Novel insights in the host-pathogen interaction of porcine toxoplasmosis

Malgorzata Jennes

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Laboratory for Immunology

Department of Virology, Parasitology and Immunology

Faculty of Veterinary Medicine

Ghent University

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Promoters: prof. dr. Eric Cox

prof. dr. Pierre Dorny

dr. Stéphane De Craeye

Malgorzata Jennes 2017

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"The ability to perceive or think differently is more important than the knowledge gained"

David Bohm

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Promoters

Prof. Dr. Eric Cox

Faculty of Veterinary Medicine, Ghent University

Prof. Dr. Pierre Dorny

Faculty of Veterinary Medicine, Ghent University

Institute of Tropical Medicine, Antwerp

Dr. Stéphane De Craeye

Scientific Institute of Public Health, Brussels

Members of the Examination Committee

Prof. Dr. Richard Ducatelle

Chairman of the Examination Committee

Faculty of Veterinary Medicine, Ghent University

Prof. Dr. Lieven De Zutter

Faculty of Veterinary Medicine, Ghent University

Dr. Katelijne Dierick

Scientific Institute of Public Health, Brussels

Dr. Brecht Devleesschauwer

Scientific Institute of Public Health, Brussels

Dr. Steven Sarazin

Faculty of Veterinary Medicine, Ghent University

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List of abbreviations

Ab: antibody
AP: apparent prevalence
β-actin
BCA: bicinchoninic acid assay
CD: cluster of differentiation
CI: confidence interval
ConA: Concanavalin A
Cp: crossing point
CT: congenital toxoplasmosis
DALY's: Disability Adjusted Life Years
DC: dendritic cell
∆ Cp: delta crossing point
dpi: days post infection
DT: Sabin-Feldman Dye Test
EDTA: ethylenediaminetetraacetic acid
ELISA: Enzyme Linked Immuno-Sorbent Assay
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
BP: guanylate-binding protein
GRA: dense granule protein
GTPase: guanosine triphosphatase
HE: hematoxyline-eosine
HRP: horseradish peroxidase
IDO: indoleamine 2,3-dioxygenase
IFA: immunofluorescent assay
IFN-γ: interferon-gamma
Ig: immunoglobuline
IL: interleukine
IPB-G: Gangji strain
IPB-LR: LR strain
IRG: immunity-related GTPase
LAT: latex agglutination
MAT: modified agglutination assay
MIC: microneme proteins
min: minute(s)
mpi: months post infection
NK: natural killer cell
NO: nitric oxide
NT: neurotoxoplasmosis

OD: optical density
OT: ocular toxoplasmosis
PBMC: peripheral blood monomorphonuclear cells
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PE: phycoerythrin
PerCP: Peridinin Chlorophyll protein
PV: parasitophorus vacuole
RT: room temperature
ROP: rhoptry protein
qPCR: real time quantitative polymerase chain reaction
SAG: surface antigen
SD: standard deviation
Se: sensitivity
Sp: specificity
TE: toxoplasmic encephalitis
Th1: T-helper 1 cell
Th2: T-helper 2 cell
TLA: *Toxoplasma*total lysate antigen
TMB: 3,3'5,5'-tetramethylbenzidine
TNF-α: Tumor Necrosis Factor
TRC: *T. gondii* induced retinochoroiditis
UI: uncertainty interval
wpi: weeks post infection

Part I

Review of the literature

Chapter 1 Toxoplasma gondii

1.1 Introduction

Toxoplasma gondii (*T. gondii*) is an obligatory intracellular parasite, present across the globe and causing an infection in all warm-blooded animals, among which are aquatic and terrestrial mammals and birds. The prevalence of toxoplasmosis in humans varies considerably between countries or ethnic groups but reaches up to 50% of the worldwide population, regardless of the continent, economic status of the countries or environmental factors such as climate, humidity or altitude (Tenter et al., 2000; Gamarra et al., 2008; Halonen and Weiss, 2009; Shapiro et al., 2010; Robert-Gangneux and Dardé, 2012; Flegr et al., 2014).

1.1.1 History

The discovery of the parasite dates to the early 20th century, when it was separately isolated from blood, spleen and liver of a gundi (*Ctenodactylus gundi*) in North Africa and a rabbit in Brasil (Nicolle and Manceaux, 1908; Splendore, 1908). The name of "*Toxoplasma gondii*" was introduced for the first time in 1909 and refers to the shape in which the parasite was observed ("toxo": an arch or bow, "plasma": life), combined with the name of the African rodent gundi (Black and Boothroyd, 2000; Weiss and Dubey, 2009a).

The first description of a human infection with severe clinical symptoms caused by the parasite was made in 1923 by Janků (1923), while the first case of fatal congenital toxoplasmosis (CT) was reported in 1939 by Abner Wolf et al. (1939). Since then the attention for the parasite and the attempts for the development of a successful vaccine significantly increased in both human and veterinary sciences. In addition to severe outcomes of the infection in human patients, the parasite was found responsible for abortions and death in ewes, and therefore for high economical losses (Dubey and Jones, 2008). Frenkel, Hutchison and Dubey revealed the full life cycle within the Felidae, the final host, and the distinct developmental stages (Frenkel et al., 1970;

Hutchison et al., 1970; Dubey, 1970b), in the second half of the 20th century by the isolation of the parasite from naturally infected hosts.

1.1.2 Taxonomy

T. gondii represents the only species within the genus *Toxoplasma*. The subsequent taxonomic groups refer to a unicellular organism with the distinct nuclear membrane and membrane bound organelles (*Eukaryota*), small submembranous cavities or alveoli (*Alveolata*), and a unique plastid-like organelle or the apicoplast (*Apicomplexa*), together with a cluster of microtubules and organelles in the apical complex (Levine, 1970; Cavallier-Smith, 1993; Cooper and Hausmann, 2015). *T. gondii* as the member of the class *Conoidasida* possesses a conoid or a motile structure, composed of fibers arranged as spiral inside the polar rings, and serving for penetration into the host cell. Further, it belongs to the subclass *Coccidiasina*, since it is an obligatory intracellular parasite of the vertebrates, with a sexual multiplication step in the gastrointestinal tract of the host, followed by spores stage in the environment and cysts in the tissues of the intermediate host (Sarcocystidae) (Kreier, 1993; Bertolino et al., 2003).
The full taxonomy is presented in Table 1.

Table 1.1 : Taxonomy of *T. gondii*
(from http://www.ncbi.nlm.nih.gov/Taxonomy/)

Domain	*Eukaryota*
Supergroup	*Chromoalveolata*
Kingdom	*Alveolata*
Phylum	*Apicomplexa*
Class	*Conoidasida*
Subclass	*Coccidiasina*
Order	*Eucoccidiorida*
Suborder	*Eimeriorina*
Family	*Sarcocystidae*
Genus	*Toxoplasma*
Species	***T. gondii***

1.1.2 Genetic diversity

Due to the high clinical relevance and the search for a successful treatment and prevention, the parasite was isolated from the naturally infected animals and humans for molecular analysis of the parasitic genome using PCR, restriction fragment length polymorphism (RFLP), or random amplified polymorphism DNA (Fuentes et al. 2001, Dardé et al. 1995, Guo et al. 1995a, Howe et al. 1997, Sibley et al. 1992). The obtained data led to genetic identification of the isolated parasites, and, therefore, allowed to distinguish three major multilocus genotypes of *T. gondii*.

The genetic lines named I, II, and III, correspond to the genetic analysis of the polymorphic surface antigen 2 locus (*SAG2*) (Howe et al. 1997) and are characterized by a different degree of virulence and pathogenicity in mice. Type I is considered as the most virulent genotype, followed by type III and II. The latter is the most prevalent genotype present in the animal reservoir and human population in Europe. Additionally, some of the strains isolated from naturally infected humans or wild and

domestic animals in South America, Africa and Asia do not fit into these three lineages, showing a highly divergent genotype or a mix of type I, II and/or III. Consequently, new genotypes were identified worldwide such as a type IV in North America, type I in Asia, type I to III in Africa, and type II, III and IV in Brazil (Ferguson, 2009; Herrmann et al., 2010; Dubey et al., 2011; Robert-Gangneux and Dardé, 2012; Turčeková et al., 2013; Dardé et al., 2014; Ajzenberg et al., 2016). The recently identified strains show often an increased virulence in mice and sometimes in humans, suggesting the existence of new atypical haplogroups (Saeij et al., 2005; Innes, 2010).

Despite the genetic heterogeneity between the strains, the *T. gondii* genome is uniformly haploid during the main developmental stages (except for the oocyst phase) and consists of three major DNA components: nuclear (87 Megabases (Mb)), mitochondrial (linear and omega shaped, 6 to 8 Mb) and apicoplastic (circular 35 Mb) (Blader and Saeij, 2009; Liting and McFadden, 2011; Ramakrishnan et al., 2012; Reiff et al., 2012).

1.2 Life cycle and the infectious stages

The life cycle of *T. gondii* is complex, since this obligatory intracellular coccidian parasite operates in a hunter-prey system that alternates between final and intermediate hosts, which are correlated with a sexual and asexual reproduction mode, respectively (Dubey, 1998b; Black and Boothroyd, 2000; Robert-Gangneux and Dardé, 2012). The life cycle consists of an entero-epithelial and extra-intestinal part, involving at least two or more hosts (Figure 1.1) (Ferguson, 2008; Robert-Gangneux and Dardé, 2012; Halonen and Weiss, 2013).

The sexual reproductive stage (or forming of gametocytes) proceeds exclusively in enterocytes of the small intestine of the Felidae, represented by domestic or wild cats and other members of the Felidae family (Ajzenberg et al., 2004; Saeij, 2005; Ferguson, 2009; Beck et al., 2009).

The asexual part of the life cycle may virtually take place in any nucleated cell of all endothermic terrestrial and aquatic animals, including domestic and wild mammals, birds and humans.

In order to accomplish the sexual multiplication and complete the life cycle, *T. gondii* has a multi-stage development, consisting of the following transmissible forms, described below: 1) the gametocytes, which are the protagonists of the sexual reproduction by anisogamy in the final host; 2) the oocysts, which are formed in the definitive host and are shed as non-sporulated, hence, not-infectious, to the environment; 3) the sporozoites, formed after sporulation of the oocysts in the environment; 4) the highly infectious tachyzoites, which are the motile and fast-multiplying form in the intermediate hosts and 5) the latent bradyzoites or the slow asexually replicating form inside tissue cysts in the final or the intermediate hosts (Kortbeek, 1999; Tenter et al., 2000; Van der Giessen et al., 2003; Sibley, 2009; Turner et al., 2013; White et al., 2014).

