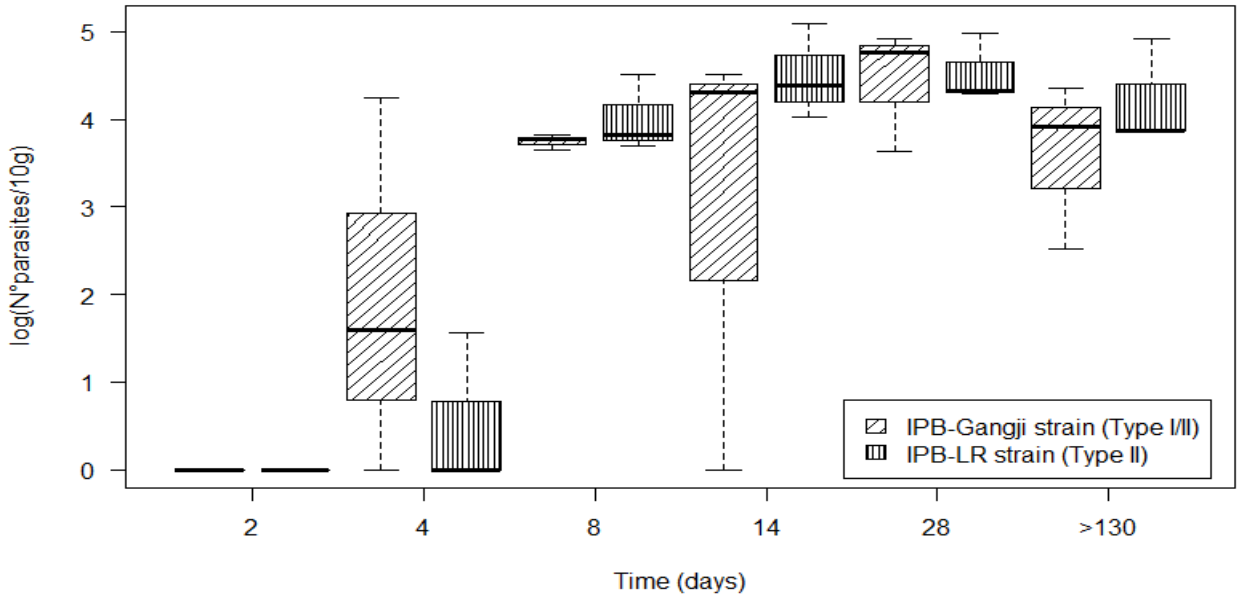

Fig.5. 2. Box and whisker plots for log (N° parasites/10g) in function of days after infection for hearts. A vertical pattern of the plots is used for the results of animals infected with the IPB-LR *T. gondii* strain (Type II) and a diagonal pattern of the plots is used for the results of animals infected with the IPB-Gangji *T. gondii* strain (Type I/II).

5.3.2 Chronic phase

All the animals in groups C2, C3 and C4 seroconverted and showed some mild clinical signs such as, fever, apathy and anorexia during the first week after infection.

5.3.2.1 Infection with tissue cysts

5.3.2.1.1 Type II strain (T. gondii IPB-LR)

During the chronic phase, in the animals infected with tissue cysts of a classical Type II strain (group C2), the parasite was found in all the tested tissues in only one of the animals. In the other two pigs of this group (pig 1 and pig 3), the parasite was found in all the tissues of the animals except for the M. gastrocnemius in pig 3 and the diaphragm in pig 1 (Figure 5.3 and suppl. Table 5.1).

The parasite burden was significantly higher in the heart when compared to the M. gastrocnemius (Ga), diaphragm (Di) and Intercostal muscles (Ic) ($P< 0.05$), but not compared to the M. psoas major (PM) and M. longissimus dorsi (LD). The highest *T. gondii* concentration was found in the brain, being significantly higher ($P< 0.05$) when compared to M. gastrocnemius, diaphragm,M. intercostal

and M. longissimus dorsi (Figure 5.3 and suppl. Table 5.1). When comparing heart and brain no significant difference was found between these two tissues.

5.3.2.1.2 Type I/II strain (T. gondii IPB-G)

The median parasite burden in the animals infected with a hybrid Type I/II strain (group C3) was not significantly different from those infected with the type II strain although they were consistently lower in all the tissues except for the Ic muscle. When comparing the different tissues of the Type I/II strain infected animals, the parasite burden was significantly higher in the brain and the heart ($P < 0.05$) compared to PM, Ga and Di and Ga and Di. The highest parasite burden was found in the heart and not in the brain as it was found in the *T. gondii* IPB-LR experimentally infected group (Figure 5.3 and suppl. Table 5.1).

5.3.2.2 Infection with oocysts

In the pigs infected with oocysts (group C4), the parasite was found in all the tissues of all the animals after 90 dpi. Additionally, the higher parasitic loads were also found in heart and brain as in the other two experimental groups (Groups C2 and C3) (Figure 5.3 and suppl. Table 5.1).

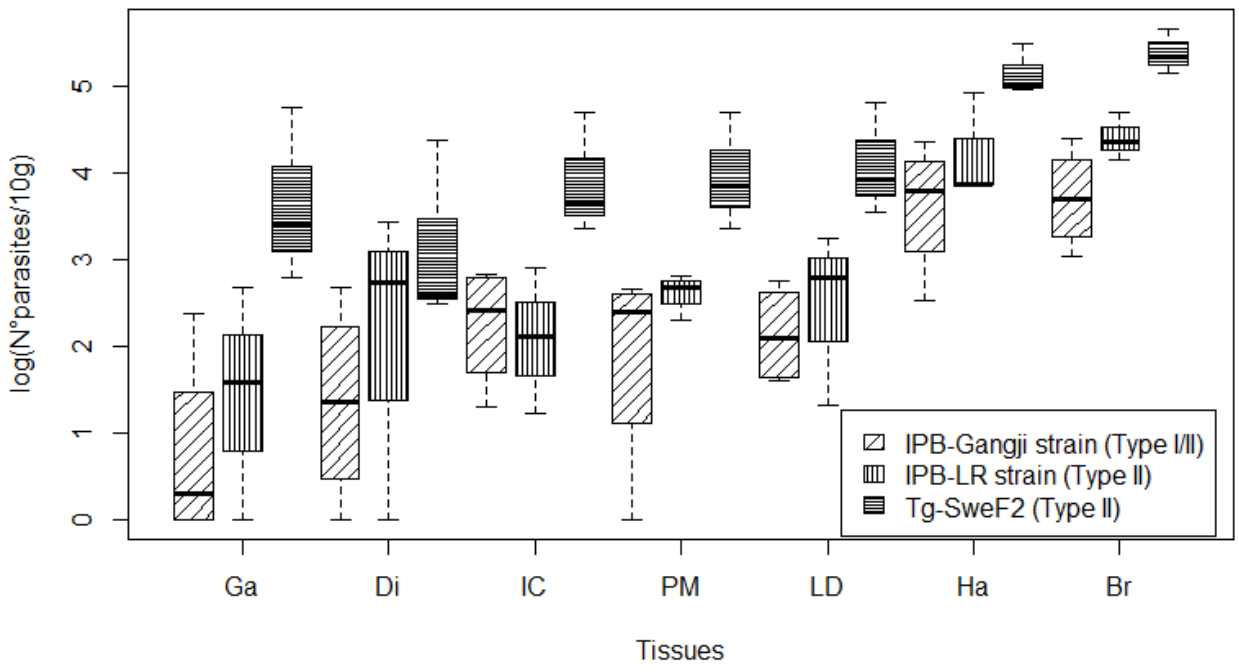

Fig. 5.3. Box and whisker plots for log (N° parasites/10g) in function of different tissues. A vertical lines pattern of the plots is used for the results of animals infected with tissue cysts of the IPB-LR *T. gondii* strain (Type II), a diagonal lines pattern of the plots is used for the results of animals infected with tissue cysts of the

IPB-Gangji *T. gondii* strain (Type I/II) and a horizontal lines pattern of the plots is used for the results of animals infected with oocysts of the Tg-SweF2 *T. gondii* strain (Type II).

5.3.3 Evolution of the parasite burden in heart and lungs during the acute and chronic phases of infection

When comparing the results during the acute and the chronic phases of infection (A1 and A2 compared with C2 and C3), in the lungs the parasite burden at day 182 after infection (chronic phase of infection: (C2 and C3) was significantly lower ($P<0.05$) compared to day 8 (acute phase of infection: (A1 and A2) confirming the decreasing trend of the parasite burden in lungs in function of time observed during the acute phase. In contrast, in the hearts, the parasite burden measured during the acute phase remained stable after 182 dpi in the chronic phase of infection.

Supplementary Table 5.1: Overview of the MC-qPCR results obtained for muscle (100g) and brain (50g) samples from experimentally infected pigs during the chronic phase of infection.

Strain/stages	Pig N°	method	PM	Ga	Di	Ic	LD	Ha	Br
IPB-LR strain/ Tissue cysts	1	MC-qPCR	32.99	33.39	-	35.15	33.05	32.63[b]	35.28[b]
		p. load	2.81	2.68	0	2.11	2.79	4.92	4.37
	2	MC-qPCR	33.39	36.76	31.06	37.87	31.66	35.92[b]	34.30[b]
		p. load	2.68	1.58	3.44	1.22	3.24	3.87	4.70
	3	MC-qPCR	34.55	-	33.25	32.69	37.57	35.89[b]	29.83
		p. load	2.30	0	2.74	2.90	1.32	3.87	4.15
IPB-G strain/ Tissue cysts	1	MC-qPCR	33.83	-	-	35.17	36.73	32.64[a]	31.85
		p. load	2.57	0	0	2.10	1.59	3.92	3.49
	2	MC-qPCR	34.79	34.34	36.18	32.92	33.15	36.52[b]	36.78[b]
		p. load	2.22	2.37	1.77	2.84	2.76	3.66	3.89
	3	MC-qPCR	33.47	39.86	38.80	33.19	33.94	34.40[b]	35.18[b]
		p. load	2.65	0.58	0.92	2.74	2.50	4.35	4.40
	4	MC-qPCR	-	-	33.39	37.65	36.42	33.88	33.31
		p. load	0	0	2.68	1.29	1.69	2.52	3.03
	Detected Mc-qPCR		**6/7**	**4/7**	**5/7**	**7/7**	**7/7**	**7/7**	**7/7**
T gSweF2/ Oocysts	1	MC-qPCR	31.26	32.53	34.00	30.40	29.56	32.50[b]	32.92[b]
		p. load	3.37	2.79	2.48	3.66	3.92	4.97	5.14
	2	MC-qPCR	29.78	31.22	33.16	31.26	30.23	32.37[b]	34.41[c]
		p. load	3.85	3.39	2.59	3.36	3.55	5.00	5.66
	3	MC-qPCR	26.77	26.47	28.17	26.73	26.35	34.11[c]	35.34[c]
		p. load	4.69	4.76	4.37	4.70	4.82	5.49	5.35
	Detected MC-qPCR		**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**

[a]: dill 1/10; [b]: dill 1/100; [c]: dill 1/1000; PM: psoas major; Ga: gastrocnemius; Di: diaphragm; Ic: intercostal; LD: longissimus dorsi; Ha: heart; Br: brain; [a:] number of positive samples in function of the total number of samples; p. load: log 10 (number of parasites per 10 g of tissue) ; -: negative result; np: not performed;

5.4. Discussion and conclusion

Pork is considered among the most important sources of *T. gondii* infection in humans (Batz et al., 2012). Although several studies have demonstrated the presence of the parasites in different tissues (Dubey et al., 1984; Dubey et al., 1986; Opsteegh et al., 2010; Juránková et al., 2013), only few studies have quantified the parasitic burden in different tissues (Opsteegh et al., 2010; Juránková et al., 2013).

In this study, for the first time, the parasitic load in the heart and the lungs was studied during the acute phase of infection following infection with tissue cysts. The heart and the lungs were selected for this study as a high parasite burden has been consistently described in the mouse and the pig models for these two tissues (Dubey, 2010; Dubey et al., 2012). The parasite was found earlier in the lungs than in the heart (2 dpi), but the parasitic load in this tissue decreased in time after reaching a maximum at day 8 post infection. Moreover, the lungs tested during the chronic phase of infection (182 dpi) showed very low or even no parasite presence in contrast to what was described by (Juránková et al., 2013) where lungs were found as the tissue with the second highest parasite burden. This difference can be explained by the earlier euthanasia of the infected animals at day 76 after infection.

On the other hand, in the heart tissue the presence of the parasite was demonstrated only from day 4 after inoculation. Remarkably, no significant variation in parasite burden was found from day 8 till day 28 post infection. When compared to the chronic phase, no significant difference was found and the parasite burden remained stable in the heart till day 182 after infection. These results confirm the importance of the heart for demonstrating the actual presence of the parasite in seropositive pigs. Although there was a trend of lower parasite burdens in pigs infected with the type I/II strain compared to the type II strain, this difference was not significant.

Considering the chronic phase of infection, the results of the present study are in agreement with previous studies identifying brain as the most infected tissue when the pigs are infected with oocysts (Opsteegh et al., 2010; Juránková et al., 2013). Overall, from all the tested tissues, heart and brain showed the highest parasitic loads and no significant difference was found between both tissues in

any of the experimental groups. These results contrast with those obtained by (Juránková et al., 2013) where the parasitic load in heart tissues was significantly lower than in the brain. The difference could be explained by the lower dose used in their experimental infections (5000 oocysts). A dose effect has been previously described in mice and pigs showing a sigmoidal relation of the probability of infection in function of the infective dose (AFSSA, 2005). However, those studies do not give much information of the quantitative parasite burden in tissues after infection with different infective doses. A recent study used a quantitative analysis of the parasite burden in pig tissues in function of the infective dose employed. Strikingly, a lower parasite burden was shown in tissues after infection with a high dose of tissue cysts from the Gangji strain than with a lower dose, while no effect was observed between high and low dose of tissue cysts from the LR strain (Jennes et al., 2017). However, these studies were performed using less sensitive and less accurate quantitative techniques.

When comparing the parasitic load resulting from infection with tissue cysts, no significant difference was observed between the two strains used (*T. gondii* IPB-LR strain (type II) and *T. gondii* IPB-Gangji (type I/II). However, although not significant, a higher parasitic load was observed in all the tissues except for Intercostal muscles after infection with the classic type II strain compared to the hybrid type I/II strain. These results are in agreement with the previous studies using less sensitive techniques (Verhelst et al., 2011; Jennes et al., 2017).

Dose-response studies performed in different animal models have been recently adapted to human *T. gondii* infections showing a clear effect of the parasite burden present in the tissues on the probability of acquiring the infection by the consumers (AFSSA, 2005; Guo et al., 2016). These results highlight the importance of accurate *T. gondii* quantitative data in the different tissues, since animals carrying a higher parasite burden in their tissues will represent a higher risk for the consumer than those with lower parasitic loads. In this study, we showed a higher parasitic load in all the tissues examined compared to previous studies (Opsteegh et al., 2010; Juránková et al., 2013), which can be explained by the higher sensitivity of the MC-qPCR and the infective dose used. Additionally, the pigs infected with oocysts (Group C4) had a significantly higher parasitic load in M. psoas major, M. gastrocnemius and M. intercostal compared to the pigs infected with tissue cysts (Groups C2 and C3). In general, the overall parasitic load in all the tissues was higher for animals infected with oocysts. These findings suggest a potential role of the transmission form on the parasite burden at slaughter age. However, special attention needs to be paid when analysing these results since the use of a different strain (*Tg-SweF2)* and the earlier euthanasia of the pigs infected with oocysts might

have also influenced the obtained parasitic loads. If confirmed, these findings indicate a higher parasite burden in pigs infected with oocysts than with tissue cysts, in contrast to what happens in the definitive host (Dubey, 2010; Dubey et al., 2012).