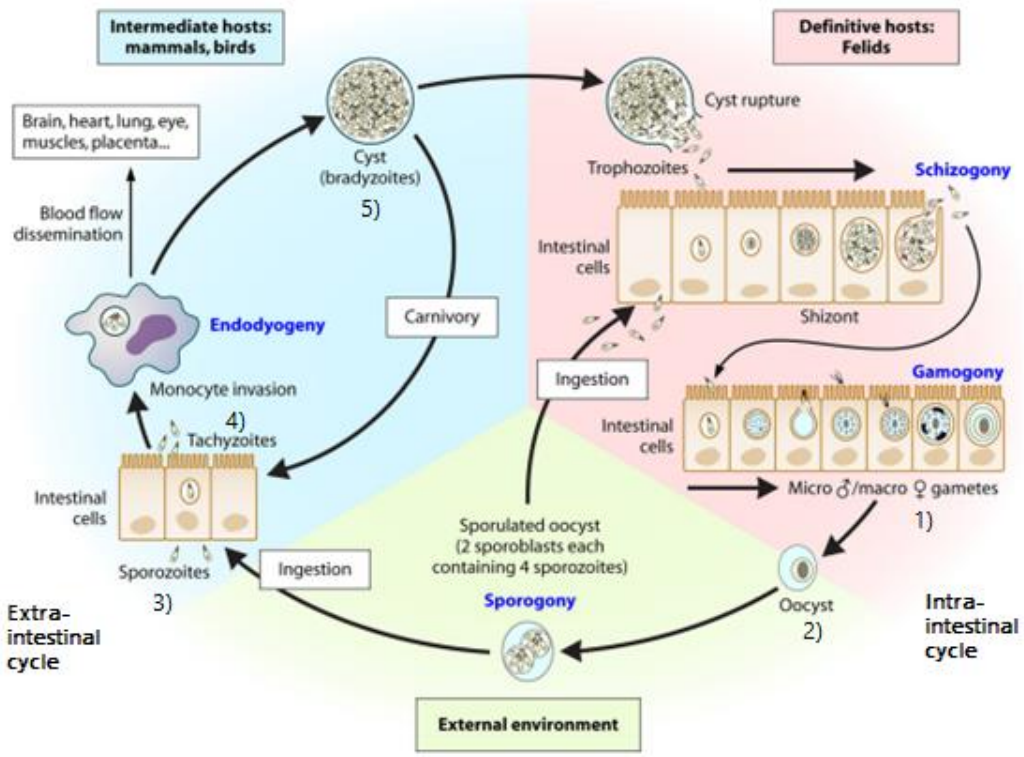


Figure 1.1 : Life cycle of *T. gondii* (adapted from: Robert-Gangneux and Dardé, 2012). The numbers refer to the transmissible forms of the parasite.

1.2.1 Entero-epithelial cycle

As indicated above, the sexual stage of the life cycle of *T. gondii* appears in the intestinal tract of the Felidae. The final host sheds oocysts upon ingestion of one of the three infectious stages: tachyzoites, bradyzoites or sporozoites. The prepatent period (the time between initial infection of the final host and the excretion of oocysts) is dependent on the parasitic stage that had been ingested: it comprises 3 to 10 days after ingestion of bradyzoites, more than 13 days after ingestion of tachyzoites and longer than 18 days after ingestion of sporulated oocysts (Dubey, 1998a; Hill et al., 2005, Dubey, 2008a). However, not each infected animal will develop toxoplasmosis with shedding of the oocysts. Since the tachyzoites are sensitive to challenging environmental conditions, and are not resistant to the action of the gastric enzymes, eventually less than 30% of the tachyzoites- or oocyst-infected cats will excrete oocysts as the result of the completed life cycle, while nearly all cats will excrete oocysts after intake of tissue cysts (Dubey et al, 1998; Dubey, 2004).

Once the cysts derived from tissues of the intermediate host are ingested, gastric enzymes will degrade the cyst wall. The released bradyzoites will then penetrate the epithelial cells of the small intestine, where several rounds of asexual replication take place, which is characterized by the development of several generations of schizonts containing merozoites (schizogony and merogony).

Schizogony proceeds within enterocytes upon colonization by the tachyzoites, which were generated by the released bradyzoites. There are five consecutive stages (A to E) of the schizogony or the asexual reproduction. Inside each schizont (also called meront) the first generation of merozoites is produced, which eventually are released from the schizont and then colonize new epithelial cells.

This event is the first step of the entero-epithelial cycle, occurring 2 days after the ingestion of the tissue cysts, and followed by the formation of male and female micro- and macrogametes, respectively (gametogony).

Gametocytes and non-sporulated oocysts

Starting from 2 days after ingestion of the parasite the first generation of merozoites colonizes new epithelial cells and, instead of becoming schizonts, proceeds with the formation of a second generation of merozoites (also called gamonts) by gametogony. These merozoites will differentiate either into a microgametocyte or a macrogametocyte. The microgametocyte (defined as microgamete after maturation) multiplies until the cell breaks, liberating the microgametes, which are motile and translocate in order to find the epithelial cell containing the macrogamete. Thereafter, the microgamete penetrates the cell and fertilization occurs, resulting in the formation of the zygote. After fertilization of the macrogamete with a microgamete a zygote is formed with a resistant oocyst wall (Hill et al, 2005; Robert Gangneux and Dardé, 2012). The multiple zygotes break the epithelial cells and form oocysts, which are simultaneously shed first into the intestinal lumen of the final host, and subsequently to the environment with the faeces. Shedding of oocysts usually starts 3 to 7 days after the ingestion of tissue cysts and can continue during 7 to 20 days post initial infection (with a variation of 2 to 10 days). On average, at least 100 million oocysts may be excreted, resulting in a severe environmental contamination. The unsporulated oocyst or zygote has a spherical shape, from 10 to 12 µm of diameter, a large nucleus with an amorphous nucleoplasm, and contains one sporoblast (Figure 1.2) (Dubey, 1998a).

Sporulated oocysts

Within five days after shedding of the oocysts to the environment the sporulation is initiated, driven by a meiosis division process resulting in a haploid sporulated oocyst. The following events proceed during the sporulation in the environment, namely the sporoblast is divided by sporogony within the oocyst in two sporoblasts with four sporozoites inside (Dubey et al., 1998a; Ferguson, 2009). The duration of sporulation varies from 1 to 24 days, and is dependent on the temperature, humidity and the presence of oxygen, as oocysts sporulate between 24 to 28 hours after excretion at

25°C, 2 to 5 days at 15°C and 21 days at 11°C (Dubey et al., 1970; Kortbeek, 1999; Van der Giessen et al., 2003; Torrey and Yolken, 2013).

Sporulated oocysts are spherical to oval in shape and have a size of 11-13 µm. They consist of two sporocysts (6-8 µm), each containing four haploid sporozoites (2 to 6 µm) (Dubey et al., 1998a; Dubey, 2009a; Ferguson, 2009; Poukchanski et al., 2013).

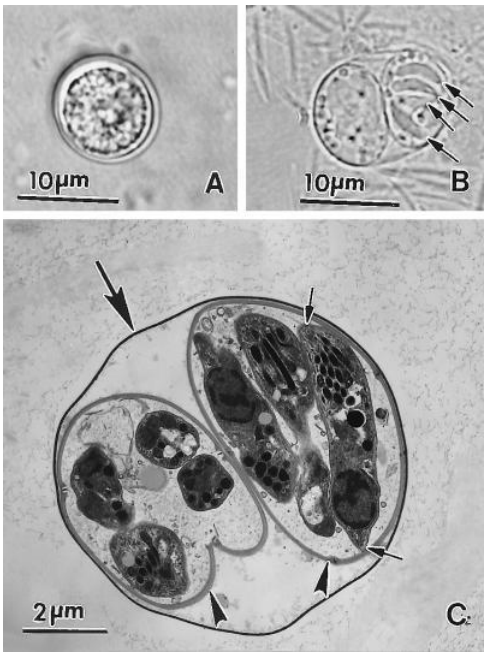


Figure 1.2 : Oocysts: A) unsporulated oocyst, B) sporulated oocyst with two sporocysts (arrows indicate four sporozoites), C) sporulated oocyst where large arrow indicates the thin oocyst wall, arrowheads show the two sporocysts and small arrows present sporozoites where one is longitudinally cut (from Dubey et al., 1998a).

The wall of a sporulated oocyst is extremely robust and multi-layered, ensuring the resistance against chemical and physical factors such as UV-light, ozone and chlorine-based products. Under humid and warm circumstances the oocysts remain infectious during 1 to 2 years in soil, or during 2 and 4.5 years in salt and in surface water, respectively (Tenter et al., 2000; Van Wormer et al., 2013). When altering the conditions to 4° C, the oocysts persist without the loss of infectious capacity up to 54 months, while in a hot and a dry environment they become inactivated already after one minute

(Dubey et al., 1998a; Ferguson, 2009; Robert-Gangneux and Dardé, 2012; Dumètre et al., 2013).

The feline host acquires a protective immunity after the primary infection, however the immunity does possibly not have a lifelong span. In some cases and especially in immunocompromised older animals with viral or bacterial co-infections, a second excretion period may occur after reinfection (Van der Giessen et al., 2003, De Craeye et al., 2008).

1.2.2 Extra-intestinal cycle

The extra-intestinal cycle of *T. gondii* is initiated by the ingestion of sporulated oocysts from the environment, or by predation of chronically infected intermediate hosts. This part of the life cycle can proceed in both the final (Felidae) and the intermediate (carnivorous and herbivorous mammals) host, including humans (Ajzenberg et al., 2004; Saeij et al., 2005; Beck et al., 2009).

The developmental stages present here are the tachyzoites and the bradyzoites, which share some morphological and physiological characteristics with the sporozoites such as the size, sickle shape and the division by endodyogeny within the host's cell. Nevertheless, the main difference between the two former parasitic forms is the speed of the multiplication, corresponding to the subsequent phases of the infection (Black en Boothroyd, 2000).