In conclusion, our results demonstrate the presence of *T. gondii* in the different tissues of pigs (intermediate hosts) and confirm that heart and brain are the predilection tissues for the direct detection of the parasite during the chronic phase of infection, independently of the transmission form and strain. During the acute phase of infection, the lungs are highly infected and they may therefore be used to demonstrate a recent infection. Overall, all the studied tissues showed a potential presence of the parasite at slaughter age posing a risk of infection for humans and other intermediate hosts consuming these tissues. Additionally, the potential role of the parasite stage on the parasite burden is suggested. However, in order to confirm these findings more experimental infections are needed using the different parasite stages originating from the same *T. gondii* strain. Also, studies on the parasite burden of naturally infected animals need to be addressed to confirm the parasite burden in pigs exposed to different doses of infection.

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Chapter VI

Molecular Epidemiology of *T. gondii* in Belgium

Adapted from the publication in preparation: Molecular epidemiology of *Toxoplasma gondii* isolates originating from Belgian pigs and humans

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Abstract

Toxoplasma gondii is a worldwide prevalent, zoonotic parasite of major importance for public health, which can infect any warm-blooded animal species, including humans. Humans can get infected by consumption of meat from a chronically infected animal, by ingestion of sporulated oocysts (resulting from the sexual replication in felids) via contaminated water, soil or vegetables and by vertical transmission through the placenta. Infection through meat consumption is estimated to account for at least 30% of human toxoplasmosis cases in developed countries and more specifically pork is considered to be responsible for 41% of foodborne human toxoplasmosis cases in the US. To better assess the role of pork as a source of infection for humans, circulating strains in both humans and pigs were isolated to perform a molecular epidemiological study. In the present study, 15 hearts from 94 sampled, originating from pigs raised in organic farms tested positive by MC-qPCR of which 9 strains were isolated by mouse bioassay, demonstrating the presence of viable parasites in animals intended for human consumption. When genotyped and compared with the human strains isolated during 2015 and 2016, a highly related structured population was demonstrated. Overall, these finding demonstrate the presence of infectious parasites in pigs intended for human consumption and the potential flow of strains from pigs to humans, reinforcing the identification of pork as a potential risk for the consumers.

6.1. Introduction

Toxoplasma gondii is an obligate intracellular cyst-forming coccidian parasite considered one of the most successful parasites worldwide (Halonen and Weiss, 2013). *T. gondii* is a zoonotic pathogen with a complex life cycle: it can infect virtually any warm-blooded animal (intermediate host), while the sexual replication only takes place in the gastrointestinal tract of the definitive hosts, which are domestic and wild Felidae (Tenter et al., 2000).

Different infection routes have been demonstrated: 1. Consumption of meat from a chronically infected animal; 2. Ingestion of sporulated oocysts (resulting from the sexual replication in felids) via contaminated water, soil or vegetables; 3. Vertical transmission through the placenta (Hide, 2016).
The consumption of raw or undercooked *T. gondii* infected meat is considered a main route of transmission for humans (Cook et al., 2000): in developed countries 50% of the infections are estimated to be meat borne (Scallan et al., 2011) and pork is considered to account for 41 % of foodborne human toxoplasmosis cases in the USA (Batz et al., 2012). Besides the wide spectrum of intermediate host and the various routes of transmission, *T. gondii* shows a very structured genetic

population in Europe and North America with three main lineages also referred to as archetypal strains (Types I, II and III). The *T. gondii* strains circulating in Europe are primarily archetypal and the type II strains are the most predominant in humans and livestock species (Dubey, 2010). However, no data are available on the genetic profile of the strains circulating in the Belgian human population or in any of the main livestock species.

The aim of this study was to identifystrains circulating in both humans and pigs and perform a molecular epidemiological study comparing the isolated strains originating from both humans and pigs to better estimate the role of pork as one of the main sources of infection for humans and the possible flow of strains from pigs to humans.

6.2. Materials and Methods

6.2.1 Porcine Toxoplasma isolates

In order to collect isolates circulating in pigs, a pilot study was designed with visits to a Belgian slaughterhouse where pigs originating from organic farms are slaughtered. Based on the preliminary results of a *T. gondii* seroprevalence study in Belgium in which the prevalence in organic farms was consistently higher than in intensive production systems (Jennes et al., unpublished results), all the samples were collected from a slaughterhouse specialized in organic pigs located in Malmedy (Wallonia, Belgium). A total of 92 pigs representing 17 Belgian organic farms were sampled during 2016. First the pigs were serologically screened for Toxoplasma antibodies, then confirmation of the presence of the parasite in heart was done by MC-qPCR, whereafter the parasite was isolated in mouse (bioassay).

6.2.1.1 Serological screening

The cardiac fluid from the 92 pigs was evaluated using the modified agglutination test (MAT) for the detection of antibodies against *T. gondii* as described by the manufacturer's instructions (Toxo-Screen DA kit, Biomerieux). Briefly, the cardiac fluid samples were diluted in PBS at a 1/40 dilution and 25µl of each dilution was applied in a round bottom microtiter plate with 25µl 2-mercaptoethanol at 0.2 mol/l. Subsequently, 50 µl of a 1/5 dilution of whole formalized tachyzoites in Bovine Albumin Borate Saline (BABS) (supplied in the kit) buffer was added, mixed for 1 minute on a plate mixer at 300 rpm and incubated overnight at room temperature. A negative and a positive control were both supplied by the manufacturer and consisted of goat serum. As an external control, positive and negative pig serum samples obtained from previous experimental infections were tested

on the same plate.

6.2.1.2 Confirmation of the presence of the parasite by MC-qPCR

In order to confirm the actual presence of the parasite and determine the parasitic load, the Magnetic capture real time PCR was performed as described (Gisbert Algaba et al., 2017). In brief, the meat samples were homogenized in the presence of lysis buffer (100 mM Tris HCL, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 40mg/l proteinase k (30 mAnson-U/mg; Amresco, Ohio, USA), pH = 8.0 ± 0.2) with a pedal homogenator (Labconsult, Brussels, Belgium) and lysed overnight at 55°C. Fat and cell debris were then removed by centrifugation and the free biotin possibly present in the crude extract was removed by adding streptavidin-coated agarose beads (binding capacity >330 nmol/ml; solulink, San Diego, USA).

The specific biotin-labelled probes against *T. gondii* (accession number: AF146527) and cellular r18S DNA were added to the biotin-free lysates and the samples were first denatured at 95 °C followed by an incubation at room temperature in order to hybridize the probes with the complementary target sequences. After hybridization, the biotin labelled probes bound to the target DNA were captured using streptavidin magnetic beads (binding capacity > 2.5 nmol/mg (Solulink, San Diego, USA) and after washing and re-suspension in elution buffer, the target DNA was released from the beads by exposure to UV light.

Finally, the qPCR was performed on the final DNA extract to detect the presence of *T. gondii* DNA and the results were analysed using the BioRad CFX manager software to obtain the crossing point (Cp) values.

6.2.1.3 Isolation of the strains, mouse bioassay

Hundred grams of cut tissue were homogenized in a blender with 150 ml sterile saline (0.9 % NaCl) supplemented with 0.25 % trypsin (Gibco) and gentamycin (0.4 mg/ml). The homogenate was then transferred to a 2 l flask and incubated in a water bath at 37°C for 1h 45 min under continuous stirring (magnetic stirrer). The digest was then filtered through gauze, transferred to a 500 ml centrifuge bottle and centrifuged at 2100 x g at RT for 20 min.

The supernatant is then removed and the pellet resuspended in 100 ml of saline with gentamycin and centrifuged at 2100 x g at room temperature for 15 min. This step was repeated once more in order to remove most of the trypsin present in the pellet. Next the final pellet was resuspended in 10 ml of PBS supplemented with gentamycin and IP injected into 5 Swiss white female mice, 1ml each.

The mice were observed daily and a humane endpoint was defined to limit the suffering of the mice in case of disease (Ethics Committee licence no: 20140704-02).

Six weeks after the inoculation, the mice were euthanized, their blood collected and tested by indirect immunofluorescence assay (IFA) as described by (Verhelst et al., 2015). The mice were observed daily and a humane-endpoint was defined to limit the suffering, in case of disease (the mice were euthanized, the lungs and, if present, ascites from the peritoneal cavity were collected and tested in qPCR).

6.2.2 Human Toxoplasma isolates

The genotypes of the 15 *T. gondii* isolates originating from cases of congenital toxoplasmosis in Belgium during 2015 and 2016 were obtained from the National Reference Center (NRC) for Congenital Infections.

6.2.3 Genotyping

All the isolates were genotyped as described by (Azjenberg, 2010). Briefly, *T. gondii* DNA was extracted from 200 µl of brain homogenate using the QIAamp DNA Mini Kit (Qiagen) according the to the manufacturer's instructions. Once extracted, a multiplex PCR was performed to amplify different microsatellite (MS) markers located in different genes (Table 6.1). These markers are grouped in two different levels of discrimination: i) typing markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, and XI.1) able to discriminate between the main *T. gondii* genotypes (I, II and III); ii) fingerprinting markers (*M48*, *M102*, *N60*, *N82*, *AA*, *N61*, and *N83*) with a high discrimination power of discrimination able to differentiate between different strains.

Table 6.1: Overview of the different microsatellites genotyping levels

Discrimination level	*Marker*	Location[a]
Typing level : $[TG/AC]_n$ or $[TC/AG]_n$	*TUB2*	IX
	W35	II
	TgM-A	X
	B18	VIIa
	B17	XII
	M33	IV
	IV.1	IV
	XI.1	XI
Fingerprinting level : $[TA/AT]_n$	*M48*	Ia
	M102	VIIa
	N60	Ib
	N82	XII
	AA	VIII
	N61	VIIb
	N83	X

[a] Location of the markers in the reference strain TgME49

6.2.4 Statistical Analysis

All the samples with an exponential-amplification curve crossing the threshold (Cp) were considered positive for *T. gondii*, samples with no amplification curve for the *T. gondii* target but amplification of the NCIAC were considered negative. As described in Gisbert Algaba et al. (2017), the limit of detection of the method is 65.4 parasites per 100 g of tissue. For each round of samples a positive control with a known number of parasites (calibrator) was performed to correct for possible deviations due to manipulation errors.

A Neighbor-Joining tree was reconstructed from microsatellite data to examine the relationships between strains collected from human and porcine isolates originated from Belgium. The tree was constructed as described by Ajzenberg et al. (2016).

6.3. Results

6.3.1 Porcine toxoplasma isolates

The pilot study yielded a total of 15 seropositive pigs (16.3 %) representing 6 organic farms. The actual presence of the parasite in hearts was confirmed by MC-qPCR in 14 pigs representing 5 organic farms (Farms: A, D, G, L and O). In Farm D and G, *T. gondii* was found in all the animals tested (D:1/ 1 and G:6/6), while in farms A, L and O only some of the tested animals were found positive (A: 1/3; L: 4/6; O: 2/3). Finally, 9 strains were successfully isolated by mouse bioassay

demonstrating the infectivity and viability of the parasite present in these samples. The results are summarized in supp. Table 6.1.

Supplementary Table 6.1: Overview of the parasitic load from naturally infected animals

Isolate	MC-qPCR (Cp)	PI load	Mouse Bioassay
ISP_A1	NA	NA	Neg
ISP_A2	NA	NA	Neg
ISP_A3	30.40	3.66	Pos
ISP_D1	29.40	3.99	Pos
ISP_G1	31.22	3.40	Neg
ISP_G2	30.49	3.63	Pos
ISP_G3	32.48	2.99	np
ISP_G4	29.02	4.11	Pos
ISP_G5	28.79	4.19	Pos
ISP_G6	28.51	4.28	Pos
ISP_L1	28.55	4.27	Pos
ISP_L2	25.48	5.26	Pos
ISP_L3	32.58	2.96	Pos
ISP_L4	31.56	3.29	Neg
ISP_L5	NA	NA	Neg
ISP_O1	28.78	4.19	Pos
ISP_O2	33.50	2.66	Neg

p. load: log 10 (number of parasites per 10 g of tissue); NA: negative result; np: not performed; pos: positive result; neg: negative result.

6.3.2 Human Toxoplasma isolates

A seroconversion was confirmed for one or more of the mice inoculated with the positive samples. The isolated strains were cryopreserved for further studies.

6.3.3 Molecular epidemiology

All the human (N=15) and pig isolated strains (N=9) were identified as archetypal type II strains (Table 6.2). Additionally, the fingerprinting analysis resulted in the identification of 3 different clusters within the pig isolates. Two different clusters were found inside one farm and the other cluster identified represented all the genotyped samples from other farm (n=2). The fourth cluster was found in two different human patients. All the data is represented in neighbor-joining tree in Figure 6.1.

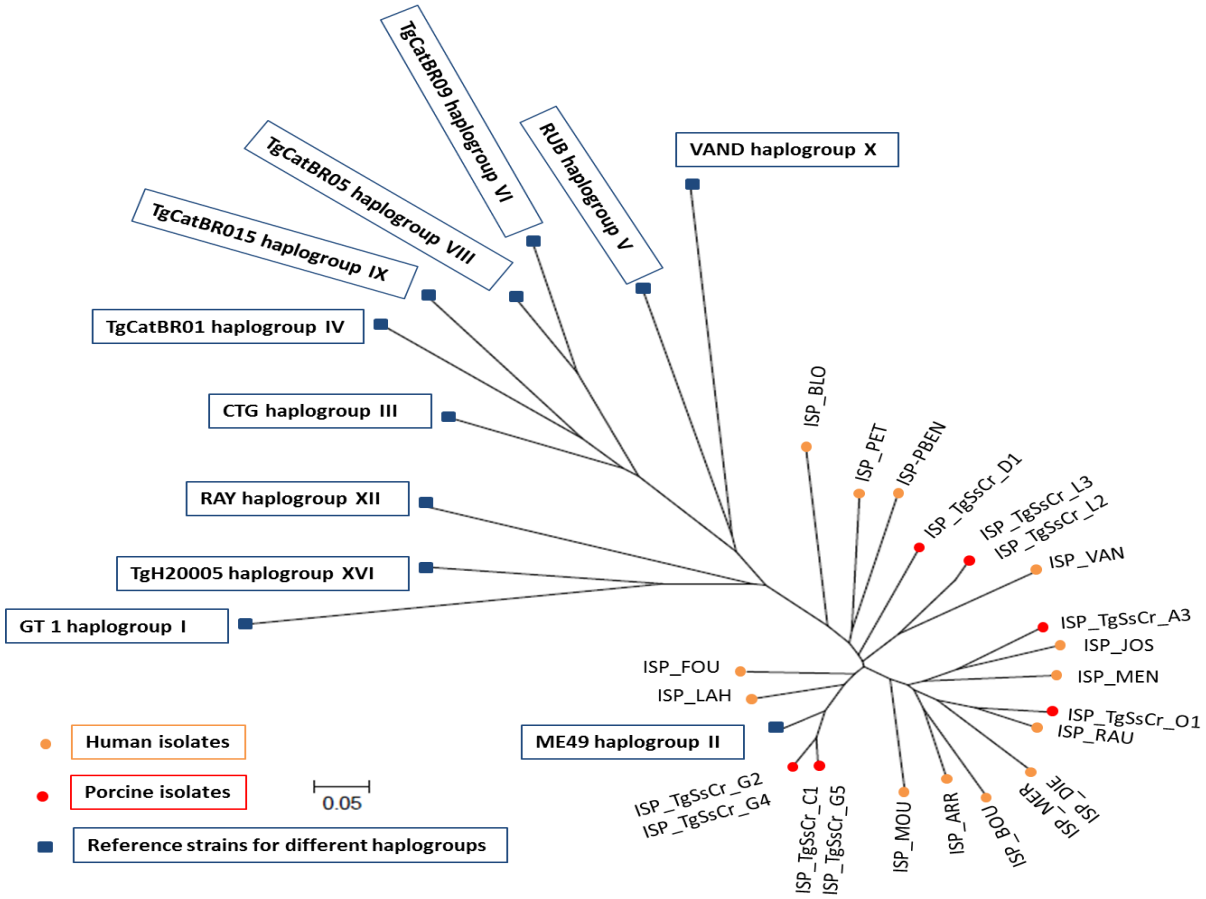

Fig 6.1: Neighbor-joining clustering of T. gondii strains based on 15 microsatellite markers. Squares represent reference strains for different haplogroups: GT1 (haplogroup I), ME49 (haplogroup II), CTG (haplogroup III), TgCatBr01 (haplogroup IV), TgCatBr05 (haplogroup VIII), TgCatBr15 (haplogroup IX), RUB (haplogroup v), VAND (haplogroup X), RAY (haplogroup XII), TgH20005 (haplogroup XVI), TgCatBr09 (haplogroup VI); the orange points represent the human isolates from this study; the red points represent the porcine isolates from this study

6.4. Discussion and conclusions

T. gondii infections in humans pose a health threat for the infected patients. The unborn fetus and immunocompromised individuals are particularly susceptible to develop the most severe consequences of the disease (Dubey, 2010; Robert-Gangneux and Dardé, 2012).

Pork is considered among the most important sources of *T. gondii* infection in humans in developed countries (Batz et al., 2012). The presence of the parasite in the different tissues of the pigs has been demonstrated by several studies (EFSA, 2016; Jennes et al., 2017; Verhelst et al., 2015; Gisbert Algaba et al., 2017). However little or no information is available on the genetics of the circulating *T. gondii* strains in pigs in the EU.

In the present study, we were able to successfully isolate and genotype nine strains originating from pigs raised in five organic farms located in Belgium. We also tried to isolate *T. gondii* strains from conventional farms with controlled housing conditions in which seropositive pigs were found, but were not successful (data not shown). All the isolated strains were identified as classical type II strains. Two different clusters were found on the same farm indicating that the animals were exposed to different strains and/or infectious sources. The other cluster identified represented all the genotyped samples from one farm indicating a common source of infection for all these animals (n=2). Studies performed in other European countries have consistently identified type II as the most prevalent in livestock; such as, 17 strains isolated from pigs in the Czech Republic (Slany et al., 2016) and 100% and 97 % of the isolates from sheep in the Netherlands and France, respectively (Halos et al., 2010; Opsteegh et al., 2010). Recently, a study performed in Belgian wildlife showed a highly clonal population of *T. gondii* circulating strains in foxes where 25 strains out of 26 were identified as type II strains and only one as a type III strain (De Craeye et al., 2011).

All the human strains isolated in the current study were also identified as type II strains and two strains had identical genotyping result suggesting a common source of infection (Table 6.2). These findings are in agreement with other studies where different collections of *T. gondii* isolates were genotyped (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002). For example, in France, 84 % the strains isolated between 1987 and 2001 were identified as type II; the other genotypes, type I and type III accounted for 8.14 % and 2.33 %, of the isolates, respectively (Ajzenberg et al., 2002).

Overall in the present study, 15 of the hearts originating from pigs raised in organic farms tested positive by MC-qPCR, demonstrating the presence of the parasite in animals intended for human consumption. These results demonstrate the higher risk that organic farms may pose to the consumer regarding *T. gondii* infections. The highly related population structure of the strains isolated during 2015 and 2016 in humans and pigs, indicates a potential flow of strains from pigs to humans and/or common source of infection (cat oocysts). These findings reinforce the identification of pork as a potential risk for the consumers.

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for their technical assistance.

Table 6.2: Overview of the genotyping results for the isolated strains

Host species	Isolate	B18	M33	TUB2	XI.1	TgM-A	W35	IV.1	B17	N61	M48	N83	N82	N60	M102	AA	Conclusion
Sus Scrofa	**TgSsCr_G2**[a]	158	169	289	356	207	242	274	336	89	231	310	111	142	174	265	Type II (15/15)
	TgSsCr_G4[a]	158	169	289	356	207	242	274	336	89	231	310	111	142	174	265	Type II (15/15)
	TgSsCr_G5[b]	158	169	289	356	207	242	274	336	95	231	310	111	142	174	265	Type II (15/15)
	TgSsCr_C1[b]	158	169	na	na	207	242	274	336	95	na	310	111	142	174	265	Type II (12/15)
	TgSsCr_L2[c]	158	169	289	356	207	242	274	336	105	213	310	109	142	174	285	Type II (15/15)
	TgSsCr_L3[c]	158	169	289	356	207	242	274	336	105	213	310	109	142	174	NA	Type II (14/15)
	TgSsCr_D1	158	169	289	356	207	242	274	336	91	221	310	113	140	174	273	Type II (15/15)
	TgSsCr_O1	158	169	289	356	207	242	274	336	95	215	308	123	140	174	263	Type II (15/15)
	TgSsCr_A3	158	169	289	356	207	242	274	336	105	215	308	119	142	174	261	Type II (15/15)
Homo sapiens	**ISP_BLO**	158	169	289	356	207	242	274	336	91	213	312	123	147	176	257	Type II (15/15)
	ISP_FOU	158	169	289	356	207	242	274	336	95	213	310	111	140	174	287	Type II (15/15)
	ISP_RAU	158	169	289	356	207	242	274	336	95	215	308	115	140	174	267	Type II (15/15)
	ISP_BOU	158	169	289	356	207	242	274	336	105	223	314	115	140	174	275	Type II (15/15)
	ISP_LAH	158	169	289	356	207	242	274	336	99	223	310	111	140	174	265	Type II (15/15)
	ISP_DIE[d]	158	169	289	356	207	242	274	336	87	217	308	115	140	174	277	Type II (15/15)
	ISP_PET	158	169	289	356	207	242	274	336	109	221	310	111	142	182	267	Type II (15/15)
	ISP_MER[d]	158	169	289	356	207	242	274	336	87	217	308	115	140	174	277	Type II (15/15)
	ISP_VAN	158	169	289	356	207	242	274	336	83	227	310	109	145	174	289	Type II (15/15)
	ISP_BEN	158	169	289	356	207	242	274	336	91	221	312	111	142	174	259	Type II (15/15)
	ISP_MEN	158	169	289	356	207	242	274	336	99	215	308	109	142	174	275	Type II (15/15)
	ISP_MOU	158	169	289	356	207	242	274	336	85	215	312	111	140	174	275	Type II (15/15)
	ISP_JOS	158	169	289	356	207	242	274	336	97	213	308	119	140	174	261	Type II (15/15)
	ISP_ARR	158	169	289	356	207	242	274	336	95	na	314	115	142	174	261	Type II (15/15)

[a]: cluster 1; [b]: cluster 2; [c]: cluster 3;[d]:cluster 4; na: not amplified

Chapter VII

General discussion and future perspectives

Toxoplasma gondii is an ubiquitous apicomplexan parasite with three different infectious stages and various routes of transmission. Its ability to infect any warm-blooded animal including humans (zoonotic) and its capacity to be transmitted between intermediate hosts bypassing the sexual replication in the definitive host (Felidae) have gained it the title of the most successful parasite worldwide. Being zoonotic and worldwide prevalent, *T. gondii* has become a parasite of global interest and there is an increasing awareness of its public health and economic importance.

To date, different strategies estimating the burden of disease in the field of public health have revealed the high importance of long time neglected diseases such as toxoplasmosis, which ranks among the most important. The key feature of these studies is the combination of mortality and morbidity into a single and easy to use metric (Devleesschauwer et al., 2014). Recently, a study performed in Europe, using the Disability-Adjusted Life Years (DALYs), which is a measure of overall disease burden, estimated that more than 180 years are lost due to congenital toxoplasmosis only in Belgium during 2013 (Smit et al., 2017). In addition a study performed in the US situates the combination *T. gondii* and pork on the top 2 of pathogen-food combinations in function of Quality-Adjusted Life Years (QALYs) (Batz et al., 2012)

Despite all these studies demonstrating the importance of *T. gondii* in human and animal health and the recommendations of the competent European food safety authorities to monitor for the presence of *T. gondii* in food, no screening programs are yet implemented in any country.

The general objective of this doctoral thesis was to study the role of pigs and their meat products in the transmission of *T. gondii* to humans. To answer this scientific question, different experimental studies were performed: 1) to develop an efficient and sensitive ISO validated molecular method for the detection of *T. gondii* in meat (**Chapter IV**); 2) to study the infection kinetics in experimentally infected pigs (**Chapter V**); 3) to detect, isolate and characterise *T. gondii* strains circulating in pigs by molecular epidemiology and to compare the genotypes with human genotypes circulating in Belgium (**Chapter VI**).

7.1 Optimization of the parasite's detection in the host tissues

In the present dissertation, we successfully optimized the MC-qPCR method (**Chapter IV)** making it more sensitive and reproducible than the previously developed methods, including the mouse bioassay (reference method). The detection of *T. gondii* infection can be performed either by

serological methods or by direct detection of the parasite. However, only direct methods will demonstrate the actual presence of the parasite in the tissues, which is needed to properly evaluate the risk of meat in human infections. To date direct detection of the parasites in the tissues is still challenging due to a low tissue cyst density which requires at least 100g of tissue to have reliable results. Consequently, commercially available DNA extraction kits have been proven to be insufficiently sensitive due to the limited amount of sample that can be tested. A major improvement of the sensitivity was achieved with a Magnetic Capture based DNA extraction, where the parasitic DNA can be selectively concentrated from a large volume of sample and the parasite burden subsequently quantified by qPCR (Opsteegh et al., 2010).

In the present study, we succeeded to develop a UV dependent release of the target DNA from the beads rendering the method more efficient and avoiding thermic treatment of the samples to elute the parasitic DNA from the beads. The thermic treatment used in the originally developed MC-qPCR may result in iron leaking from the beads with subsequent inhibition in the qPCR. This elegant approach allowed us to overcome the sensitivity and variability issues shown in previous studies where MC-qPCR was still less sensitive than mouse bioassay. Moreover, we subjected the new method to an ISO 17025 validation to objectively confirm its robustness and reliability for routine use in a diagnostic lab. We achieved this by adding the co-capture of cellular r18S which helps to track the extraction step and serves as a non-competitive PCR inhibition control. The robustness of the modified MC-qPCR method was clearly reflected in the results of the validation process with a coefficient of variation (CV) lower than 5%, the higher sensitivity of the MC-qPCR compared to the mouse bioassay (Sensivity MC-qPCR: 94.12% Sensitivity mouse bioassay: 86.49%), the broad range of matrices that can be analysed and a lower limit of detection than the previous methods (65.4 Tachyzoites or 1 cyst per 100 g of tissue).

Overall, our results demonstrate the potential of the modified MC-qPCR as a reliable alternative with regards to the mouse bioassay for archetypal *T. gondii* strains (Type I, Type II and Type III). This method can be used in monitoring programs as it overcomes the main drawbacks of the mouse bioassay: ethically questionable, expensive and time consuming. While the turnaround time (TAT) of mouse bioassay is at least 48 days, the MC-qPCR can give results within only 2 days after reception of the samples keeping in line with the short shelf-life of meat and most of the meat products. Additionally, a higher number of samples can be simultaneously processed which also represents an advantage for monitoring program.

Whereas the MC-qPCR has a shorter TAT (2 days) and is cheaper than the bioassay, a major disadvantage of this technique is the lack of information provided on the viability of the parasites as further discussed in point 7.4.2. Importantly, the costs of this technique are still too high for routine testing; they are estimated to be 100€ per sample, including the costs of personnel.

Besides these two disadvantages, this method can be potentially used to consider *T. gondii*-free the tested meat at high certainty level, which represents a major advantage for sensitive target population groups, such as immunocompromised patients or pregnant women who would assumedly agree to buy more expensive but safer food products. Freezing meat products in order to make them safe is another option, but presents some issues such as keeping the cold chain and the lower organoleptic quality of frozen products.

7.2 Study the infection kinetics of experimentally infected pigs

We used the newly upgraded and ISO 17025 validated MC-qPCR (**Chapter IV)** to demonstrate a high parasite burden in different muscular tissues of experimentally infected animals confirming the risk of pork as a source of infection for humans when consumed raw or undercooked (**Chapter V**). The MC-qPCR allows the study of the quantitative distribution of the parasite through the carcasses in different animal species and the assessment of the risk of infection for the consumer of the different animal tissues. In the present study, the lungs and heart were identified as the most parasitized tissues independently of the infectious stqge and strain used during the acute and chronic phase of infection, respectively. These two tissues are of pivotal importance to demonstrate an actual presence or absence of the parasite in the animals. To date, in pigs, the parasite burden has been only studied following experimental infections with oocysts or tissue cysts but a comparison of the parasite burden in the tissues after an infection with either one of the two infectious stages had not yet been done. Here, we demonstrate a consistently higher parasitic load in all the tested tissues except for the diaphragm in animals infected with oocysts in comparison to infection with tissue cysts. As a result of the experimental infections, diaphragm has been shown to carry one of the lowest parasite burden from all the tested tissues making it an inappropriate tissue to demonstrate the presence of the parasite in pigs.

An effect of the genetic profile of the *T. gondii* inoculum on the parasitic load in some tissues was observed in previous studies. Indeed, infection with a hybrid Type I/II strain (*T. gondii* ISP-Gangji) resulted in a more pronounced clearance of the parasite in some tissues when compared to a classical

type II strain (Verhelst et al., 2011; Jennes et al., 2017). Remarkably, infection with the same strains showed a small or negligible effect on the parasite burden in the pig's tissues in our study. The existence of many different strains of *T gondii*, the large variation in the individual host response to a *T. gondii* infection and the different infection protocols make interpretation and comparison of results difficult.

In conclusion, these findings confirm the importance of pork as a source of infection for humans. All the tissues tested (M. gastrocnemius, M. longissimus dorsi, diaphragm, intercostal muscles, M. psoas major, heart and brain) showed the presence of the parasite. Heart and brain were the most parasitized tissues during the chronic phase of infection (predilection tissues for the detection of the parasite during the chronic phase) whereas heart and lungs were most parasitized during the acute phase of infection (predilection tissues during the acute phase of infection). Additionally, pigs infected with oocysts may pose a higher risk for the consumer due to the higher parasite burden in the tissues following infection, as found in this study.

7.3 Molecular epidemiology of T. gondii in Belgium

We applied succesfully a combined strategy (serological and molecular) to demonstrate the actual presence of infectious *T. gondii* in the tissues of naturally infected pigs intended for human consumption and the potential flow of strains from pigs to humans (**Chapter VI**). Furthermore, we aimed to identify the circulating strains in both humans and pigs and perform a molecular epidemiological study comparing the isolated strains. Besides the wide spectrum of intermediate host and the various routes of transmission, *T. gondii* shows a very structured genetic population in Europe and North America with three main lineages also referred to as archetypal strains (Types I, II and III) with the type II strains as the most predominant in humans and livestock species. However, no data were available on the genetic profile of the strains circulating in the Belgian human population or in any of the main livestock species.