Tachyzoites

The tachyzoites are the first and asexual stage of the extra-intestinal cycle, representing the acute phase of the infection. They are the rapid multiplying stage in any nucleated cell of the intermediate hosts and in non-intestinal epithelial cells of the definite host. Their name refers to the speed of replication (Greek for 'fast') (Dubey, 2008a).

A tachyzoite has a half-moon shape, is 2 to 6 µm long and contains different organelles such as the nucleus, the endoplasmatic reticulum with associated Golgi apparatus, mitochondria, the apicoplast, involved in protein and fatty acids synthesis, and multiple

inclusion bodies (Figure 1.3) (Dubey et al., 1998b). In comparison with the sporozoites, there are more abundant secretory organelles such as micronemes, rhoptries and amylopectin granules in tachyzoites, which secrete proteolytic enzymes, necessary for the host's cell penetration. These organelles also contribute to an appropriate environment for growth and development of the parasite in the infected cells (McFadden, 2011). Tachyzoites lack any motility organ like cilia or flagella, but they can move by rotation, undulation, flexing or gliding (Chiappino et al., 1984).

The tachyzoites differentiate from sporozoites upon ingestion of the oocyst, or from bradyzoites within the tissue cysts, and, when released, subsequently penetrate any nucleated cell, multiply and disseminate further.

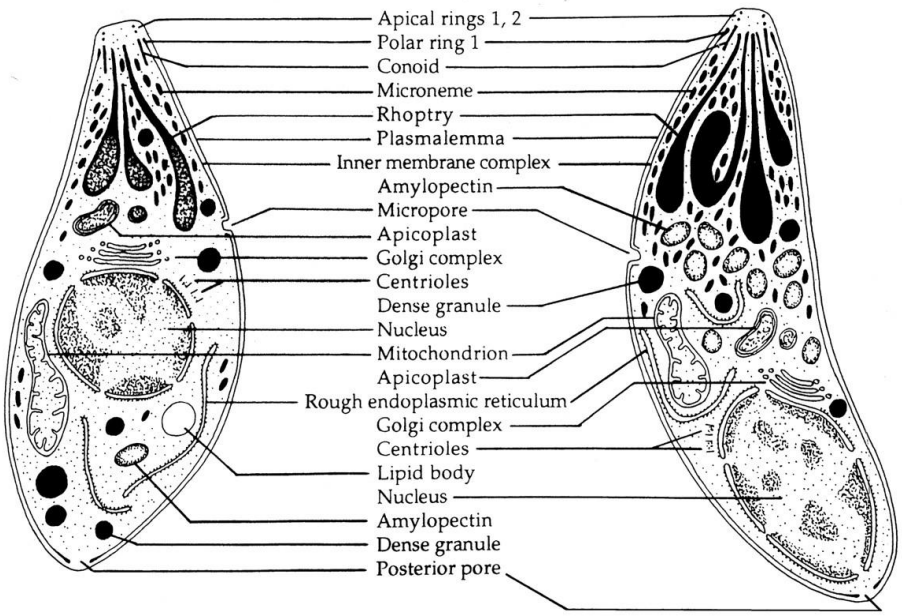


Figure 1.3 : Tachyzoite (left) and bradyzoite (right) (from Dubey et al., 1998b).

When the host's cell is being invaded by phagocytosis or by an active penetration, initial recognition facilitated by surface antigens (SAGs) leads to adhesion of the parasite to the cell via MIC proteins. Soon after that stage a parasitophorous vacuole (PV) is established, by rhoptry proteins (ROP) secretion and conoid movements, and consisting of the host's and parasitic components. Upon forming the PV, replication of

the parasitic DNA follows every six to nine hours, prior to cell division (Kafsack et al., 2007).

Tachyzoites multiply very rapidly in all nucleated cells by endodyogeny, with daughter cells still in the original cell during the acute phase of the infection (Dubey et al., 1998b; Black and Boothroyd, 2000; Robert-Gangneux and Dardé, 2012).

The multiplication continues until the cell bursts, releasing the tachyzoites to the blood stream in order to disseminate the infection, with the preference for the central nervous system, eye, skeletal muscles and heart. The process leads indirectly to destruction of the initially infected cells and invasion of the others (Dubey et al., 1998b; Black and Boothroyd, 2000; Montoya and Liesenfeld, 2004).

In the final stage of the division and 1 to 2 weeks (7 to 10 days) after the initial infection, the tachyzoites convert to bradyzoites and generate tissue cysts (see further Bradyzoites and tissue cysts) (Van der Giessen et al., 2003; Ferguson, 2009).

Bradyzoites and tissue cysts

Bradyzoites are the slow multiplying stage of *T. gondii*, and their name is associated with the prolonged speed of replication (Greek for 'slow') (Dubey, 2008a). They are present in the chronic phase of the infection, showing a very low level of metabolic activity; they can persist life-long within the host (Kortbeek, 1999; Black and Boothroyd, 2000; Dubey, 2008a).

Bradyzoites are sickle shaped, 7 by 1.5 µm in size, and are enclosed per hundreds within a tissue cyst. The bradyzoite is morphologically very similar to tachyzoite; among others, it is slightly thinner and less susceptible to degradation by proteolytic enzymes than a tachyzoite (Figure 1.3). Additionally, the prepatent period in cats after infection with bradyzoites is shorter than for tachyzoites: 3-10 and >10 days, respectively (Dubey, 2004; Hill et al., 2005; Dubey, 2008a).

Despite the morphological and physiological differences, bradyzoites also multiply by endodyogeny inside the tissue cyst (Dubey et al., 1998b; Black and Boothroyd, 2000; Robert-Gangneux and Dardé, 2012).

The tissue cysts develop intracellularly within the nucleated cell cytoplasm of the intermediate or final hosts. In reference to the infected cell type and the age of the cyst, a young tissue cyst has a 5 µm diameter and contains two bradyzoites, while more mature cyst can contain multiple hundreds or thousands of them, having a spheroidal (70 µm of diameter) or elongated (100 µm of diameter) shape, when present in the brain or in the intramuscular tissue, respectively (Figure 1.4) (Dubey et al., 1998b; Robert-Gangneux and Dardé, 2012).

Tissue cysts reside predominantly lifelong in neural and muscular tissues like brain, eyes and skeletal and cardiac muscles, causing a very limited and local inflammatory response of the host. The tissue cyst wall is composed of host cell and parasite materials (Dubey et al., 1998b; Ferguson, 2009). Reactivation of the resting tissue cysts might occur in individuals with a temporarily or permanently compromised immune system (Dubey et al., 1998b; Black and Boothroyd, 2000; Van der Giessen et al., 2003; Montoya and Liesenfeld, 2004).

As described earlier, due to the predation or ingestion of raw or undercooked infected meat, the tissue cysts reach the digestive tract of the final or the intermediate hosts, where cysts are disrupted liberating bradyzoites. These bradyzoites initiate the asexual cycle in the intermediate host by subsequently infecting the intestinal epithelium of the new host, differentiating into tachyzoites and disseminating in the body, or they give rise to several generations of merozoites for the sexual multiplication in the final host, resulting in the gametocytes production, followed by oocyst shedding (Black and Boothroyd, 2000; Dubey, 2009a; Innes, 2010; Robert-Gangneux and Dardé, 2012).

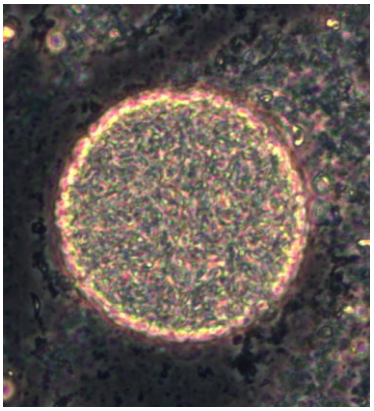


Figure 1.4 : Tissue cyst with bradyzoites (courtesy of dr S. De Craeye, National Reference Laboratory for Toxoplasmosis, WIV-ISP, Brussels, Belgium).

1.3 Toxoplasmosis as a zoonotic disease

As indicated above, *T. gondii* is widespread in all warm-blooded animals (carnivores and herbivores). The infection is initiated within the final host and is passed onto a variety of the intermediate hosts including humans via several transmission routes (Figure 1.5). To date, not much data is described on the pathogenesis or clinical symptoms of the natural acute or chronic infection in pigs. Although the thesis mainly discusses the porcine toxoplasmosis and the associated immune responses and the dissemination of the parasite, the well-known human infection served as a model to study the corresponding events in the porcine host.

1.3.1 Transmission routes between and within the hosts

From the evolutionary point of view, the original infection path, representing a full life cycle of the parasite, is limited to two hosts: members of the Felidae family and small animals such as rodents or birds. The intermediate host ingests the oocysts spread in the environment with the faeces of the cat and, depending on the virulence of the strain, dies due to acute toxoplasmosis or survives after subclinical infection but becomes chronically infected. The tissue cysts developed during the chronic phase

contain bradyzoites, which reach the final host by predation of the chronically infected rodent and by this close the life cycle of *T. gondii*.

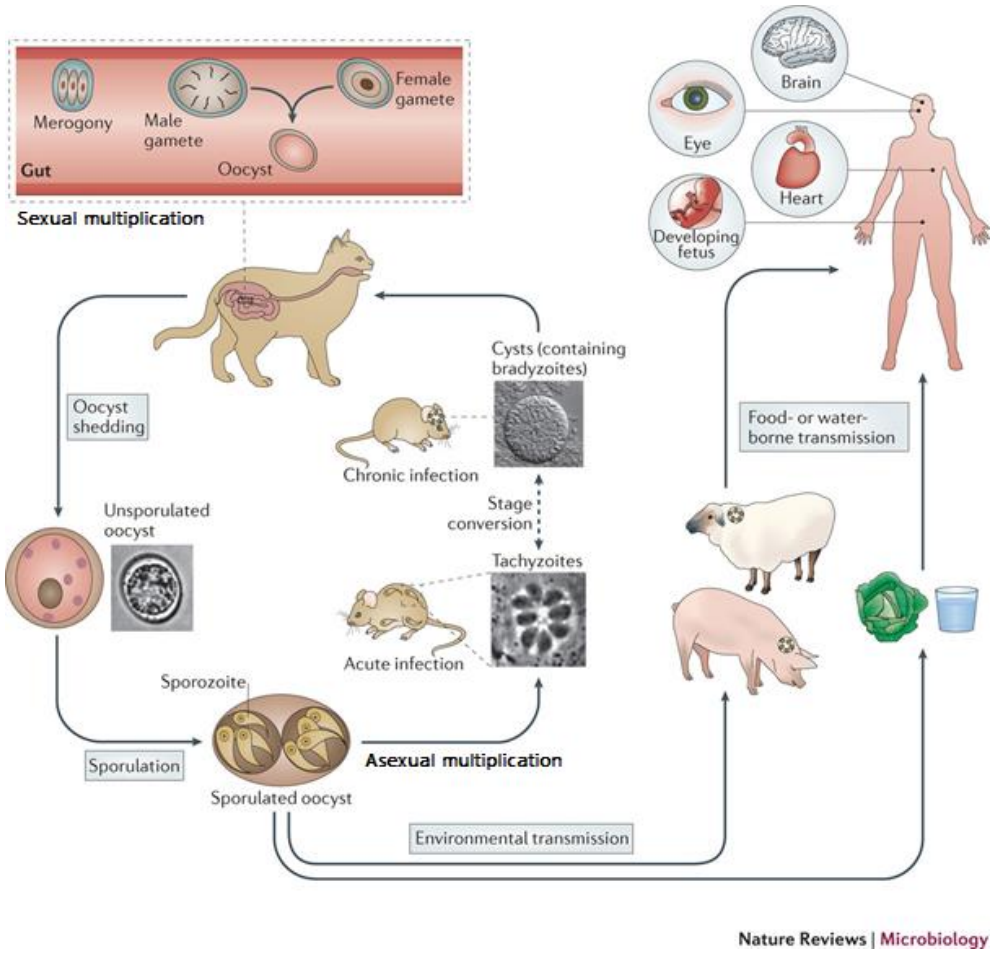


Figure 1.5 : The life cycle of *T. gondii* (adapted from: Hunter and Sibley, 2012).

Also other mammals next to small rodents, including humans, can accidentally ingest the oocysts due to contamination of grass, vegetables or water with cat feces, and serve as such as intermediate hosts for the parasite. Due to the fact that oocysts are shed in a very high amount, heavily contaminating the environment, together with their prolonged viability, ingestion of the oocysts is a major infection source for humans (Opsteegh et al., 2016b). An alternative infection route for the carnivorous mammals and humans is the predation or consumption of the chronically infected intermediate hosts such as wild and domestic animals. In this case, the ingestion of the tissue cysts present in the muscles and organs will lead to the conversion of bradyzoites into

tachyzoites in the intestines followed by systemic dissemination, and may result in the development of a new generation of latent tissue cysts.

Both these sources of infection refer to a horizontal *T. gondii* transmission between hosts by means of oocysts or bradyzoites. In addition, a vertical transmission within a female host is also possible. Congenitally acquired infection occurs, when the mother undergoes a primary infection during pregnancy. In this case circulating tachyzoites, resulting from the acute phase, cross the placental barrier and infect the foetus (Ferguson, 2009; Jones and Dubey, 2012).

1.3.2 Human toxoplasmosis

As mentioned earlier, according to global estimations, 30 to 50% of the world human population is infected by *T. gondii* (Tenter et al., 2000; Flegr et al., 2014). The lowest seroprevalence (10-30%) is reported in the following regions: Northern Europe, North America, South East Asia, and Sahelian countries of Africa; in the Central and Southern European countries a moderate seroprevalence (30-50%) is observed, while Latin America and tropical Africa seem to show the highest seroprevalence (80%) of *T. gondii* in humans (Innes, 2010; Robert-Gangneux and Dardé, 2012).

Within the European human population subclinical infection is estimated to be present in 50%-80% of inhabitants, with an incidence rate of 0.56 per 100,000 population of confirmed reported cases, and the frequency of 2-8 maternal seroconversions per 1000 pregnancies (EFSA report, 2012; Opsteegh et al., 2016b).

In Belgium, the seroprevalence in pregnant women or women at childbearing age was 50% and 63%, respectively, in the past 25 years (Luyasu et al., 1997; Carlier et al., 2012; Flegr et al., 2014), while the rate of the seroconversion during pregnancy is estimated at 3-10 per 10,000 live births (Breugelmans et al., 2004).

Horizontal transmission

The horizontal route of infection may occur via accidental oocysts ingestion due to the environmental contamination, via the consumption of the infected animal products

containing bradyzoites, or by accidental transmission of the acute, reactivated or chronic forms of the parasite via blood, tissue or organ transplants.

As described earlier, domestic or wild Felidae shed big amounts of oocysts into the environment during a short period of time upon the primary (or in rare cases reactivated) infection (Dubey, 1995a). Human toxoplasmosis via oocysts originating from the direct contact with sporulated, hence infectious, oocysts follows e.g. manipulation of the faeces when emptying the litter box, or indirectly via contaminated soil, water or vegetables.

Due to a long survival of oocysts in mild environmental temperature and humid conditions, the direct contact with soil, during e.g. gardening or playing in sandpits, seems to be an important risk factor, contributing to 6-17% of primary human infections, as reported in a case-control study in Europe (Cook et al., 2000, Petersen et al., 2010).

The oocysts of *T. gondii* remain viable for an extensive period of time not only in soil, but also in ground, surface or seawater, in which the parasitic DNA has been already detected (Yang, et al., 2009; Mazzillo et al., 2013; Verant et al., 2014). Additionally, aquatic mammals and invertebrate biological organisms may passively carry the parasite. The human consumption of shellfish, such as, oysters, clams and mussels is consequently a potential source of human infection (Esmerini et al., 2010; Robert-Gangneux and Dardé, 2012).

Bringing both infection sources together, contaminated water and soil serve as the transfer medium for oocysts to consumable plants, vegetables and fruits. The lack of hygienic measures when processing fresh food increases the risk of *T. gondii* infection incidence in humans (Afonso et al., 2008; Robert-Gangneux and Dardé, 2012).

The second mode of horizontal transmission of the infection to humans is by the consumption of raw, undercooked, cured and derived meat products.

In European countries any source of meat from wild and domesticated mammals and birds is considered as a potential source of *T. gondii* infection. Meat consumption of undercooked or cured meat is responsible for 30-63% foodborne human

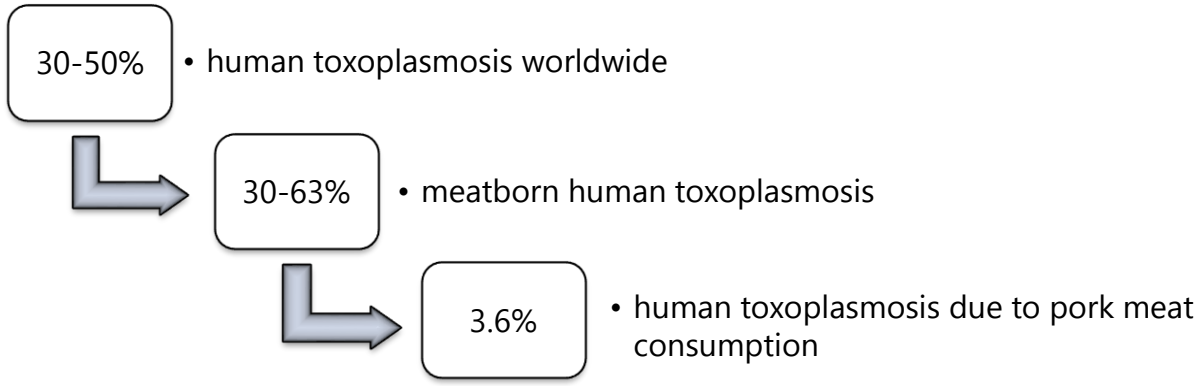
toxoplasmosis cases worldwide (Table 1.2), although this can vary, in reference to the local culinary habits, and to the prevalence of the parasite in meat-producing animals (Cook et al., 2000; Kijlstra and Jongert, 2008; Robert-Gangneux and Dardé, 2012). Based on a recent EFSA report (Opsteegh et al., 2016b), the ranking of the animal species ordered by diminishing incidence is as follows: sheep (48.1%), horses (27.8%), goats (13.3%), pigs (3.6%) and bovines (3.2%). However, new trends in farming of different species, tending to animal-friendly organic herds or bio-farms with free-ranging animals will presumably lead to a rise in *T. gondii* prevalence in animals and, subsequently, in the human population (Hill et al., 2010; Dubey et al., 2012b). Next to the origin of the meat, both the storage conditions and the processing method can play an important role in the viability of the tissue cysts. Fresh meat products, meat pieces that underwent freezing at temperatures above -12°C, temperatures lower than -12°C but for shorter than 3 days, or products heated at the core for lower than 67°C or manufactured by insufficient processing (such as cured with too low salt concentration) or by low temperature smoking, can all contribute to food-borne human toxoplasmosis (Cook et al., 2000; Hill et al., 2006; Bayarri et al., 2010; Petersen et al., 2010; Sullivan and Jeffers, 2012).

It is worth mentioning that other animal products than meat can also serve as a source of infection. In rare documented cases, the consumption of raw milk containing tachyzoites from recently infected goats, sheep or camels can lead to acquired toxoplasmosis in humans (Jones et al., 2009, Gembremedhin et al., 2014; Boughattas, 2015a, 2015b).

The last (and least frequent) mode of horizontal transmission of *T. gondii* towards humans is transplantation, where the infection can be passed on to a human patient by donation of blood, bone marrow, retina or a solid organ containing cysts. Donors undergoing the acute phase of subclinical infection when donating blood or bone marrow, spread tachyzoites, while seropositive individuals serving as donors for visceral organs can potentially transfer tissue cysts. Heart transplants are more prone to house tissue cysts in comparison with internal organs such as liver, lungs or kidneys due to

the fact that muscles commonly favour parasite encystment; moreover the heart is known to be one of the predilection sites during systemic dissemination of the tachyzoites in the intermediate host. In addition, the immunosuppressive treatment prior to transplantation makes the receiving patient more vulnerable upon the transfusion or surgery, what frequently leads to reactivation of dormant bradyzoites in the donated organ (Tenter et al., 2000; Barsoum, 2004; Robert-Gangneux and Dardé, 2012).