Based on the results obtained from the experimentally infected animals (**Chapter V)** and the preliminary results of a *T. gondii* seroprevalence study in Belgium in which the prevalence in organic farms was consistently higher than in intensive production systems (Jennes et al., unpublished results), we decided to collect samples from a slaughterhouse specialized in organic pigs located in Malmedy (Wallonia, Belgium). In order to reduce the costs and the efficiency of the study, first the pigs were serologically screened for Toxoplasma antibodies, then confirmation of the presence of the parasite in the heart of seropositive pigs was done by MC-qPCR, whereafter the parasite was isolated

in mice (bioassay). The human strains were isolated by the National Reference Center (NRC) for Congenital Infections in Belgium during 2015 and 2016.

In the present study, 14 of 94 hearts originating from pigs raised in organic farms tested positive by MC-qPCR of which nine were isolated in mice, demonstrating the presence of viable parasites in animals intended for human consumption. When genotyped and compared to the human strains, a highly related structured population was demonstrated with all the isolated strains for both species classified as Type II strains. Indeed, the strains isolated from humans and pigs show a very similar genetic profile suggesting a probable flow of strains from pigs to humans or a common source of infection (cat oocysts).

Remarkably, this combined approach was also used to try to isolate strains from industrial pig farms where the controlled housing conditions makes contact of the animals with the parasite more challenging. Although positive serological results can be found in these industrial farms, we were not able to isolate any strains (unpublished data), probably due to the lower prevalence of infected animals in those farms, a lower parasite burden as it is thought that animals are mostly infected by ingestion of chronically infected rodents (lower parasite burden after infection with tissue cysts, **Chapter V**) and the larger size of the herds which makes the sampling more challenging.

These findings, point out the farm management and especially the housing conditions as most important factors allowing *T. gondii* infections in pigs. Currently, there is a trend moving towards more animal-friendly production systems (organic farms) and farms where the pigs have outdoors access allowing more exposure to the parasite. In addition, and as a result of globalisation, nowadays pork is imported from countries where the hygienic and management farm measures can be less stringent than in the EU and where the animals can be exposed to atypical strains that can be more virulent to the pork consumers than the current European strains.

To prevent this, different measures can be adopted such as the implementation of the MC-qPCR to identify parasites in tissues from pigs raised outdoors; avoiding the use of contaminated tissues for meat products consumed fresh and the obligation of freezing *T. gondii* seropositive pig carcasses before consumption. Additionally, the obligation to freeze pork products originating from outside the EU to avoid the incursion of atypical strains inside our borders can be considered.

7.4 Unresolved questions

Today, more than hundred years after *T. gondii* was first described in 1908 and despite the intense research resulting in the elucidation of its complete life cycle, the different infectious routes and stages, the determination of its wide intermediate host spectrum and its phenotypic and genotypic features, many questions remain unresolved. These questions are summarized in Figure 7.1

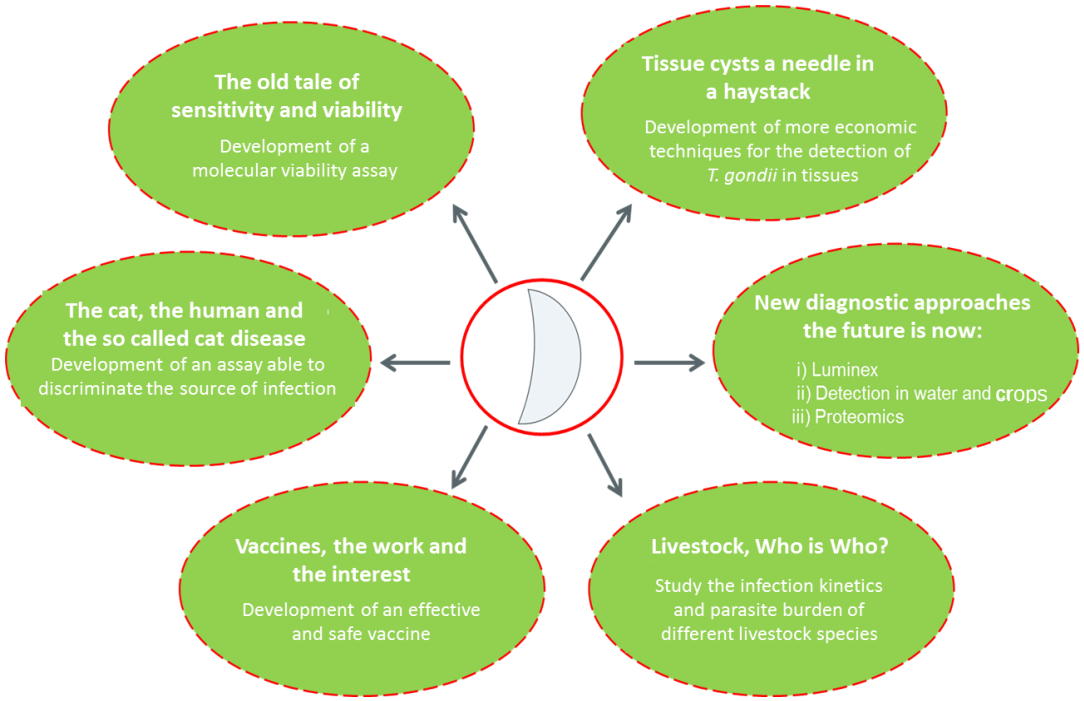

Fig. 7.1 Overview of the main unresolved questions

7.4.1 The cat, the human and the so-called cat disease

Since the elucidation of the final life cycle of *T. gondii* pointing felids as the definitive hosts, *T. gondii* infection in humans has been associated with cats and their shedding of millions of infectious oocysts in the environment (Torrey and Yolken, 2013). Actually, some of the few known registered *T. gondii* outbreaks have been epidemiologically associated with infection with oocysts: i) a group of three women in Victoria, Canada whose infection in 1994-1995 was attributed to oocysts contaminating the drinking water of the city, ii) a group of 39 individuals who likely got infected by ingestion of aerosolized oocysts in Georgia, Atlanta in 1977. However, the origin of these infection could not be laboratory assessed (Hill et al., 2011).

Recently, the identification and cloning of a sporozoite stage-specific recombinant antigen (TgERP) has enabled to determine the oocyst source of infection in function of the presence/absence of Ab against this stage-specific antigen (Hill et al., 2011). However, as TgERP is only expressed during the early phase of infection by sporozoites, the Ab titers will decrease in function of time rendering the differentiation of the source of infection in old or chronic infections difficult. In the study of Hill et al. (2011), the presence of Ab against TgERP was detected in 63.2 % individuals in the acute stage of infection (103/163) and in 17.6 % individuals in the chronic stage of infection (31/176). Brazilian scientists identified and cloned a different sporozoite stage-specific recombinant antigen (CCp5A), which is also able to discriminate between an infection with oocysts or tissue cysts but poses the same issues regarding chronic infections (Santana et al., 2015).

The development of a sporozoite-specific detection method is a major breakthrough in the understanding of the epidemiology of *T. gondii* infections in humans and animals. Once the main source of infection is established, more targeted strategies can be applied to minimize the number of human and animal infections. However, it appears that the results from those studies seem to be difficult to reproduce). The development of a commercially available and standardized sporozoite detecting serological test may overcome this problem.

7.4.1 Tissue cysts, a needle in a haystack

Since the transmission of *T. gondii* by meat consumption was first suggested (Weinman and Chandler, 1954) and confirmed to also occur in humans (Desmonts et al., 1965), carnivorism has been considered as one of the main routes of infections of *T. gondii* for humans in developed countries (Cook et al., 2000; Scallan et al., 2011; Batz et al., 2012; Opsteegh et al., 2016).

Tissue cysts are the result of the extra-intestinal phase of the parasite in both the definitive and the intermediate hosts and they can remain lifelong in the tissues of the host. Resistant to any antibiotic, the concentration and size of the cysts in the tissues is extremely variable depending of the dose of infection, the immune system of the infected host species and even of individuals within a species. Sometimes the homogeneity and density of tissue cysts in the tissues is so low that the direct detection of *T. gondii* in tissues represents a challenge. To overcome this sensitivity problem very sensitive techniques have been developed such as, the MC-qPCR described in **Chapter IV**. However, the high cost of this technique makes it difficult to implement in routine diagnostic practices. Therefore, the development of a very sensitive technique with a reasonable prize is one of

the challenges for the near future.

7.4.2 The old tale of sensitivity and viability

Even though very sensitive, the Mc-qPCR described in **Chapter IV** is only able to demonstrate the presence of the parasitic DNA in the tissues of the host but does not give any information on the viability of the parasites and thus their ability to infect other hosts. In this study, the correlation between the mouse-bioassay and the MC-qPCR was compared using the Cohen's kappa index resulting in a substantial agreement between both methods (Cohen's Kappa: 0.74). However, the different results observed with these two methods can be explained either by the higher sensitivity of the MC-qPCR compared to the mouse bioassay or by the fact that some of the parasites found with MC-qPCR were no longer viable and thus unable to initiate an infection in the inoculated mice.

To the best of our knowledge, this is one of the unresolved questions that needs to be addressed in the near future, eg by the development of a sensitive molecular detection RNA-based method able to address viability of the parasites. However, the new method will need to have a sensitivity comparable to MC-qPCR or at least equivalent to the mouse bioassay. Such a test would greatly reduce the number of experimental animals used in the frame of toxoplasmosis diagnosis.

7.4.3 Livestock, Who is Who?

Toxoplasma gondii infection in humans has been consistently associated with the consumption of raw or undercooked meat of the main livestock species (Dubey, 2010). However, which species account for the majority of the human infections varies between studies and countries. For example, 41% of the cases of foodborne human toxoplasmosis in the United States are estimated to be caused by the consumption of pork, which can thus be considered as a main source of infection for humans (Batz et al., 2012). However, in the Netherlands, it was estimated that only 11 % of meat borne *T. gondii* infections in humans are due to pork, while the majority (68%) of the meat borne infections could actually be attributed to beef consumption (Opsteegh et al., 2011). Remarkably, although cattle are more exposed to *T. gondii* oocysts than pigs because of their more intense contact with the environment, tissue cysts seem not to persist long in most of the cattle tissues, suggesting a clearance of the parasite in infected animals (Opsteegh et al., 2016). In pigs, *T. gondii* is thought to remain lifelong in the tissues of the animals (Dubey, 2010).

As explained above, the parasite burden in different tissues differs from one livestock species to

another. In pigs and sheep, the heart has been described as the predilection tissue to find the parasite and the presence of the parasite has been described in many tissues of experimentally and naturally infected animals. On the other hand, a recent study suggests the liver to be a better tissue to demonstrate the presence of *T. gondii* in cattle (Opsteegh et al., 2016).

7.4.4 New diagnostic approaches, the future is now

Science is continuously evolving and with it the approach towards diagnosis and treatment of infectious diseases. The diagnosis of toxoplasmosis, including the detection of antibodies against *T. gondii* and the demonstration of the presence of *T. gondii* in the tissues, have improved considerably during the last decades as shown in previous sections of this chapter **(Chapter VII)**. However, there is still room for improvement. Here we summarize a few new developments in this field that may impact our understanding of *T. gondii* in the near future.

Detection of T. gondii oocysts in water

As already explained in Chapters I and II, felids shed large numbers of oocysts following infection with *T. gondii*. These resistant stages play a pivotal role in spreading the disease to a wide range of intermediate hosts, including humans. Although the number of feral cats in urban areas has decreased significantly in developed countries during the last decades, as a result of communal spay/neuter programmes, keeping domestic cats is becoming increasingly popular as cats easily fit in our busy modern lives (Walden, 2015). A study in California has estimated that among owned cats, 44% defecates outside at least 75% of the time, and 8% uses both a litter box and defecates outdoors between 25% and 75% of the time. Furthermore, it was also estimated that 87% of the cat owners dispose the litter boxes in the normal garbage, while 4% discard the content near their property and 9% flush the litter in the toilet (Torrey and Yolken, 2013). Many of these oocysts will potentially end up in the surface water and the sea as confirmed with the findings of accumulation of *T. gondii* oocysts in bivalves and *T. gondii* infections in sea mammals (Palos Ladeiro M., 2014; van de Velde et al., 2016).

Up to day, sensitive and efficient detection of *T. gondii* oocysts in water is still challenging due to the low density of oocysts and the lack of a commercially available immunomagnetic separation (IMS) technique. However, bivalves with their ability to accumulate oocysts and their high capacity to filter big volumes of water (up to 100 litres a day for oysters) can become a natural concentrator of oocysts present in water samples. The final detection of the oocysts could then be performed by testing these bivalves by qPCR. This alternative approach can become a good alternative to detect *T. gondii*

oocysts in large volumes of water.

Detection of T. gondii in vegetables and crops

Crops and vegetables can be contaminated with oocysts in different ways, during primary production (direct shedding of oocysts, contaminated irrigation water, etc.), during transport and market processing or directly by the food handlers (Caradonna et al., 2017). As it happens with the oocysts in water, a low density of oocysts and the lack of a specific concentration method makes the direct detection of oocysts in fruits, vegetables and crops very challenging. However, with a similar approach to the one described in Chapter IV (MC-qPCR), a specific concentration of *T. gondii* DNA is possible once the sample is lysed. For this, disrupting the cell wall and the oocysts walls pose the main challenge. This obstacle could be eventually overcome with the use of specific enzymes to disrupt the cell wall and different cycles of freezing and thawing in liquid nitrogen to lyse the extremely resistant oocyst walls.

Luminex, date and source of the infection

Luminex is a new technique which uses microspheres that can be coated with different types of biomolecules:

- Specific antigens in order to detect antibodies against concrete targets
- Specific antibodies in order to detect concrete antigens
- Specific DNA sequences in order to capture a target DNA sequence

This technique can be used to estimate more accurately the date and source of infection. During the early phase of infection different antigens are expressed by sporozoites as already mentioned in point 7.4.1, and also some antigens common in bradyzoites and sporozoites are chronologically expressed by *T. gondii* enabling the estimation of the date of infection. First, the surface antigens (SAG) make contact with the host cell plasma membrane and once attached MIC proteins are excreted. Subsequently rhoptry neck proteins (ROP) and apical membrane antigen 1 (AMA1) and dense granule proteins (GRA) account for the final steps of the penetration and the formation of the parasitophorous vacuole (PV) (De Craeye, 2012). An overview of the process and the proteins implied during the host cell invasion is shown in Figure 7.2.

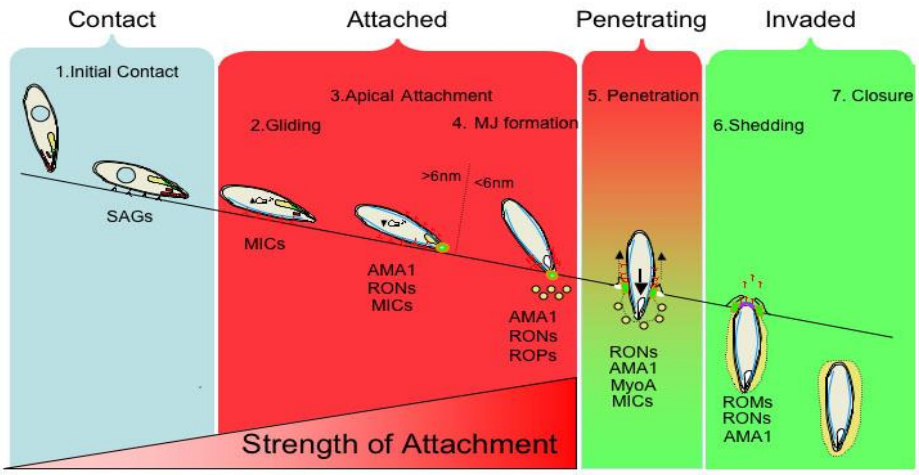

Fig.7.2 Overview of the cell penetration process by *T. gondii*, adapted from De Craeye, 2012.