Table 1.2 : The sources of human toxoplasmosis (Cook et al. 2000; Kijlstra and Jongert, 2008; Robert-Gangneux and Dardé, 2012 EFSA report 2016).



Vertical transmission

As a consequence of a primary infection during pregnancy, the tachyzoites raised during the acute phase disseminate, can cross the placental barrier and colonize the maternal and fetal tissues in on average 30% of the cases (Robert-Gangneux and Dardé, 2012). The placental barrier against *T. gondii* is more efficient during the first 3 months than during the second or third trimester (Tait and Hunter, 2009). The frequency of CT prevalence increases with the proceeding gestational stage: an infection of the mother in early pregnancy carries a low risk for congenital infection (6-8%) and increases over 30-45% during the second trimester until 60-90% in the last three months (Dunn et al., 1999, Dubey, 2010).

The severity of the malformations of the foetus resulting from the congenital infection are clearly inversely correlated with the gestation stage (see further 1.3.3) (Tenter et al., 2000; Pfaff et al., 2007; Dubey, 2010; Milewska-Bobula et al., 2015).

1.3.3 Clinical signs and symptoms

The course of *T. gondii* infection in an intermediate host such as a human or a domestic pig is predominantly subclinical. A symptomatic infection accompanied by the clinical signs with a broad range in severity, occurs in certain groups of patients. These groups are at a higher risk and, consistently, develop a more severe disease outcome. Seronegative pregnant women and newborns with CT, and patients with a temporary compromised or permanently impaired immune system could be included to this category (Weiss and Dubey, 2009a).

Subclinical toxoplasmosis in immunocompetent individuals

In immunocompetent individuals a subclinical, hence, asymptomatic infection is expected in more than 80% of the cases (Robert-Gangneux and Dardé, 2012; Halonen and Weiss, 2013; Halsby et al., 2014). However, even in healthy immunocompetent patients and presumably depending on the strain and dose, some atypical symptoms are reported, frequently associated with flu or mononucleosis, such as: sore throat and lymphadenopathy of a. o. cervical lymph nodes, fever, headache, myalgia and arthralgia, general malaise and weakness, anorexia due to abdominal pain (Walker et al., 1990; Hill and Dubey, 2002; Ho-Yen, 2003; Weiss and Dubey, 2009a; Galli-Tsinopoulou et al., 2010; Taila et al., 2011; Anand et al., 2012; Cuomo et al., 2013; Machala et al., 2013; Chaudhry et al., 2014). These clinical features are limited in time and self-resolving, but under certain circumstances more severe outcomes may be presented, such as splenomegaly, lymphadenitis, pneumonia, hepatitis, polymyositis or myocarditis (Nunura et al., 2010; Abhilash et al., 2013; Cuomo et al., 2013).

Occasionally, *T. gondii* induced retinochoroiditis (TRC) or ocular toxoplasmosis (OT) can also result from a postnatally acquired infection, but not only from CT as generally assumed. In addition, OT-patients carry the risk of recurrence of the symptoms (Theodossiadis et al., 1995; Baglivo and Safran, 2003; Stanford et al., 2005; Flegr et al., 2014; Undseth et al., 2014). Finally, latent toxoplasmosis in immunocompetent humans is nowadays considered as an important risk factor for the development of neuropsychiatric disorders, associated with behavioural changes, such as schizophrenia, Parkinson's disease and suicidal intentions (Yolken et al., 2009; Lester, 2010; Miman et al., 2010). The reason for these would lie in increased dopamine and decreased tryptophan and serotonin levels as a consequence of *T. gondii* metabolism within the neural tissues (Träskman et al. 1981; Müller et al., 2009; Okusaga et al., 2012; Flegr, 2013; Wong et al., 2013).

In general, once the patient acquires humoral and cellular immunity, the protection is life-long and the symptoms fade away. Nevertheless, clinical signs were described in patients upon re-infection with a strain of a different genotype, indicating that cross-immunity was not sufficient in providing a full protection (Elbez-Rubinstein et al;, 2009; Valdès et al., 2011).

Congenital toxoplasmosis in the foetus or newborn

The most serious consequences of a *T. gondii* infection are associated with the congenital transmission from the primary infected pregnant mother to her foetus. As mentioned before, in early pregnancy the parasite is less capable of crossing the placental barrier; opposite to that, the clinical manifestations in the first trimester have the greatest impact on fetal development, showing a decreasing severity over the gestational time (Dunn et al., 1999; Cook et al., 2000; Hill and Dubey, 2002).

Infection of the foetus during the first weeks of the pregnancy can lead to abortion, stillbirth or severe abnormalities in the cerebral tissues, among which are hydro- or microcephaly, intracranial calcifications, psychomotoric and mental retardation, epileptic seizures or deafness (Dubey, 2010; Chaudhry et al., 2014; Flegr et al., 2014).

The ocular tissues are also frequently affected leading to unilateral or bilateral microphthalmia, cataract, glaucoma, strabismus, nystagmus, retinochoroiditis, optic and retinal neuritis or blindness (Yamamoto et al., 2000; Miller et al., 2009; Robert-Gangneux and Dardé, 2012; Paquet and Yudin, 2013). However, it is important to mention that the ocular symptoms of congenital toxoplasmosis can occur years after prenatal infection, during childhood or in the early adulthood (Butler et al., 2013; Chaudhry et al., 2014).

Maternal infection in a later stadium of the pregnancy leads to mild or asymptomatic CT in 85% of the neonates at birth, and when followed up for the effectiveness of the treatment until early childhood (Dunn et al., 1999; Foulon et al., 1999).

Cerebral and extra-cerebral toxoplasmosis in immunocompromised individuals

Cerebral toxoplasmosis is a life threatening illness in immunocompromised individuals. This category includes HIV-carriers, leukaemia and lymphoma patients, or people undergoing an immunosuppressive therapy for cancer or for organ transplantation (Tait and Hunter, 2009; Robert-Gangneux and Dardé, 2012; Flegr et al., 2014). Toxoplasmosis develops here most frequently as the reactivation of a latent infection, rather than as a primary infection, except for organ donations between a chronically infected donor and a seronegative receptor (Israelski and Remington, 1993, Derouin and Pelloux, 2008).

Toxoplasmic encephalitis (TE) or neurotoxoplasmosis (NT) has the highest prevalence in HIV-infected patients due to the impairment and deficiency of the T-cell populations, and is associated with headache, lethargy, incoordination, loss of memory, epilepsy, obsessive-compulsive disorders and dementia (Correia et al., 2013; Flegr et al., 2014). In NT patients, also other organs can be involved, among which are the eyes (retinochoroiditis or OT) lungs (pneumonitis, bronchiolitis or pulmonary toxoplasmosis or PT), heart (myocarditis, pericarditis), liver (hepatosplenomegaly, hepatitis), pancreas, bone marrow, bladder, lymph nodes, kidney, spleen and skin (Rabaud et al., 1994; Anand et al., 2012. Robert-Gangneux and Dardé, 2012).

In humans undergoing immunosuppressive therapy for receiving organ transplants, the risk of reactivation of the latent *T. gondii* infection is related to the duration and level of immunosuppression. Additionally, as indicated earlier, heart transplantations are associated with a higher risk of the transmission of the disease in comparison with other transplants, since muscles, and heart in particular, are the predilection tissues for hosting the cysts (Carruthers and Suzuki, 2007).

The pathogenesis and associated clinical signs of porcine *T. gondii* infection are discussed in Chapter 3.

1.4 Diagnostic methods in humans and animals

The diagnosis of an acute or chronic *T. gondii* infection in humans or animals is established by the combination of different techniques. The clinical features are, although with some exceptions, not exclusive for this parasite. It is therefore recommended to apply a single or multiple diagnostic assay(s) to obtain the highest specificity (Sp) and sensitivity (Se) (or the analytical sensitivity: the ability of a test to detect a target analyte (e.g. an antibody or antigen), which is usually expressed as the minimum detectable concentration of the analyte). The final choice will depend on the type of sample available and the aim of the assay (detection versus isolation). Among the commonly used techniques serological tests are the first choice for an initial screening of antibodies against the parasitic antigens. Direct detection of the parasite in smears of blood or biopsy sections is performed by microscopic examination. Detection and isolation of the viable parasite from host's tissues is possible with bioassay or cell cultures, while the presence of the parasitic DNA can be determined by molecular techniques such as Real Time-PCR or Magnetic Capture PCR (Montoya and Liesenfeld, 2004).

1.4.1 Serological techniques

Serological confirmation of *T. gondii* infection by immunoglobulin (Ig) M and/or IgG antibody detection is indicative for a recent or former exposure of the host to the parasite (Mancianti et al., 2010; Bhattacharwa et al., 2013; Chahed Bel-Ochi et al., 2013). The isotype IgM antibodies appear during a primary infection, starting from the first week post infection (pi), and they are followed by the appearance of IgG isotype antibodies (Kortbeek, 1999; Hill and Dubey, 2002). The titer of IgM gradually decreases from 1 month pi, while that of IgG is just about to rise exponentially. IgM antibodies can persist up to one year after infection; their presence can be interpreted as a recent exposure to the parasite. The IgM detection alone as a tool for the prenatal diagnosis of CT has therefore a limited diagnostic value, but it can be used in addition to another assay (Kortbeek, 1999; van der Giessen et al., 2003).

IgG starts to appear from 3 weeks pi and progressively increases until 3 months pi. While the titer of IgM gradually and relatively quickly decreases over time, IgG persists at a high concentration in blood, urine and cerebrospinal liquid. Therefore, IgG assures a long-term humoral immunity to a greater extent than IgM, and it serves as a marker of chronic infection (Dubey, 1998a). Under certain circumstances, IgG can serve as the detection tool of recent *T. gondii* infection: though not via a single but via a paired samples measurement. Such analysis requires two samples, taken with 2-4 weeks interval to measure seroconversion or an increase in the IgG titre (Van Knapen, 1986; Hill and Dubey, 2002).

IgG avidity assay is performed in pregnant women with high titers of both conventional isotypes, or in pregnant women with a recent seroconversion to estimate the time-point of the primary infection (Figure 1.6) (Roberts et al., 2001; Sukthana, 2006; Pourabolghasem et al., 2011; Smets et al., 2016). As the maturation of the IgG avidity proceeds with the increasing interval from the infection, a low-avidity IgG may also persist throughout the pregnancy as the result of the anti-*Toxoplasma* treatment with spiramycin (Lefevre-Pettazzoni et al., 2007; Meroni et al., 2009).

Additionally, next to IgM and IgG detection, an increase in IgA levels can be used as the marker of the recent infection. The elevation of IgA titers follow shortly the initial IgM rise and persist at the detectable levels for 6 to 7 months pi. The use of IgA as a diagnostic tool is, however, controversial, since its detection can vary from case to case, being affected by the patients' heterogeneity and their immune status; it also depends strongly on the time point of the blood collection, sensitivity of the applied assay and the used antigen (Roberts et al., 2001; Suzuki et al., 2001, Nascimento et al., 2008, Murat et al., 2013).

For the discrimination of CT from the passively transferred maternal immunity, IgA and IgM are detected in the new born child, since these isotypes, opposite to IgG, cannot cross the placental barrier and, thus, can only arise from an active infection during pregnancy (van der Giessen et al., 2003). The recognition pattern of the parasitic antigens can be visualized and compared via immunoblotting, and when different between mother and child it is significant for a congenital infection (Hill and Dubey, 2002; Rorman et al., 2006).

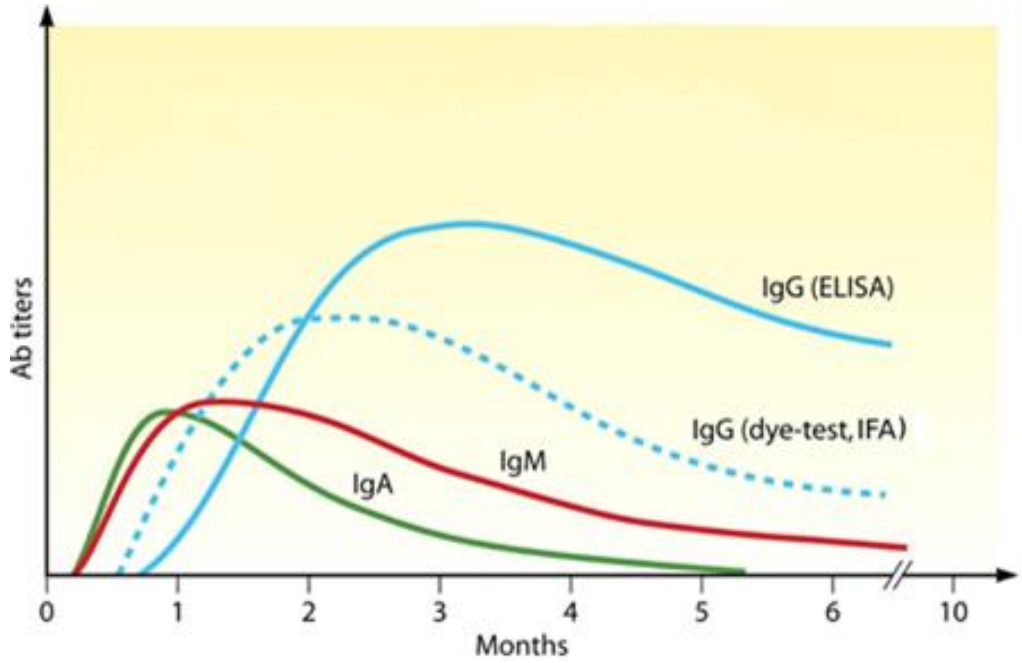


Figure 1.6 : Antibodies titer in the course of the primary infection (adapted from: Robert-Gangneux and Dardé, 2012). ELISA: Enzyme-linked immunosorbent assay; dye-test: Sabine-Feldman Dye Test (DT); IFA: Indirect Immunofluorescence Assay.

Sabine – Feldman Dye Test (DT)

This technique is defined as the "gold standard" for *T. gondii* detection in serum of humans due to the highest sensitivity and specificity close to 100% (Willis et al., 2002). Viable tachyzoites, harvested from intraperitoneally infected mice, actively take up methylene blue dye from the culture medium when incubated with the serum of the patient and activated by the complement and present antibodies. Parasites eliminated by complement-mediated lysis (due to the presence of antibodies and complement in serum and plasma of patient, respectively) do not take up the dye and remain colourless under inverted microscopy observation (Reiter – Owona et al., 1999; Kijlstra, 2004; Rorman et al., 2006).

Agglutination assays

These techniques detect macroscopically visual agglutination of the cells and do not require microscopic inspection (Murat et al., 2013).

In the direct agglutination serial dilutions of serum are brought together with a suspension of whole tachyzoites in U-shaped wells. Parasites dispersed over the bottom of the well stand for a positive reaction, while sedimentation of the individual parasites indicate a negative reaction. Direct agglutination can detect all kind of immunoglobulins at the same time and thereby is only used as a screening method (Tlamçani et al., 2013).

Modified agglutination test (MAT) is a modification of the direct agglutination developed by Desmonts and Remington (1980) to increase the test sensitivity and, indirectly, specificity up to 83% and 90%, respectively (Dubey et al., 1995b). This method uses formalin–preserved whole tachyzoites and a pre-treatment of serum samples with mercaptoethanol to selectively denature IgM, which tends to give a strong agglutination due to a high avidity, and to detect IgG instead. By this, the specificity of the assay also increases, as IgG shows a higher affinity to the antigens, in comparison with the IgM (Dubey et al., 1995b; Willis et al., 2002. Dubey, 2010).

In the indirect haemagglutination test *T. gondii* tachyzoites soluble antigen is coated on tanned red blood cells that are then agglutinated in the presence of antibodies in the serum sample (Figure 1.7) (Murat et al., 2013). The assay performs better, when applied on human samples (Se: 100% and Sp: 98.5%) than on porcine serum (Se: 30% and Sp: 98%), (Dubey et al., 1995b; Liu et al., 2015).

In the latex agglutination test (LA) the same soluble antigen is coated on latex particles and subsequently incubated with the serum in order to observe agglutination (Dubey, 2010). Despite the optimal assay characteristics in tests on human samples (Se: 86-94% and Sp: 100%; Liu et al., 2015), porcine samples may yield fals negative results due to the low sensitivity (Se: 46% and Sp: 97%; Dubey at al., 1995b).

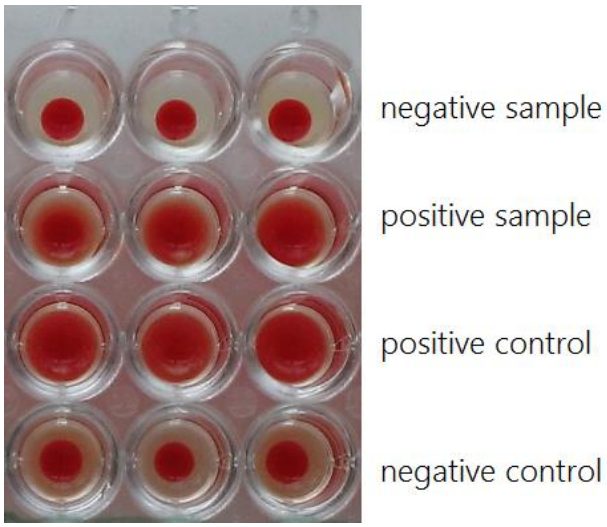


Figure 1.7: Positive and negative results of the haemagglutination test (Laboratory for Immunology, Faculty of Veterinary Medicine, Ghent).

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay is a useful and powerful method to detect small concentrations (in the range of picogram) of antibodies or proteins in solution (Murphy et al., 2008). The main advantages of this technique are the possibility of using different parasitic antigens (both native or recombinant), the prominent sensitivity and specificity (Se: 78-94% and Sp: >85%, depending on the study) and the high

throughput of the samples, together with the titration or quantification of the detected antibodies.

The high flexibility of the ELISA system allows substantial variation in protocols (e.g. direct or indirect antigen ELISA, single or double sandwich ELISA), in antigen used for capturing of the parasite-specific antibodies and in detection of antibodies (total antigen-specific Ig's or rather of different isotypes (IgA, IgE, IgG, IgM)) and this in the biological fluids of different species.

The primary used antigen mixture was derived from viable and homogenized tachyzoites of the RH-strain (referring to the initials of the first infected patient) and named as Total Lysate Antigen (TLA) (Hughes et al., 1982; Hughes et al., 1986). TLA preparation implies producing tachyzoites by culturing the parasite in cell lines or in mice, and has therefore a potential bio-safety risk for the operator. The inoculation of mice has severe consequences for the animals, since mice develop an acute toxoplasmosis with fatal peritonitis upon inoculation. On top of that, the collected ascites contains cellular material as the result of infection but also the innate immune response. Cell line cultivation leads to *T. gondii* lysate collection via an animal-friendly, but still bio-hazardous method. Nevertheless, it does not contain immune cell rests, but may still contain host cell residues. Furthermore, its composition can vary significantly between laboratories or production rounds, what makes the ELISA's using TLA as a capture antigen difficult to standardize and evaluate (Liu et al., 2015).

A better option is the use of recombinant antigens, relatively easily produced at high concentrations in bacterial cells as a vector. The main advantages of the recombinant antigenic proteins is the predefined and homogenized composition and a significant time and work reduction (Holec-Gąsior, 2011). The antigens frequently used in the serological assays are P35, matrix protein (MAG), different dense granule proteins (GRAs), microneme proteins (MICs) or surface antigens (SAGs) (Kotresha and Noordin, 2010; Holec-Gąsior, 2013).

However, the combination of multiple parasitic antigens in one serologic assay such as TLA or chimeric antigens, instead of a single recombinant protein, should ensure a

more efficient antibodies detection by the higher number of available epitopes for the antibody binding and because not all recombinant antigens are expressed in equal amounts in the course of the infection (Basso et al., 2013; Bokken et al., 2015).