Hence, the use of beads coated with these specific antigens and the antigens described in point 7.4.1, will help to date the *T. gondii* infection and characterize the infectious stage. The combination of this technique with an exhaustive epidemiological analysis might result in the identification of the concrete source of infection.

Proteomics and the ion trap spectrometry

T. gondii is considered to be able to express at least 6500 different proteins and it is estimated that only 30% of the *T. gondii* proteome has been detected (Xia, et al., 2008; Fritz et al., 2012). However, the majority of studies has been limited to tachyzoites and not much information exists on the proteomics of the other two infectious stages (bradyzoites and sporozoites). The proteomic description of these other stages may enable a better understanding of the *T. gondii* biology.

Nowadays, various analytical disciplines contribute to the elucidation of the proteomic potential of *T. gondii*, but mass spectrometry has become the method of choice for analysis of complex protein samples (Trajkovic, 2014).

Although polar, nonvolatile and thermally unstable, peptides eluted from the liquid chromatography system can be analyzed with an appropriate sample introduction such as electrospray ionization (ESI) as it transfers peptides to the gas phase without degradation (Trajkovic, 2014).

This technique can allow the identification of *T. gondii* proteins involved in the penetration of the host cells, the transformation into bradyzoites, the tissue cyst formation, etc., with the additional value of giving more information such as post-translational modifications (PTM) (Trajkovic, 2014).

7.4.5 Vaccines, the work and the interest

Since the discovery of the consequences for public health deriving from *T. gondii* infections, the development of a vaccine has been the foundation stone of several research studies. During the last decades several vaccines have been developed using different approaches:

- The use of recombinant DNA technology to produce specific recombinant antigens in bacteria
- The lysis of the parasites making a pool of the expressed parasite antigens to induce immunity
- The use of attenuated mutant strains which will give immunity to the infected hosts with no associated clinical signs
- The use of naked DNA vaccines consisting of plasmid DNA into which the relevant gene or genes of the parasitic agent has been inserted

When developing a vaccine three main aims have to be considered: i) Limit the acute parasitemia and protect against congenital toxoplasmosis; ii) Reduce the number of tissue cysts found in the different tissues of the intermediate and definitive hosts; iii) Reduce to a minimum or even prevent the shedding of oocysts by cats.

Up to date, only one vaccine is commercially available (Toxovax®) in some countries for veterinary use. It consists of attenuated live parasites. Toxovax is used to limit the incidence of abortion in sheep and it has been demonstrated to be effective for at least 18 months. However, the vaccination needs to be repeated every two years and it does not eliminate the parasite (Kur et al., 2009). Unfortunately, Toxovax cannot be used in humans because it is an attenuated live vaccine and can regain virulence resulting in iatrogenic infections (Kur et al., 2009).

Once an effective and safe vaccine preventing congenital transmission and the oocyst shedding by cats is developed, the major objective will be to convince and commercialize this vaccine. Fortunately, currently there is an increasing awareness of the Public Health impact of human and animal toxoplasmosis and several studies are performed showing the economic loss associated with this worldwide prevalent parasite. Therefore, the implementation of vaccination and educational programs can be successfully applied in order to decrease the incidence of this long time neglected disease.

Reference list

References

Abdul-Fattah, M.M., Sanad, M.M., Darwish, R.A., Yousef, S.M., 1992. Is immuno-protection against *Toxoplasma* strain specific? J. Egypt. Soc. Parasitol. 22, 77–82.

Ajzenberg, D., 2011. Unresolved questions about the most successful known parasite. Expert Rev. Anti Infect. Ther. 9, 169–171.

Ajzenberg, D., Bañuls, A.L., Su, C., Dumètre, A., Demar, M., Carme, B., Dardé, M.L., 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. Int. J. Parasitol. 34, 1185–1196.

Ajzenberg, D., Cogné, N., Paris, L., Bessières, M.-H., Thulliez, P., Filisetti, D., Pelloux, H., Marty, P., Dardé, M.-L., 2002. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. J. Infect. Dis. 186, 684–689.

Ajzenberg, D., Collinet, F., Mercier, A., Vignoles, P ., Dardé, M.-L. 2010. Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. J. Clin Microbiol. 48, 4641-4645.

Aroussi, A., Vignoles, P., Dalmay, F., Wimel, L., Dardé, M.-L., Mercier, A., Ajzenberg, D., 2015. Detection of *Toxoplasma gondii* DNA in horse meat from supermarkets in France and performance evaluation of two serological tests. Parasite 22, 14.

Ajzenberg, D., Lamaury I., Demar M., Vautrin C., Cabié A., Simon S., Nicolas M., Desbois-Nogard N., Boukhari R., Riahi H., 2016. Performance Testing of PCR Assay in Blood Samples for the Diagnosis of Toxoplasmic Encephalitis in AIDS Patients from the French Departments of America and Genetic Diversity of *Toxoplasma gondii*: A Prospective and Multicentric Study." PLoS Neglected Tropical Diseases 10, no. 6 .

Aspinall, T.V., Marlee, D., Hyde, J.E., Sims, P.F.G., 2002. Prevalence of *Toxoplasma gondii* in commercial meat products as monitored by polymerase chain reaction – food for thought? Int. J. Parasitol. 32, 1193–1199.

Azab, M.E., el-Shenawy, S.F., el-Hady, H.M., Ahmad, M.M., 1993. Comparative study of three tests (indirect haemagglutination, direct agglutination, and indirect immunofluorescence) for detection of antibodies to *Toxoplasma gondii* in pregnant women. J. Egypt. Soc. Parasitol. 23, 471–476.

Bártová, E., Sedlák, K., 2011. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in slaughtered pigs in the Czech Republic. Parasitology 138, 1369–1371.

Basso, W., Handke, M., Sydler, T., Borel, N., Grimm, F., Sidler, X., Deplazes, P., 2015. Involvement of *Toxoplasma gondii* in reproductive disorders in Swiss pig farms. Parasitol. Int. 64, 157–160.

Batz, M.B., Hoffmann, S., Morris, J.G.J., 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. J. Food Prot. 75, 1278–1291.

Blewett, D.A., Miller, J.K., Harding, J., 1983. Simple technique for the direct isolation of toxoplasma tissue cysts from fetal ovine brain. Vet.Rec. 112, 98-100.

Berger-Schoch, A.E., Bernet, D., Doherr, M.G., Gottstein, B., Frey, C.F., 2011. *Toxoplasma gondii* in Switzerland: a serosurvey based on meat juice analysis of slaughtered pigs, wild boar, sheep and cattle. Zoonoses Public Health 58, 472–478.

Beringer, T., 1992. Ist die Toxoplasmose-Diagnostik im Rahmen der Schwangerschaftsvorsorge sinnvoll? Geburtshilfe Frauenheilkd. 52, 740–741.

Bertranpetit, E., Jombart, T., Paradis, E., Pena, H., Dubey, J., Su, C., Mercier, A., Devillard, S., Ajzenberg, D., 2017. Phylogeography of *Toxoplasma gondii* points to a South American origin. Infect. Genet. Evol. 48, 150–155.

Breugelmans, M., Naessens, A., Foulon, W., 2005. Prevention of toxoplasmosis during pregnancy – an epidemiologic survey over 22 consecutive years. J. Perinat. Med. 32, 211–214.

Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. J. Clin. Microbiol. 27, 1787–1792.

Butler, N.J., Furtado, J.M., Winthrop, K.L., Smith, J.R., 2013. Ocular toxoplasmosis II: clinical features, pathology and management. Clin. Experiment. Ophthalmol. 41, 95–108.

Buxton, D., Maley, S.W., Wright, S.E., Rodger, S., Bartley, P., Innes, E.A., 2007. *Toxoplasma gondii* and ovine toxoplasmosis: New aspects of an old story. Vet. Parasitol., Special section: Food-borne Parasitic Zoonosis 149, 25–28.

Caradonna, T., Marangi, M., Del Chierico, F., Ferrari, N., Reddel, S., Bracaglia, G., Normanno, G., Putignani, L., Giangaspero, A., 2017. Detection and prevalence of protozoan parasites in ready-to-eat packaged salads on sale in Italy. Food Microbiol. 67, 67–75.

Cardoen, S., Van Huffel, X., Berkvens, D., Quoilin, S., Ducoffre, G., Saegerman, C., Speybroeck, N., Imberechts, H., Herman, L., Ducatelle, R., 2009. Evidence-based semiquantitative methodology for prioritization of foodborne zoonoses. Foodborne Pathog. Dis. 6, 1083–1096.

Carruthers, V.B., Sibley, L.D., 1997. Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. Eur. J. Cell Biol. 73, 114–123.

Chiappino, M.L., Nichols, B.A., O'Connor, G.R., 1984. Scanning electron microscopy of

Toxoplasma gondii: parasite torsion and host-cell responses during invasion. J. Protozool. 31, 288–292.

Cook, A.J., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of toxoplasma infection in pregnant women: European multicentre case-control study. European Research Network on Congenital *t*oxoplasmosis. BMJ 321, 142–147.

Dardé, M.L., 1996. Biodiversity in *Toxoplasma gondii*. Curr. Top. Microbiol. Immunol. 219, 27–41.

Dardé, M.L., Bouteille, B., Pestre-Alexandre, M., 1992. Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. J. Parasitol. 78, 786–794.

da Silva, A.V., Langoni, H., 2001. The detection of *Toxoplasma gondii* by comparing cytology, histopathology, bioassay in mice, and the polymerase chain reaction (PCR). Vet.Parasitol. 97, 191-198.

De Craeye, 2012. *Toxoplasma gondii*, a successful and Underestimated Foodborne Parasite. PhD thesis, Faculty of Veterinary Medicine, Ghent University, Belgium

De Craeye, S., Speybroeck, N., Ajzenberg, D., Darde, M.L., Collinet, F., Tavernier, P., Van, G.S., Dorny, P., Dierick, K., 2011. *Toxoplasma gondii* and *Neospora caninum* in wildlife: common parasites in Belgian foxes and Cervidae? Vet.Parasitol. 178, 64-69.

De Paschale, M., Agrappi, C., Clerici, P., Mirri, P., Manco, M.T., Cavallari, S., Viganò, E.F., 2008. Seroprevalence and incidence of *Toxoplasma gondii* infection in the Legnano area of Italy. Clin. Microbiol. Infect. 14, 186–189.

Demar, M., Hommel, D., Djossou, F., Peneau, C., Boukhari, R., Louvel, D., Bourbigot, A.-M., Nasser, V., Ajzenberg, D., Darde, M.-L., Carme, B., 2012. Acute toxoplasmosis in immunocompetent patients hospitalized in an intensive care unit in French Guiana. Clin. Microbiol. Infect. 18, E221–E231.

Desmonts, G., Couvreur, J., Alison, F., Baudelot, J., Gerbeaux, J., Lelong, M., 1965. Epidemiological Study on toxoplasmosis; the Influence of Cooking of Butcher's Meat on the Frequency of Human Infection. Rev Franqaise Etudes Clin Biol 10, 952–8.

Devleesschauwer, B., Maertens de Noordhout, C., Smit, G.S.A., Duchateau, L., Dorny, P., Stein, C., Van Oyen, H., Speybroeck, N., 2014. Quantifying burden of disease to support public health policy in Belgium: opportunities and constraints. BMC Public Health 14, 1196.

Dhumne, M., Sengupta, C., Kadival, G., Rathinaswamy, A., Velumani, A., 2007. National seroprevalence of *Toxoplasma gondii* in India. J. Parasitol. 93, 1520–1521.

Djokic, V., Blaga, R., Aubert, D., Durand, B., Perret, C., Geers, R., Ducry, T., Vallee, I., Djurkovic Djakovic, O., Mzabi, A., Villena, I., Boireau, P., 2016. *Toxoplasma gondii* infection in pork produced in France. Parasitology 143, 557–567.

Dorny, P., Speybroeck, N., Verstraete, S., Baeke, M., De, A.B., Berkvens, D., Vercruysse, J., 2002. Serological survey of *Toxoplasma gondii*, feline immunodeficiency virus and feline leukaemia virus in urban stray cats in Belgium. Vet. Rec. 151, 626–629.

Dubey, J., Jones, J., 2008. *Toxoplasma gondii* infection in humans and animals in the United States. Int. J. Parasitol. 38, 1257–1278.

Dubey, J.P., 1998. Advances in the life cycle of *Toxoplasma gondii*. Int. J. Parasitol. 28, 1019–1024.

Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin. Microbiol. Rev. 11, 267-299.

Dubey, J.P., 2010. Toxoplasmosis of animals and humans. Second edition. CRC press. Taylor & Francis Group. ISBN/ 978-1-4200-9236-3 5harback

Dubey, J.P., Lago, E.G., Gennari, S.M., Su, C., Jones, J.L., 2012. Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. Parasitology 139, 1375–1424.

Dubey, J.P., Miller, N.L., Frenkel, J.K., 1970. The *Toxoplasma gondii* oocyst from cat feces. J. Exp. Med. 132, 636–662.

European Food Safety Authority (EFSA). Scientific Opinion of the Panel on Biological Hazards: Surveillance and Monitoring of Toxoplasma in Humans, Food and Animals - Scientific Opinion of the Panel on Biological Hazards." *EFSA Journal* 5, no. 12 (December 2007): 583.

Estes, M.C., Sevall, J.S., 2003. Multiplex PCR using real time DNA amplification for the rapid detection and quantitation of HTLV I or II. Mol.Cell Probes 17, 59-68.

Evengård, B., Petersson, K., Engman, M.L., Wiklund, S., Ivarsson, S.A., Teär-Fahnehjelm, K., Forsgren, M., Gilbert, R., Malm, G., 2001. Low incidence of toxoplasma infection during pregnancy and in newborns in Sweden. Epidemiol. Infect. 127, 121–127.

Eyles, D.E., Coleman, N., 1953. Synergistic effect of sulfadiazine and daraprim against experimental toxoplasmosis in the mouse. Antibiot. Chemother. Northfield Ill 3, 483–490.

Fehlhaber, K., Hintersdorf, P., Krüger G., 2003. Prävalenz von *Toxoplasma gondii*. Fleishforschung und Entwicklung. 83, 97-99.

Ferguson, D.J.P., 2002. *Toxoplasma gondii* and sex: essential or optional extra? Trends Parasitol. 18,

355–359.

Figueiró-Filho, E. A., Lopes A. H. A., Senefonte, F. R. A., 2005. Toxoplasmose aguda: estudo da frequencia, taxa de transmissao vertical e relaçao entre os testes diagnósticos materno-fetais em gestantes em estado da regiao Centro-Oeste do Brasil. Rev. Bras. Ginecol. Obstet. 27: 442-449

Flegr, J., Havlícek, J., Kodym, P., Malý, M., Smahel, Z., 2002. Increased risk of traffic accidents in subjects with latent toxoplasmosis: a retrospective case-control study. BMC Infect. Dis. 2, 11.