Indirect Immunofluorescence assay (IFA)

In this assay the recognition of the whole tachyzoites, coated on a glass slide, is detected by the use of secondary antibodies, labeled with a fluorescent dye such as fluorescein isothiocyanate (FITC), Texas Red, Peridinin Chlorophyll protein (PerCP), rhodamine and phycoerythrin (PE) (Murphy et al., 2008; Elgert, 2009; Odell and Cook, 2013) and results can be observed using a fluorescence microscope (Figure 1.8) (Storch, 2000; Murphy et al., 2008). The assay scores high in terms of Se (75%) and Sp (100%), (Udonsom et al., 2010). Confocal fluorescence microscopy, which employs computer-aided techniques to produce an ultrathin optical section of a cell or tissue, gives a higher resolution allowing analysis of cellular components (Murphy et al., 2008; Elgert, 2009).

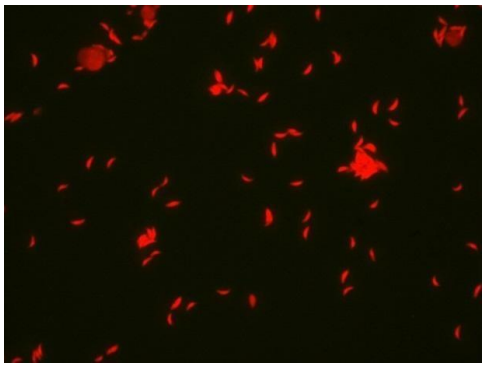
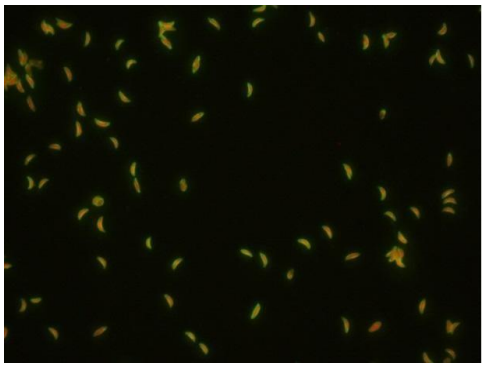
A)  B) 

Figure 1.8 : Immunofluorescence microscopy image of fixed tachyzoites incubated with: A) negative porcine serum and B) positive porcine serum (courtesy of Dr S. De Craeye, National Reference Laboratory for Toxoplasmosis, WIV-ISP, Brussels).

1.4.2 Microscopy

Tachyzoites can be detected via light or fluorescence microscopy in blood smears, in the amniotic fluid or the cerebrospinal liquid in case of CT in a newborn or in cryosections made from biopsy samples from infected individuals. The techniques include a direct staining with Giemsa dye or the combination of a primary and an enzyme-conjugated or fluorescent secondary antibodies (Figure 1.9) (Tabei, 1982; Bottone, 1991). By the use of antibodies against stage-specific antigens it is possible to make a differentiation between tachyzoites and bradyzoites and other related Protozoa (McAllister et al., 1996).

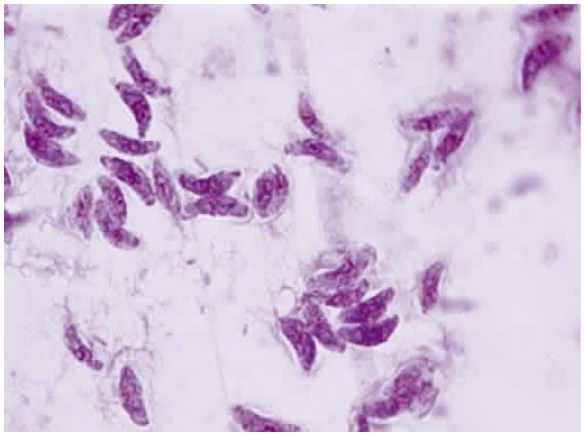


Figure 1.9 : Light microscopy image of Giemsa-stained tachyzoites collected from intraperitoneally infected mice (adapted from: Duane's Clinical Ophtalmology, Volume 4, chapter 46 by K. F. Tabbara).

Tissue cysts in hosts samples such as cerebral suspension can be observed by contrast-phase microscopy (Figure 1.10) or visualized by staining with Giemsa and Hematoxylin-Eosin (HE).

Opposite to tachyzoites and bradyzoites, oocysts exhibit a blue autofluorescence and, therefore, do not need to be stained prior to detection under a ultra-violet light source (Lindquist et al., 2003).

Finally, confocal fluorescence microscopy makes it possible to study the ultra-structural subcellular morphology of different stages of *T. gondii* (Melzer et al., 2010).

Summarizing, microscopy as a diagnostic method has many advantages among which is the ease of use, the short processing time, high specificity and the relatively low price of the equipment and reagents. However, the main drawback of this technique is the limited sensitivity due to small density of the parasite in the tissues or in non-concentrated body secrets/excretes and liquids from the placenta or neural system (Dubey, 1998b).

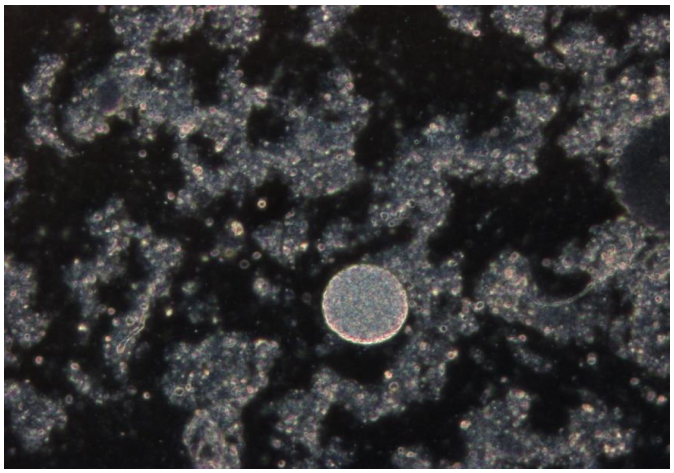


Figure 1.10 : Phase-contrast microscopy image of a tissue cyst in the cerebral suspension from a *T. gondii* (IPB-Gangji strain) infected mouse (courtesy of dr S. De Craeye, National Reference Laboratory for Toxoplasmosis, WIV-ISP, Brussels, Belgium).

1.4.3 Detection of the viable parasite

In this group of techniques the patient's sample is transferred to the intermediate (mouse) or final host (cat) in order to proceed with the life cycle of the parasite and enable the detection, or is cultivated *in vitro* to stimulate the spontaneous multiplication.

Bioassay

Clinical toxoplasmosis can be diagnosed by the intra-peritoneal or subcutaneous inoculation of seronegative animals (Swiss mice) with the patient's samples such as blood, urine, cerebrospinal liquid, biopsy samples from the lymph nodes or muscles.

Swiss mouse strain, being a very homogenous and well-established, does not show a reasonable susceptibility towards *T. gondii* (with the exception of the highly virulent strains such as RH) but develops an optimal immune response and is therefore suitable for the bioassay and strain maintance. The tachyzoites in the liquid samples can be directly injected upon washing and pelleting of the cell debris. The tissue should be first homogenized and enzymatically treated using pepsin or trypsin, in order to digest the wall of the tissue cysts and release the bradyzoites, which are less susceptible to the enzymatic activity of pepsin. Depending on the virulence and the dose of the inoculated strains, some mice develop acute toxoplasmosis shortly after infection and should be euthanized according to the human end points. This is mainly the case for the genotype I strains; less virulent strains (genotype II) lead to chronic asymptomatic infection (Jacobs and Melton, 1954; Derouin et al., 1987; Johnson, 1988). In that case the infection in mice is individually detected after 4 to 6 weeks by serological assay (IFA) and PCR (blood, lungs, brain) or light microscopy to demonstrate the presence of the tissue cysts.

In addition to mice, also cats may be used in bioassay. This technique is particularly interesting when the estimated concentration of the tissue cysts is too low for the mice inoculation (Dubey, 2010). The cats are serologically followed up upon inoculation to detect seroconversion; the positive result of the assay is obtained by isolation of the excreted oocysts from the faeces by flotation techniques and the detection by PCR or microscopy (Dubey et al., 1970; Kourenti et al., 2003; Lindquist et al., 2003). The bioassay in cats is more sensitive than in mice due to several reasons: much bigger amounts of the test sample can be fed, cats show evolutionary a higher susceptibility to the ingested tissue cysts than mice, and, as the result of a successful infection, the number of shed oocysts is very high, even when inoculated with a very low virulent sample (Dubey, 1995; Dubey, 2008).

Though considered as a gold standard test for the detection of viable *T. gondii* because of its high sensitivity (1 cysts per 100 g of sample) and a very high specificity (nearly 100%) in mice and 100% in cats (Rothe et al., 1985), bioassay remains controversial due

to the excessive use of laboratory animals, long detection time, handling of hazardous material, possible contamination of the environment with the shed oocysts and for being labour-intensive. The assay may yield false negative results, when the parasite does not survive the handling procedure or when the infected sample is processed too late to initiate an infection with the viable parasite and evoke a response in the inoculated animal.

Cell cultures

In vitro cultivation of tachyzoites incorporated into a fast growing cell culture is a promising alternative for the use of mice in bioassay (Hughes et al., 1985; da Costa Silva et al., 2012; Wu et al., 2012). The parasite can already be detected from two days after inoculation. However, the assay seems to have a lower sensitivity and causes the reduction of virulence, together with a decreasing expression of some parasitic antigens, by which this technique is still under discussion (Foulon et al., 1999; Degirmenci et al., 2011).

1.4.4 Molecular techniques

PCR

The polymerase chain reaction (PCR) allows the denaturation and amplification of a specific DNA sequence by the use of thermostable DNA polymerases to produce thousands of copies of the replicated DNA fragment (Murphy et al., 2008). The relatively high specificity and sensitivity of the PCR technique to detect small amounts of DNA allows detection of *T. gondii* nucleic acids using DNA targets such as 35 repetitive B1 gen or 300-repetitive 529 – bp DNA sequence (Burg et al., 1989; Homan et al., 2000). PCR is commonly used for detection of *T. gondii* in CT (from amniotic fluid, placenta, cord blood, fetal blood and peripheral maternal blood), in ocular toxoplasmosis (ocular fluids) and in samples from immunosuppressed patients (cerebral biopsy, cerebrospinal fluid, lymph nodes, and others) (Murat et al., 2013; Liu

et al., 2015). In veterinary medicine it is mainly used for the confirmation of *T. gondii* infection in the abortion protocol in ewes (Wastling et al., 1993). The technique is very sensitive as an individual tachyzoite in the samples can be detected. This is especially important for the diagnosis in immunocompromised patients, where the antibody production against the parasite is impaired (Montoya and Liesenfeld, 2004). The biggest drawback is the high risk of contamination during processing, and, hence, obtaining false positive results. However, opposite to the conventional PCR, real-time PCR (see further) eliminates the possible risk of contamination and increases the assay characteristics (Liu et al., 2015).