Foulon, W., Pinon, J.M., Stray-Pedersen, B., Pollak, A., Lappalainen, M., Decoster, A., Villena, I., Jenum, P.A., Hayde, M., Naessens, A., 1999. Prenatal diagnosis of congenital toxoplasmosis: a multicenter evaluation of different diagnostic parameters. Am. J. Obstet. Gynecol. 181, 843–847.

Frenkel, J.K., 1974. Pathology and pathogenesis of congenital toxoplasmosis. Bull. N. Y. Acad. Med. 50, 182–191.

Frenkel, J.K., Ruiz, A., Chinchilla, M., 1975. Soil Survival of *Toxoplasma* Oocysts in Kansas and Costa Rica. Am. J. Trop. Med. Hyg. 24, 439–443.

Fritz, H.M., Bowyer, P.W., Bogyo, M., Conrad, P.A., Boothroyd, J.C., 2012. Proteomic Analysis of Fractionated *Toxoplasma* Oocysts Reveals Clues to Their Environmental Resistance. PLOS ONE 7, e29955.

Fuente, M. C., Bovone, N. S., Cabral, G. E., 1997. Profilaxis de la toxoplasmosis prenatal. Medicina. 57: 155-160

Gamble, H.R., Dubey, J.P., Lambillotte, D.N., 2005. Comparison of a commercial ELISA with the modified agglutination test for detection of *Toxoplasma* infection in the domestic pig. Vet. Parasitol. 128, 177-181.

Garcia, J.L., Gennari, S.M., Machado, R.Z., Navarro, I.T., 2006. *Toxoplasma gondii:* detection by mouse bioassay, histopathology, and polymerase chain reaction in tissues from experimentally infected pigs. Exp. Parasitol. 113, 267-271.

García-Bocanegra, I., Simon-Grifé, M., Dubey, J.P., Casal, J., Martín, G.E., Cabezón, O., Perea, A., Almería, S., 2010. Seroprevalence and risk factors associated with *Toxoplasma gondii* in domestic pigs from Spain. Parasitol. Int. 59, 421–426.

Gardner, I.A., Greiner, M., Dubey, J.P., 2010. Statistical evaluation of test accuracy studies for *Toxoplasma gondii* in food animal intermediate hosts. Zoonoses Public Health 57, 82-94.

Gisbert Algaba, I., Geerts, M., Jennes, M., Coucke, W., Opsteegh, M., Cox, E., Dorny, P., Dierick, K., De Craeye, S., 2017. A more sensitive, efficient and ISO 17025 validated Magnetic Capture real time PCR method for the detection of archetypal *Toxoplasma gondii* strains in meat. Int. J. Parasitol.

Goldstein, E.J.C., Montoya, J.G., Remington, J.S., 2008. Management of *Toxoplasma gondii* Infection during Pregnancy. Clin. Infect. Dis. 47, 554–566.

Grigg, M.E., 2001. Success and Virulence in *Toxoplasma* as the Result of Sexual Recombination Between Two Distinct Ancestries. Science 294, 161–165.

Guo, M., Buchanan, R.L., Dubey, J.P., Hill, D.E., Lambertini, E., Ying, Y., Gamble, H.R., Jones, J.L., Pradhan, A.K., 2015. Qualitative Assessment for *Toxoplasma gondii* Exposure Risk Associated with Meat Products in the United States. J. Food Prot. 78, 2207–2219.

Halonen, S. K., & Weiss, L. M. (2013). Toxoplasmosis. Handbook of Clinical Neurology, 114, 125–145.

Halová, D., Mulcahy, G., Rafter, P., Turčeková, L., Grant, T., de Waal, T., 2013. *Toxoplasma gondii* in Ireland: seroprevalence and novel molecular detection method in sheep, pigs, deer and chickens. Zoonoses Public Health 60, 168–173.

Havelaar, A., Martyn H., Kirk D., Torgerson P. R., Herman J., Gibb, Hald T., Lake R.J., Praet N., et al. 2010. World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. PLoS Medicine 12, no. 12.

Hide, G., 2016. Role of vertical transmission of *Toxoplasma gondii* in prevalence of infection. Expert Rev. Anti Infect. Ther. 14, 335–344.

Hill, D., Coss, C., Dubey, J.P., Wroblewski, K., Sautter, M., Hosten, T., Muñoz-Zanzi, C., Mui, E., Withers, S., Boyer, K., Hermes, G., Coyne, J., Jagdis, F., Burnett, A., McLeod, P., Morton, H., Robinson, D., McLeod, R., 2011. Identification of a Sporozoite-Specific Antigen from *Toxoplasma gondii*. J. Parasitol. 97, 328–337.

Hill, D.E., Chirukandoth, S., Dubey, J.P., Lunney, J.K., Gamble, H.R., 2006. Comparison of detection methods for *Toxoplasma gondii* in naturally and experimentally infected swine. Vet. Parasitol. 141, 9-17.

Hill, D.E., Haley, C., Wagner, B., Gamble, H.R., Dubey, J.P., 2010. Seroprevalence of and risk factors for *Toxoplasma gondii* in the US swine herd using sera collected during the National Animal Health Monitoring Survey (Swine 2006). Zoonoses Public Health 57, 53–59.

Hill, D., Dubey, J.P., 2002. *Toxoplasma gondii*: transmission, diagnosis and prevention. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 8, 634–640.

Hoffmann, Sandra and Tobenna D. Anekwe. Making Sense of Recent Cost-of-Foodborne-Illness Estimates, EIB-118, U.S. Department of Agriculture, Economic Research Service, September 2013.

Hofhuis, A., van Pelt W., van Duynhoven Y. T. H. P., Nijhuis C. D. M., Mollema L., van der Klis F. R. M., Havelaar A. H. Kortbeek L. M., 2011. "Decreased Prevalence and Age-Specific Risk Factors for Toxoplasma gondii IgG Antibodies in The Netherlands between 1995/1996 and 2006/2007. Epidemiology and Infection 139, no. 4: 530–38.

Holland, G.N., 2003. Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease. Am. J. Ophthalmol. 136, 973–988.

Homan, W.L., Vercammen, M., De Braekeleer, J., Verschueren, H., 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int. J. Parasitol. 30, 69–75.

Hosein, S., Limon, G., Dadios, N., Guitian, J., Blake, D.P., 2016. *Toxoplasma gondii* detection in cattle: A slaughterhouse survey. Vet. Parasitol. 228, 126–129.

Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172, 1561–1566.

Ho-Yen. 2001. Infection in the immunocompetent. In: Joynson, D.H., Wreghitt, T.G. (Eds), Toxoplasmosis. A comprehensive clinical guide. Cambridge University Press.

Hunter, C.A., Sibley, L.D., 2012. Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. Nat. Rev. Microbiol. 10, 766–778.

Hutchison, W.M., Dunachie, J.F., Siim, J.C., Work, K., 1969. Life cycle of *Toxoplasma gondii*. Br. Med. J. 4, 806.

Israelski, D.M., Chmiel, J.S., Poggensee, L., Phair, J.P., Remington, J.S., 1993. Prevalence of Toxoplasma infection in a cohort of homosexual men at risk of AIDS and toxoplasmic encephalitis. J. Acquir. Immune Defic. Syndr. 6, 414–418.

Jaqueti, J., Hernández-García, R., Nicolás, D., Martínez-Hernández, D., Navarro-Gallar, F., García-Esteban, R.J., 1991. Serology against *Toxoplasma gondii* in pregnant women. Development of prevalence rates in the course of 4 years. Rev. Clin. Esp. 188, 278–280.

Jeannel, D., Niel, G., Costagliola, D., Danis, M., Traore, B.M., Gentilini, M., 1988. Epidemiology of toxoplasmosis among pregnant women in the Paris area. Int. J. Epidemiol. 17, 595–602.

Jennes, M., De Craeye, S., Devriendt, B., Dierick, K., Dorny, P., & Cox, E., 2017. Strain- and Dose-Dependent Reduction of *Toxoplasma gondii* Burden in Pigs Is Associated with Interferon-Gamma Production by CD8+ Lymphocytes in a Heterologous Challenge Model. Frontiers in Cellular and Infection Microbiology, 7, 232.

Jennes, M., 2017. Novel insights in the host-pathogen interaction of porcine toxoplasmosis. PhD thesis, Faculty of Veterinary Medicine, Ghent University, Belgium

Jenum, P.A., Kapperud, G., Stray-Pedersen, B., Melby, K.K., Eskild, A., Eng, J., 1998. Prevalence of *Toxoplasma gondii* specific immunoglobulin G antibodies among pregnant women in Norway. Epidemiol. Infect. 120, 87–92.

Jokelainen, P., Simola, O., Rantanen, E., Näreaho, A., Lohi, H., Sukura, A. 2012. Feline toxoplasmosis in Finland. J. of Veterinary Diagnostic Investigation. 24, 115-1124.

Jones, J. L., Kruszon-Moran D., Rivera H.N., Price C., Wilkins P. 2014. *Toxoplasma gondii* Seroprevalence in the United States 2009–2010 and Comparison with the Past Two Decades. The American Journal of Tropical Medicine and Hygiene 90, no. 6: 1135–39.

Juránková, J., Basso, W., Neumayerová, H., Baláž, V., Jánová, E., Sidler, X., Deplazes, P., Koudela, B., 2014. Brain is the predilection site of *Toxoplasma gondii* in experimentally inoculated pigs as revealed by magnetic capture and real-time PCR. Food Microbiol. 38, 167–170.

Khan, U.A., Su, C.L., Sibley, D., Paulsen, I.T., Ajioka, J.W., Taylor, S., Sibley, L.D., 2007. Genetics and Genome Organization of *Toxoplasma gondii*.

Kijlstra, A., Eissen, O.A., Cornelissen, J., Munniksma, K., Eijck, I., Kortbeek, T., 2004. *Toxoplasma gondii* infection in animal-friendly pig production systems. Invest. Ophthalmol. Vis. Sci. 45, 3165–3169.

Kittleson, M.M., Kobashigawa, J.A., 2013. *Toxoplasma gondii*: Good, Bad, or Indifferent? Exposure in the Heart Transplant Recipient: Good, Bad, or Indifferent? Transplant. J. 96.

Klun, I., Djurković-Djaković, O., Katić-Radivojević, S., Nikolić, A., 2006. Cross-sectional survey on *Toxoplasma gondii* infection in cattle, sheep and pigs in Serbia: seroprevalence and risk factors. Vet. Parasitol. 135, 121–131.

Kodjikian, L., Hoigne, I., Adam, O., Jacquier, P., Aebi-ochsner, C., Aebi, C., Garweg, J.G., 2004. Vertical transmission of toxoplasmosis from a chronically infected immunocompetent woman. Pediatr. Infect. Dis. J. 23, 272–274.

Kortbeek, L.M., De Melker, H.E., Veldhuijzen, I.K., Conyn-Van Spaendonck, M.A.E., 2004. Population-based Toxoplasma seroprevalence study in The Netherlands. Epidemiol. Infect. 132, 839–845.

Kur, J., Holec-Gąsior, L., Hiszczyńska-Sawicka, E., 2009. Current status of toxoplasmosis vaccine development. Expert Rev. Vaccines 8, 791–808.

Ladas, I.D., Rallatos, C.L., Kanaki, C.S., Damanakis, A.G., Zafirakis, P.K., Rallatos, G.L., 1999. Presumed Congenital Ocular toxoplasmosis in Two Successive Siblings. Ophthalmologica 213, 320–322.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159-174

Lester, D., 2010. Brain parasites and suicide. Psychol. Rep. 107, 424.

Liu, Q., Wei, F., Gao, S., Jiang, L., Lian, H., Yuan, B., Yuan, Z., Xia, Z., Liu, B., Xu, X., Zhu, X.-Q., 2009. *Toxoplasma gondii* infection in pregnant women in China. Trans. R. Soc. Trop. Med. Hyg. 103, 162–166.

Lopes, A.P., Dubey, J.P., Neto, F., Rodrigues, A., Martins, T., Rodrigues, M., Cardoso, L., 2013. Seroprevalence of *Toxoplasma gondii* infection in cattle, sheep, goats and pigs from the North of Portugal for human consumption. Vet. Parasitol. 193, 266–269.

Lorenzi, H., Khan, A., Behnke, M.S., Namasivayam, S., Swapna, L.S., Hadjithomas, M., Karamycheva, S., Pinney, D., Brunk, B.P., Ajioka, J.W., Ajzenberg, D., Boothroyd, J.C., Boyle, J.P., Dardé, M.L., Diaz-Miranda, M.A., Dubey, J.P., Fritz, H.M., Gennari, S.M., Gregory, B.D., Kim, K., Saeij, J.P.J., Su, C., White, M.W., Zhu, X.-Q., Howe, D.K., Rosenthal, B.M., Grigg, M.E., Parkinson, J., Liu, L., Kissinger, J.C., Roos, D.S., David Sibley, L., 2016. Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. Nat. Commun. 7, 10147.

Luft, B.J., Hafner, R., Korzun, A.H., Leport, C., Antoniskis, D., Bosler, E.M., Bourland, D.D., Uttamchandani, R., Fuhrer, J., Jacobson, J., 1993. Toxoplasmic encephalitis in patients with the acquired immunodeficiency syndrome. Members of the ACTG 077p/ANRS 009 Study Team. N. Engl. J. Med. 329, 995–1000.

Luft, B.J., Remington, J.S., 1992. Toxoplasmic encephalitis in AIDS. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 15, 211–222.

Makuwa, M., Lecko, M., Nsimba, B., Bakouetela, J., Lounana-Kouta, J., 1992. Toxoplasmose et la femme enceinte au Congo, bilan de 5 ans de depistage. (1986-1990). Med. Afr. Noire. 39: 493-495

Maldonado, Y.A., Read, J.S., 2017. Diagnosis, Treatment, and Prevention of Congenital toxoplasmosis in the United States. Pediatrics 139, e20163860.

Mangen, M. J., Bouwknegt M., Friesema I. H. M., Haagsma I. H. M., Kortbeek L. M., Tariq L., Wilsonv, van Pelt W., Havelaar A.H. 2015. Cost-of-Illness and Disease Burden of Food-Related Pathogens in the Netherlands, 2011. International Journal of Food Microbiology 196, no. Supplement C: 84–93.

Mariuz, P.R., Luft, B.J., 1992. Toxoplasmic encephalitis. AIDS Clin. Rev. 105–130.

Masala, G., Porcu, R., Madau, L., Tanda, A., Ibba, B., Satta, G., Tola, S., 2003. Survey of ovine and

caprine toxoplasmosis by IFAT and PCR assays in Sardinia, Italy. Vet. Parasitol. 117, 15–21.

McFadden, G.I., 2011. The apicoplast. Protoplasma 248, 641–650.

Messier, V., Lévesque, B., Proulx, J.-F., Rochette, L., Libman, M.D., Ward, B.J., Serhir, B., Couillard, M., Ogden, N.H., Dewailly, É., Hubert, B., Déry, S., Barthe, C., Murphy, D., Dixon, B., 2009. Seroprevalence of *Toxoplasma gondii* Among Nunavik Inuit (Canada). Zoonoses Public Health 56, 188–197.

Moens, B., Lopez, G., Adaui, V., Gonzalez, E., Kerremans, L., Clark, D., Verdonck, K., Gotuzzo, E., Vanham, G., Cassar, O., Gessain, A., Vandamme, A.M., Van, D.S., 2009. Development and validation of a multiplex real-time PCR assay for simultaneous genotyping and human T-lymphotropic virus type 1, 2, and 3 proviral load determination. J. Clin. Microbiol. 47, 3682-3691.

Morris, A., Croxson, M., 2004. Serological evidence of *Toxoplasma gondii* infection among pregnant women in Auckland. N. Z. Med. J. 117, U770.

Nogareda, F., Le Strat Y., Villena I., De Valk H., Goulet V. 2014. Incidence and Prevalence of *Toxoplasma gondii* Infection in Women in France, 1980-2020: Model-Based Estimation." Epidemiology and Infection 142, no. 8: 1661–70.

Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A., van der Giessen, J., 2010. Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. Int. J. Food Microbiol. 139, 193–201.

Opsteegh, M., Prickaerts, S., Frankena, K., Evers, E.G., 2011. A quantitative microbial risk assessment for meatborne *Toxoplasma gondii* infection in The Netherlands. Int. J. Food Microbiol. 150, 103–114.

Opsteegh M, Schares G, Blaga R and van der Giessen J on behalf of the consortium, 2016. Experimental studies of of *Toxoplasma gondii* in the main livestock species (GP/EFSA/BIOHAZ/2013/01) Final report. EFSA supporting publication 2016:EN-995, 161 pp.

Opsteegh M, Schares G, Blaga R and van der Giessen J on behalf of the consortium, 2016. Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat (GP/EFSA/BIOHAZ/2013/01) Final report. EFSA supporting publication 2016:EN-996, 294 pp.

Ozgonul, C., Besirli, C.G., 2017. Recent Developments in the Diagnosis and Treatment of Ocular toxoplasmosis. Ophtalmic research Vol. 57, No. 1.

Palos Ladeiro, M., Aubert, D., Villena, I., Geffard, A., Bigot, A., 2014. Bioaccumulation of human waterborne protozoa by zebra mussel (*Dreissena polymorpha*): interest for water biomonitoring. Water Res. 48, 148–155.

Petersen, E., 2007. Toxoplasmosis. Semin. Fetal. Neonatal Med. 12, 214–223.

Pfaff, A.W., Abou-Bacar, A., Letscher-Bru, V., Villard, O., Senegas, A., Mousli, M., Candolfi, E., 2007. Cellular and molecular physiopathology of congenital toxoplasmosis: The dual role of IFN-γ. Parasitology 134, 1895–1902.

Poirotte, C., Kappeler, P.M., Ngoubangoye, B., Bourgeois, S., Moussodji, M., Charpentier, M.J.E., 2016. Morbid attraction to leopard urine in Toxoplasma-infected chimpanzees. Curr. Biol. CB 26, R98-99.

Pomares, C., Montoya, J.-G., 2016. Laboratory diagnosis of congenital toxoplasmosis. J. Clinical Microbiology. 2448-2454.

Prusa, A.-R., Kasper, D.C., Sawers, L., Walter, E., Hayde, M., Stillwaggon, E., 2017. Congenital toxoplasmosis in Austria: Prenatal screening for prevention is cost-saving. PLoS Negl. Trop. Dis. 11.

Renold, C., Sugar, A., Chave, J.P., Perrin, L., Delavelle, J., Pizzolato, G., Burkhard, P., Gabriel, V., Hirschel, B., 1992. Toxoplasma encephalitis in patients with the acquired immunodeficiency syndrome. Medicine (Baltimore) 71, 224–239.

Robert-Gangneux, F., Dardé, M.-L., 2012. Epidemiology of and Diagnostic Strategies for toxoplasmosis. Clin. Microbiol. Rev. 25, 264–296.

Rosso, F., Les, J.T., Agudelo, A., Villalobos, C., Chaves, J.A., Tunubala, G.A., Messa, A., Remington, J.S., Montoya, J.G., 2008. Prevalence of infection with *Toxoplasma gondii* among pregnant women in Cali, Colombia, South America. Am. J. Trop. Med. Hyg. 78, 504–508.

Ryu, J.S., Min, D.Y., Ahn, M.H., Choi, H.G., Rho, S.C., Shin, Y.J., Choi, B., Joo, H.D., 1996. Toxoplasma antibody titers by ELISA and indirect latex agglutination test in pregnant women. Korean J. Parasitol. 34, 233–238.

Sabin, A.B., Feldman, H.A., 1948. Dyes as Microchemical Indicators of a New Immunity Phenomenon Affecting a Protozoon Parasite (Toxoplasma). Science 108, 660–663.

Sabin, A.B., Warren, J., 1942. Therapeutic Effectiveness of Certain Sulfonamides on Infection by an Intracellular Protozoon (Toxoplasma). Proc. Soc. Exp. Biol. Med. 51, 19–23.

Santana, S.S., Gebrim, L.C., Carvalho, F.R., Barros, H.S., Barros, P.C., Pajuaba, A.C.A.M., Messina, V., Possenti, A., Cherchi, S., Reiche, E.M.V., Navarro, I.T., Garcia, J.L., Pozio, E., Mineo, T.W.P., Spano, F., Mineo, J.R., 2015. CCp5A Protein from *Toxoplasma gondii* as a Serological Marker of Oocyst-driven Infections in Humans and Domestic Animals. Front. Microbiol. 6.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. Emerg.

Infect. Dis. 17, 7–15.

Sever, J.L., Ellenberg, J.H., Ley, A.C., Madden, D.L., Fuccillo, D.A., Tzan, N.R., Edmonds, D.M., 1988. Toxoplasmosis: Maternal and Pediatric Findings in 23,000 Pregnancies. Pediatrics 82, 181–192.

Sheffield, H.G., Melton, M.L., 1970. *Toxoplasma gondii*: the oocyst, sporozoite, and infection of cultured cells. Science 167, 892–893.

Shwab, E.K., Zhu, X.-Q., Majumdar, D., Pena, H.F.J., Gennari, S.M., Dubey, J.P., Su, C., 2014. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. Parasitology 141, 453–461.

Sibley, L.D., 2009. Development of forward genetics in *Toxoplasma gondii*. Int. J. Parasitol. 39, 915–924.

Sindoni, L., Cananzi, F., Ciano V., 1989. Indagine sieroepidemiologica sulla prevalenza di anti-corpi antiToxoplasma in ovini, caprini e bovini stanzianti in alcune zone della Sicilia: valutanzione dei tests utilizzati. Arch. Vet. Ital. 40: 118-127

Smit, G.S.A., Padalko, E., Van Acker, J., Hens, N., Dorny, P., Speybroeck, N., Devleesschauwer, B., 2017. Public Health Impact of Congenital Toxoplasmosis and Cytomegalovirus Infection in Belgium, 2013: A Systematic Review and Data Synthesis. Clin. Infect. Dis. 65, 661–668.

Sobanski V, Ajzenberg D, Delhaes L, Bautin N, Just N. 2013. Severe toxoplasmosis in immunocompetent hosts: be aware of atypical strains. Am J. Respir Crit Care Med. 187 (10) 1143-1145.

Staggs, S. E., See, M. J., Dubey, J. P., & Villegas, E. N., 2009. Obtaining Highly Purified *Toxoplasma gondii* Oocysts by a Discontinuous Cesium Chloride Gradient. JoVE, 33, 1420.

Su, C., Evans, D., Cole, R.H., Kissinger, J.C., Ajioka, J.W., Sibley, L.D., 2003. Recent Expansion of Toxoplasma Through Enhanced Oral Transmission. Science 299, 414–416.

Su, C., Howe, D.K., Dubey, J.P., Ajioka, J.W., Sibley, L.D., 2002. Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. Proc. Natl. Acad. Sci. U. S. A. 99, 10753–10758. doi:10.1073/pnas.172117099

Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30, 1217–1258.

Terazawa, A., Muljono, R., Susanto, L., Margono, S.S., Konishi, E., 2003. High Toxoplasma antibody prevalence among inhabitants in Jakarta, Indonesia. Jpn. J. Infect. Dis. 56, 107–109.

Torrey, E.F., Yolken, R.H., 2013. Toxoplasma oocysts as a public health problem. Trends Parasitol.

29, 380–384.

Trajkovic, S., 2014. Methods development in biological mass spectrometry: application in glycoproteomics. Theses Diss.Chem.

Valdès, V., Legagneur, H., Watrin, V., Paris, L., Hascoët, J.-M., 2011. Congenital toxoplasmosis due to maternal reinfection during pregnancy]. Arch. Pediatr. Organe Off. Soc. Francaise Pediatr. 18, 761–763.

van der Giessen, J. W. B., Kortbeek, L. M., Valkenburgh, S. M., 2003. Advies Toxoplasmose van de interdepepartamentale werkgroep Zoonosen. Ministerie van Economische Zaken: Nederlandse Voedsel- en Warenautoriteit

van de Velde, N., Devleesschauwer, B., Leopold, M., Begeman, L., IJsseldijk, L., Hiemstra, S., IJzer, J., Brownlow, A., Davison, N., Haelters, J., Jauniaux, T., Siebert, U., Dorny, P., De Craeye, S., 2016. *Toxoplasma gondii* in stranded marine mammals from the North Sea and Eastern Atlantic Ocean: Findings and diagnostic difficulties. Vet. Parasitol. 230, 25–32.

van Knapen, F., Kremers, A.F., Franchimont, J.H., Narucka, U., 1995. Prevalence of antibodies to *Toxoplasma gondii* in cattle and swine in The Netherlands: towards an integrated control of livestock production. Vet. Q. 17, 87–91.

Verhelst, D., De, C.S., Jennes, M., Dorny, P., Goddeeris, B., Cox, E., 2015. Interferon-gamma expression and infectivity of Toxoplasma infected tissues in experimentally infected sheep in comparison with pigs. Vet. Parasitol. 207, 7-16.

Villari, S., Vesco, G., Petersen, E., Crispo, A., Buffolano, W., 2009. Risk factors for toxoplasmosis in pigs bred in Sicily, Southern Italy. Vet. Parasitol. 161, 1–8. 9

Walden, Liz. “Worldwide Pet Ownership Statistics | Most Common Pets Around the World.” PetSecure, May 13, 2015. https://www.petsecure.com.au/pet-care/a-guide-to-worldwide-pet-ownership/.

Walpole, I.R., Hodgen, N., Bower, C., 1991. Congenital toxoplasmosis: a large survey in western Australia. Med. J. Aust. 154, 720–724.

Watson, J.D., Crick, F.H., 1953. The structure of DNA. Cold Spring Harb. Symp. Quant. Biol. 18, 123–131.

Webster, J.P., 2007. The Effect of *Toxoplasma gondii* on Animal Behavior: Playing Cat and Mouse. Schizophr. Bull. 33, 752–756.

Weinman, D., Chandler, A.H., 1954. Toxoplasmosis in swine and rodents; reciprocal oral infection and potential human hazard. Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med. N. Y. N 87, 211–216.

Weiss, L.M., Dubey, J.P., 2009. Toxoplasmosis: A history of clinical observations. Int. J. Parasitol. 39, 895–901.

Wilking, H., Thamm M., Stark K., Aebischer T., Seeberv, 2016. Prevalence, Incidence Estimations, and Risk Factors of *Toxoplasma Gondii* Infection in Germany: A Representative, Cross-Sectional, Serological Study. Scientific Reports 6: 22551.

Woldemichael, T., Fontanet, A.L., Sahlu, T., Gilis, H., Messele, T., Rinke de Wit, T.F., Yeneneh, H., Coutinho, R.A., Van Gool, T., 1998. Evaluation of the Eiken latex agglutination test for anti-Toxoplasma antibodies and seroprevalence of Toxoplasma infection among factory workers in Addis Ababa, Ethiopia. Trans. R. Soc. Trop. Med. Hyg. 92, 401–403.

Wolf, A., Cowen, D., Paige, B., 1939. Human toxoplasmosis: occurrence in infants as an encephalomyelitis verification by transmission to animals. Science. 89: 226-227,

Xia, D., Sanderson, S.J., Jones, A.R., Prieto, J.H., Yates, J.R., Bromley, E., Tomley, F.M., Lal, K., Sinden, R.E., Brunk, B.P., Roos, D.S., Wastling, J.M., 2008. The proteome of *Toxoplasma gondii*: integration with the genome provides novel insights into gene expression and annotation. Genome Biol. 9, R116.

Yamamoto, J.H., Lima Vallochi, A., Silveira, C., Kalil Filho, J., Nussenblatt, R.B., Cunha-Neto, E., Gazzinelli, R.T., Belfort, R., Vicente Rizzo, L., 2000. Discrimination between Patients with Acquired toxoplasmosis and Congenital toxoplasmosis on the Basis of the Immune Response to Parasite Antigens. J. Infect. Dis. 181, 2018–2022.

Summary

Toxoplasma gondii is a zoonotic parasite of global interest. Nowadays, there is an increasing awareness worldwide of its public health and economic importance. In humans, the majority of infections are asymptomatic or, on rare occasions cause mild flu-like symptoms. However, infection may produce congenital disease if a primary infection occurs in pregnant women, potentially leading to abortion, fetal abnormalities, perinatal death and reduced quality of life in surviving children. Additionally, toxoplasmosis can result in severe disease in immunocompromised people and serious eye disease in otherwise healthy individuals. In livestock, infection with *T. gondii* may be associated with reproductive disorders, reduced weight gain and weak progeny, with consequently, important economic losses for producers. Among the different routes of transmission to humans the consumption of undercooked or raw meat is very important. Pork is considered to be among the main sources of human infection. This thesis aims at generating knowledge on the role of pigs in the transmission of the parasite by developing an improved detection method of *T. gondii* in pig tissues, studying the distribution and survival of the parasite in experimentally infected pigs, and by isolating and genotyping *T. gondii* strains from Belgian pigs.

The literature study (**Part I**) provides a wide overview of the currently published research regarding the most important features of *T. gondii* considered as one of the most successful parasites worldwide. The first chapter (**Chapter I**) describes the morphology and biology of the different infectious stages as well as *T. gondii's* complex life cycle with intermediate and definitive hosts. In addition, a short overview of the molecular epidemiology is given.

Chapter II describes the main routes of transmission between the intermediate and definitive hosts or between intermediate hosts along with the significant impact of the parasite on humans and livestock health and its economic importance for Public Health.

Finally, in **Chapter III** the different approaches and techniques for diagnosis of *T. gondii* are described, emphasizing on their advantages and limitations in order to demonstrate recent or old infections and presence or absence of the parasite in the tissues.

The experimental part (**Part II**) starts by situating the main problems studied in the present dissertation and as such by giving the aims of this PhD study. It continues with a detailed description of the different experimental studies performed to answer these objectives.

The general objective of this study was to study the role of pork and other meat products products as a source of *T. gondii* infection in humans. More in detail, the specific objectives were to develop an efficient and sensitive ISO validated molecular method for the detection of *T. gondii* in various tissues, to study the infection kinetics in experimentally and naturally infected pigs to better assess the role of pork as a source of infection and finally to detect, isolate and study the circulating *T. gondii* strains in Belgian pigs.

In **Chapter IV** we present an efficient and ISO 17025 validated MC-qPCR method for the detection of archetypal *T. gondii* strains in meat, published in the International Journal for Parasitology (Gisbert Algaba, et al., 2017). The method combines the hybridization of specific, biotinylated probes with the capture of those probes with streptavidin coated paramagnetic beads and the release of the target DNA from the beads by exposure to UV light. That way, *T. gondii* DNA can be selectively concentrated from a large volume of sample increasing significantly the sensitivity of the technique. The results showed a more sensitive (99% LOD of 65.4 tachyzoites per 100 g of meat and sensitivity: 94.12 %), economic and reliable method than the reference bioassay method. Actually, they demonstrate the potential of this method as full alternative for the mouse bioassay for screening of various tissues and meat, with the additional advantage of being quantitative.

In **Chapter V** we describe the effect of different *T. gondii* strains and infectious stages on the parasitic load in pig carcasses (Gisbert Algaba et al., accepted). Here we used the MC-qPCR to quantitatively address the presence of the parasite (parasitic load) during the acute (0-28 days post infection) and chronic (3-4 months post infection) phases of infection along with a comparison of the parasite burden in function of the different infectious stages and strains used in experimentally infected pigs. The highest burden of infection was found in heart and lungs during the acute phase of infection while heart and brain were identified as the most parasitized tissues during the chronic phase of infection, independently of the infectious form and the strain used. Remarkably, a higher parasite burden was measured in different tissues following infection with oocysts when compared with a tissue cyst infection during the chronic phase of infection and additionally no significantly different parasite burden resulted from infection with two different strains.

Chapter VI describes the molecular epidemiology of *T. gondii* isolates originating from Belgian pigs and humans (Gisbert Algaba et al., submitted). In this study we focus on the detection and isolation of different *T. gondii* strains in pigs and the comparison of these strains with those isolated in the frame of the National Reference Centre for Congenital Infections in Belgium. Overall, in the

present study, 14/92 hearts originating from pigs raised in organic farms tested positive by MC-qPCR, demonstrating the presence of the parasite in animals intended for human consumption. Moreover, the highly related population structure of the strains isolated during 2015 and 2016 in humans and pigs (24/24 type II strains) indicates a potential flow of strains from pigs to humans. These findings reinforce the identification of pork as a potential risk for the consumers and demonstrate the higher risk that pigs from organic farms pose to the consumer regarding *T. gondii* infections

In **Chapter VII**, a general discussion is provided stressing the contribution of the presented studies to a better understanding and awareness of human and animal toxoplasmosis and more specifically, the identification of pork and its pivotal role as a source of infection for humans using the MC-qPCR, a very sensitive molecular method able to detect only one tissue cyst per 100 g of meat. However, this method is not able to differentiate between viable and non-viable parasites which is discussed along with the main unresolved questions and new potential approaches for the detection of *T. gondii* in this chapter.

In conclusion, the present dissertation provides a new assay for the detection of *T. gondii* that can replace the mouse bioassay. The test allowed to determine the parasite distribution in the host tissues during the acute and chronic phases of infection as well as the effect of infection with different strains and infectious stages of the parasite on the parasite burden. Furthermore, a molecular epidemiology study showed a highly related structured population with all the isolates classified as Type II strains in both human and pigs.

Samenvatting

Toxoplasma gondii is een zoönotische parasiet met een wereldwijde impact. De laatste jaren is het bewustzijn omtrent het economisch en volksgezondheidsbelang van deze parasiet danig toegenomen. Humane infecties verlopen meestal asymptomatisch of gaan in zeldzame gevallen gepaard met milde griepachtige symptomen. Congenitale aandoeningen kunnen echter optreden wanneer de primo-infectie plaatsvindt tijdens de zwangerschap. Dit kan aanleiding geven tot abortus, foetale abnormaliteiten, perinatale sterfte en een verminderde levenskwaliteit van de overlevende kinderen. Verder kunnen ernstige symptomen waargenomen worden bij immunodeficiënte patiënten, als ook oogaandoeningen bij gezonde patiënten. Infecties met *T. gondii* bij nutsdieren kunnen gepaard gaan met reproductiestoornissen, verminderde gewichtstoename en zwakkere nakomelingen. Dit leidt tot significante economische verliezen voor de producenten.
Een van de belangrijke transmissieroutes naar de mens is de consumptie van onvoldoende verhitte of rauw vlees. Varkensvlees wordt aanschouwd als een van de hoofdbronnen voor humane infectie. Deze thesis heeft als doel meer kennis te verwerven omtrent de rol van varkens bij de transmissie van de parasiet. Om dit te realiseren werd een verbeterde detectiemethode voor *T. gondii* in varkensweefsels ontwikkeld, werd de verspreidings- en overlevingscapaciteiten van de parasiet onderzocht in experimenteel geïnfecteerde varkens en werden *T. gondii* stammen geïsoleerd uit Belgische varkens en vervolgens gegenotypeerd.

De literatuurstudie (**Deel 1**) schetst een ruim overzicht van het reeds gepubliceerd onderzoek omtrent de voornaamste kenmerken van *T. gondii* die deze parasiet tot één van de meest succesvolle parasieten ter wereld maakt. Het eerste hoofdstuk (**Hoofstuk I**) beschrijft de morfologie en biologie van de verschillende infectieuze vormen van *T. gondii*, als ook de complexe levenscyclus met tussen- en eindgastheren. Hierop volgt een kort overzicht van de moleculaire epidemiologie.

Hoofdstuk II beschrijft de voornaamste transmissieroutes tussen enerzijds tussen- en eindgastheren en anderzijds tussengastheren onderling. Verder wordt de significante impact van de parasiet op de gezondheid van mens en dier beschreven en de economische belangen geassocieerd met de volksgezondheid.

Tot slot wordt in **Hoofdstuk III** de verschillende aanpakken en technieken voor de diagnose van *T. gondii* beschreven, met aandacht voor de voordelen en beperkingen bij het aantonen van recente of oude infecties, en de aan- of afwezigheid van de parasiet in de weefsels.

Het experimenteel gedeelte (**Deel II**) van deze thesis begint met een situering van de

hoofdproblemen behandeld in deze thesis en de doelstellingen van dit doctoraatsonderzoek. Verder worden de verschillende experimentele studies die een antwoord bieden aan deze doelstellingen in detail beschreven.

De algemene doelstelling van deze thesis is de studie naar de rol van varkens en varkensvleesproducten in de transmissie van *T. gondii* naar de mens. De specifieke doelstellingen waren de ontwikkeling van een efficiënte en gevoelige ISO-gevalideerde moleculaire methode voor de detectie van *T. gondii* in verschillende weefsels, de studie van de infectiekinetieken in experimenteel en natuurlijk besmette varkens zodat de rol van varkensvlees als infectiebron beter ingeschat kan worden en tot slot de detectie, isolatie en typering van de circulerende stammen in de varkenspopulatie.

In **Hoofdstuk IV** wordt een efficiënte en ISO 17025-gevalideerde MC-qPCR methode voorgesteld voor de detectie van achetypische *T. gondii* stammen in vlees, deze methode werd gepubliceerd in de International Journal for Parasitology (Gisbert Algaba, et al.,2017). Deze methode combineert de hybridisatie van specifieke, gebiotinyleerde proben met het vangen van die proben met streptavidin-coated paramagnetische beads. Vervolgens wordt het target-DNA losgekoppeld van de beads door blootstelling aan UV-licht. Op deze manier kan *T. gondii*-DNA op selectieve wijze worden geconcentreerd in een groot staalvolume, waardoor de gevoeligheid van de techniek significant toeneemt. De resultaten toonden aan dat de methode gevoeliger (99% LOD van 65.4 tachyzoïten per 100 g vlees en sensitiviteit: 94.12 %), economischer en betrouwbaarder is ten opzichte van de referentiemethode, namelijk de bio-assay. Deze resultaten demonstreren het potentieel van deze methode als alternatief voor de bio-assay op basis van proefmuizen voor de screening van verschillende weefsels en vlees. Daarenboven bezit de MC-qPCR methode een extra voordeel, namelijk de mogelijkheid tot kwantificatie.

Hoofdstuk V beschrijft het effect van verschillende *T. gondii* stammen en infectieuze vormen op de parasitaire belading van varkenskarkassen (Gisbert Algaba et al., aanvaard). In deze studie wordt de MC-qPCR gebruikt voor de kwantificatie van de parasiet (parasitic load) tijdens de acute (0-28 dagen na infectie) en chronische (3-4 maand na infectie) fase van de infectie. Tevens werd de parasitaire belading vergeleken in functie van de verschillende infectieuze vormen en stammen gebruikt tijdens de experimentele infectie van de varkens. De hoogste parasitaire belading werd teruggevonden in het hart- en longweefsel tijdens de acute fase van de infectie. Terwijl tijdens de chronische fase de hoogste belading werd teruggevonden in hart- en hersenweefsel. De gebruikte

infectieuze vorm en stam hadden bij beide waarnemingen geen invloed.
Er werd een opmerkelijk hogere parasitaire belading gemeten in de verschillende weefsels tijdens de chronische fase na infectie met oöcysten in vergelijking met weefselcysten. Geen significante verschillen in parasitaire belading werden waargenomen door het gebruik van de twee verschillende *T. gondii* stammen.

Hoofdstuk VI beschrijft de moleculaire epidemiologie van *T. gondii* isolaten afkomstig van Belgische patiënten en varkens (Gisbert Algaba et al., ingediend). In deze studie wordt gefocust op de detectie en isolatie van de verschillende *T. gondii* stammen in varkens en de vergelijking van deze stammen met deze geïsoleerd in het kader van het Nationaal Referentie Centrum voor Toxoplasmose in België. In de huidige studie testten 14 van de 92 harten afkomstig van varkens, opgekweekt op bio-boerderijen, positief aan de hand van de MC-qPCR. Dit toont de aanwezigheid aan van de parasiet in dieren bestemd voor humane consumptie. De sterk gerelateerde populatiestructuur van de stammen geïsoleerd gedurende 2015 en 2016 uit mensen en varkens (24/24 type II strains) geeft de mogelijkse stroom van stammen weer vanuit de varkens naar de mens. Deze bevindingen versterken de identificatie van het varken als potentieel risico voor de consument en illustreert het hoger risico dat varkens van bio-boerderijen vormen voor de consument met betrekking tot *T. gondii* infectie.

Hoofdstuk VII omvat de algemene discussie waarin de bijdrage van de gepresenteerde studies voor een beter inzicht en bewustzijn van de humane en veterinaire toxoplasmosis wordt benadrukt. In het bijzonder wordt de identificatie van het varken en zijn sleutelrol als humane infectiebron besproken gebruikmakend van de MC-qPCR, een heel gevoelige moleculaire methode (sensitiviteit: 94.12%) in staat om één weefselcyste per 100 gram vlees te detecteren. Deze methode is echter niet in staat te differentiëren tussen levende en dode parasieten. Andere belangrijke vragen en nieuwe potentiële aanpakken voor de detectie van *T. gondii* worden in dit hoofdstuk beschreven.

Dit doctoraatsonderzoek stelt een nieuwe assay voor die de detectie van *T. gondii* aan de hand van de bio-assay kan vervangen. De assay maakt de het mogelijk om de verspreiding van de parasiet over de weefsels van de gastheer vast te stellen zowel tijdens de acute, als de chronische fase van de infectie. Bovendien is het mogelijk het effect van verschillende stammen en infectieuze vormen van de parasiet op de parasitaire belading van de weefsels te bestuderen. Verder toonde de moleculaire epidemiologische studie een sterk gerelateerde populatiestructuur aan. Waarbij alle humane en varkensisolaten werden geclassificeerd als type II stammen.

Curriculum Vitae

Ignacio Gisbert Algaba was born on December 22nd 1988 in Valencia, Spain. He started his studies in the Faculty of Veterinary Sciences UCH-CEU in Valencia, Spain in September 2006. After graduating as a DVM in June 2012, he was awarded a Leonardo Grant for young graduates to obtain his first work experience as a research assistant in the Department of Veterinary Public Health at the Faculty of Veterinary Medicine of Ghent University, Belgium. There, he acquired a broad knowledge in microbiology and several molecular techniques and assisted PhD students with their research in different food pathogens such as, *Campylobacter*, *Yersinia* and *Salmonella*.

In 2014, he was enrolled as a PhD student at the Department of Virology, Parasitology and Immunology of the Faculty of Veterinary Medicine, Ghent University in the framework of the "TOXOSAFE project" (RF 13/6274), funded by the Belgian Federal Public Service for Health, Food Chain Safety and Environment. The TOXOSAFE project was focused on multiple aspects of the natural and experimental infection with *Toxoplasma gondii* in pigs. As a PhD student, Ignacio performed his research at the Scientific Institute of Public Health (WIV-ISP) in Brussels. In addition to his doctoral studies, he was actively involved with the daily working of the Toxoplasmosis department at the WIV-ISP as part of the NRC for congenital diseases in Belgium.

Ignacio Gisbert Algaba is an author and co-author of several publications in peer-reviewed international journals and he actively participated in national and international conferences, presenting his research.

Scientific publications

Seliwiorstow, T., Baré J., Van Damme I., **Gisbert Algaba I.,** Uyttendaele M., De Zutter L., 2016. Transfer of Campylobacter from a Positive Batch to Broiler Carcasses of a Subsequently Slaughtered Negative Batch: A Quantitative Approach. *Journal of Food Protection* 79, no. 6: 896–901.

Gisbert Algaba, I., Geerts, M., Jennes, M., Coucke, W., Opsteegh, M., Cox, E., Dorny, P., Dierick, K., De Craeye, S., 2017. A more sensitive, efficient and ISO 17025 validated Magnetic Capture real time PCR method for the detection of archetypal Toxoplasma gondii strains in meat. Int. J. Parasitol.

Gisbert Algaba I., Verhaegen B., Jennes M., Raman M., Coucke W., Cox E., Dorny P., Dierick K., De Craeye S., 2018. Pork as a source of transmission of Toxoplasma gondii to humans: a parasite burden study in pig tissues after infection with different strains of Toxoplasma gondii in function of time and different parasite stages (accepted for publication *in Int. J. Parasitol*).

Gisbert Algaba I., Murat J. B., Coucke W., Mercier A., Cox E., Dorny P., Dierick K., De Craeye S. Molecular epidemiology of *Toxoplasma gondii* isolates originating from Belgian pigs and humans (In preparation).

Jennes M., Rahman M., **Gisbert Algaba I.,** Nguyen U., De Craeye S., Dierick K., Dorny P., Cox E. In vitro interferon-gamma induction by TLA-fractions in an acute and chronic model of *Toxoplasma gondii* infection in pigs (In preparation).

Rahman M., Jennes M., **Gisbert Algaba I**., Devriendt B., Dorny P., Dierick K., De Craeye D., Cox E. Identification of novel vaccine antigens for immunization against *Toxoplasma gondii* (in preparation)

Abstracts, posters and oral presentations

Gisbert Algaba I*., De Craeye S.*, Cox E., Dorny P., Dierick K. An improved and ISO 17025 accredited direct detection method for *T. gondii* in meat. EMOP 12th Congress, 20-24 July, Turku, Finland. Oral presentation

Jennes M., Rahman M., De Craeye S., **Gisbert Algaba I.,** Dorny P., Dierick K., Cox E. Lymphocyte subsets activation by *Toxoplasma gondii* antigens. 3rd International Veterinary Congress, 18-20 August 2016, London, UK. Oral presentation.

Gisbert Algaba I., Verhaegen B., Delbrassinne L., Botteldoorn N., De Craeye S. Demonstration of the presence of *T. gondii* in bio pigs intended for human consumption in Belgium. 4th Apicowplexa congress, 11-14 October 2017, Madrid, Spain. Poster

Gisbert Algaba I., Cox E., Dorny P., Dierick K., De Craeye S. Zoonoses parasitaires: transmission de *T. gondii* du porc à l'homme ? 10ème symposium annuel, Recherche contractuelle- DG Animaux, Végétaux et Alimentation, 25-26 October 2017, Brussels, Belgium. Oral presentation

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