Real-time quantitative PCR (qPCR)

Real-time quantitative PCR (qPCR) implies the same amplification and detection process as conventional PCR with a high specificity and sensitivity, but above that it allows also quantification of specific DNA sequences (Murat et al., 2013; Rahumatullah et al., 2015).

This technique uses the same targets as conventional PCR: a target molecule is copied once at each cycle and data are captured by the thermal cycling. The amount of target molecules present in the sample is the limiting factor of this reaction (Tevfik, 2006). In qPCR fluorescent specific and non-specific probes are used to label PCR products from a series of dilutions of a standard and the samples. The total fluorescent signal is subsequently measured during the exponential phase of the reaction, where the mean fluorescence is directly proportional to the amount of target in the sample and the concentration of the parasite is calculated based on the standard values (Fraga et al., 2008).

Magnetic Capture (MC)-PCR

In the conventional PCR the high sensitivity of the technique cannot, however, guarantee that the parasite, which is not always equally distributed in the samples (except for blood), will be successfully detected. The biggest challenge is then the

detection of the parasitic DNA in relatively small, in terms of size, biopsy of autopsy samples. Therefore, successful attempts have been undertaken to concentrate the parasitic DNA using magnetic beads coated with probes against common targets, and subsequently to perform a quantification by qPCR (Opsteegh et al., 2010; Algaba et al., 2017). In that way, not 1 g of a tissue as for PCR or qPCR, but 100 g can be processed, increasing the potential chance of the detection of *T. gondii*. This technique is very valuable in the field of food safety and zoonosis prevention from production animals.

1.5 Treatment and prevention

The only fully effective medical drugs for the treatment of toxoplasmosis in humans are the sulfonamides, such as sulfadiazine, sulfamethazine or sulfamerazine, and pyrimethamine. They work synergistically by the inhibition of the metabolic pathway of aminobenzoic acid and folic acid, as they diffuse across the parasitic cell membrane to reach the cytoplasm. Unfortunately, they are only useful in case of acute parasitaemia, but they are not capable of eliminating the existing infection by affecting the bradyzoites in tissue cysts (Dubey, 2010).

The commonly used treatment for CT is spiramycin, because it accumulates strongly in the tissues such as placenta, but without reaching the foetus by crossing the placental barrier. In case of encephalitis the combinations of frequently used drugs are pyrimethamine-sulfadiazine, trimethoprim-sulfamethoxazole and pyrimethamine-clindamycin, each acting with a high efficacy but with no significant difference between them (Wei et al., 2015).

The main aims of the prevention by vaccination in multi-species is the reduction of the circulating tachyzoites during acute infection and, therefore, inhibition of congenital transmission, limitation of the tissue cysts within the intermediate hosts and eradication of the environmental contamination by the cat. Although numerous potential vaccine candidates have been experimentally tested in animals in the last decades with varying degrees of success, and one vaccine is currently commercially available for sheep, there are no known effective and registered products for the use in humans (Kortbeek, 1999;

Montoya and Lieesenfeld, 2004; Buxton et al., 2007). The control strategy by vaccination has evolved from classic vaccines containing a single or a combination of parasitic antigens, among which are surface or excretion/secretion proteins, to DNA vaccines (Vercammen et al., 2000; Letscher-Bru et al., 2003; Jongert et al., 2008; Li, et al., 2011; Cao et al., 2015; Wagner et al., 2015). Another possibility is the use of attenuated viable strains, but the efficiency is still species-dependent and associated with the manipulation risk by the operator (Katzer et al., 2014; Burrells et al., 2015). Recent data on the use of vaccine candidates are promising but further research is necessary to prove their efficacy in different species and in humans.

In addition to medical treatment and control of infection by vaccination, the efforts should be focused on the implementation of prevention methods in the daily human life, in particular in pregnant women or immunocompromised individuals (Kortbeek, 1999; Hill and Dubey, 2002; van der Giessen et al., 2003; Breugelmans et al., 2004; Montoya and Liesenfeld, 2004; Opsteegh et al; 2015).

As the current preventive trends are mainly focused on the reduction of the congenital toxoplasmosis due to the severity of the outcomes on a short- and long-term, the primary prevention methods should include a monthly follow-up during the pregnancy and providing the information to pregnant women on the possible infection sources and the corresponding preventive measures. Such a preventive information-based strategy has been proven to be successful in the reduction of seroconversion incidence from 1.43% to nearly 0.1% observed between 1979 and 2001 (Breugelmans et al., 2004). In brief, the advises should cover proper heating of all meat, avoiding the consumption of raw meat (or diary) products, hand hygiene for gardening and other soil contact, washing of vegetables and fruits consumed raw, and precaution measures for cleaning of the cat litter box and avoid contact with possibly contaminated water (Breugelmans et al., 2004; Opsteegh et al., 2015).

Since domesticated or feral cats will always be present in and around the human population, the cat is presumed to be the main source of infection for humans, and this mostly indirect via contaminated environment or infected intermediate hosts.

Therefore, pregnant women and immunodeficient individuals should avoid cleaning the litter box and have contact with potentially contaminated soil or vegetables.

Further, it is well established that food-borne toxoplasmosis by the consumption of infected raw or not well-baked meat or the ingestion of oocysts with the drink water and vegetables are the main sources of food-borne infection for humans. Therefore, the general precautions include hygienic measures, which should be taken during and after processing the food from animal origin, such as washing hands and kitchen material or avoiding contact with raw or unprocessed meat. It is recommended to store the meat during at least 3 days at a core temperature of -12°C, or to prepare it afterwards at minimum 67°C during 10 minutes to inactivate the parasite. When storing at + 4°C the bradyzoites survive approximately 3 weeks, while more than 10 days at -6.7°C. While working in the garden or collecting vegetables for consumption, one should wash their hands with water thoroughly, and wear gloves to avoid direct contact with the oocysts (Dubey, 2010).

Cats should not be fed with uncooked meat or viscera but with frozen, cooked, canned or dry feed. Dead animals on farms or in the slaughterhouses, as well as the placental and fetal tissues in case of abortion in sheep, should be immediately removed to avoid cannibalism in pigs and scavenging by other animals (Hill and Dubey, 2002; Sukthana, 2006; Jones and Dubey, 2012; Robert-Gangneux and Dardé, 2012).

A more detailed description of the risk factors and prevention methods on farm level will be discussed in Chapter 3.

Chapter 2 Pathogenesis, innate and acquired immune responses during *T. gondii* infection in intermediate hosts

2.1 General pathogenesis

T. gondii infection in an intermediate host initiates a series of events, activating different components of the immune system during the acute and chronic stages of acquired toxoplasmosis. The acute phase of the infection is characterized by the rapid and excessive multiplication, followed by the thorough dissemination of the parasite within the host. These events trigger acute innate immune mechanisms by activating Natural Killer cells (NK's), macrophages and granulocytes. Subsequently, cellular and humoral adaptive immune responses take over in an attempt to combat the latent stages of the parasite or at least prevent the stage conversion into the more virulent form. Nevertheless, the host's immune responses are complex and intended to keep up the delicate balance between the invasion strategies of the parasite and the defense mechanisms of the host.

Since few studies notified the natural immune events of acquired porcine *T. gondii* infection, the author based this chapter on widely described mouse and/or human studies. The immune responses induced by some experimental infections in pigs are highlighted in Chapter 3.

As described in Chapter 1, upon ingestion of the sporulated oocysts or the tissue cysts and the subsequent action of the gastric enzymes, the parasites are released into the intestinal lumen. From there the sporozoites or bradyzoites, respectively, invade the intestinal epithelial cells and rapidly multiply, transforming into the virulent stage of the tachyzoites. The infected enterocytes burst open and discharge a new generation of daughter cells. Newly released tachyzoites proceed by penetrating the surrounded cells and forming the PV from the parasitic and host's plasma membranes. When released outside to the extracellular matrix or lamina priopria, the parasite may be taken up by antigen presenting cells, namely dendritic cells (DC's) or macrophages (Figure 2.1). From there the acute phase of the infection is initiated, with the release of cytokines (IL-12 and TNF-α) (Miller et al., 2009; Cohen and Denkers, 2014). These

cytokines initiate the next innate and adaptive immune responses by activating corresponding cell populations such as NK's and T lymphocytes, respectively (see 2.2) (Suss-Toby et al., 1996; Carruthers, 2002; Kasper et al., 2004; Gregg et al., 2013; Cohen and Denkers, 2014). The recognition of the parasite by immune cells involves a certain set of Toll Like Receptors (TLR's) in combination with the matching parasitic antigens to initiate the pathways of the intrinsic activation.

Despite (or as the result of) the rapidly initiated innate host's defense, *T. gondii* crosses the trans epithelial barrier of the intestine, subsequently disseminates via the lymph vessels and further via the systemic circulation to the peripheral tissues, escaping from the initiated gut immune response (Dubey, 1998b). This unusual distribution appears to proceed preferably within the monocytes, which not only have the capacity of reaching the lymph organs but also can migrate and reside into the internal organs, which particularly favours the parasite survival and persistence during the chronic phase of the infection (see 2.3).

It is important to notice that the ingested stage of the parasite seems not to influence the immune response in the intermediate host. The interaction of the host with the infectious stages of the parasite (oocysts and tissue cysts) appears to last too short to induce a different pattern of the acquired immune response. The wall of the oocyst or tissue cyst is equally digested by the gastric enzymes, leading to the release of the encapsulated parasites. No evidence has been found so far that the pattern of immune response is different upon ingestion of one of these forms (Miller et al., 2009). However, the extent of the immune reaction following the inoculation might be indeed greater when ingesting bradyzoites, since a single oocysts contains only 8 sporozoites, while there might be hundreds to thousands single bradyzoites within a tissue cysts, each of them being able to transform into a tachyzoite (Dubey, 2010).

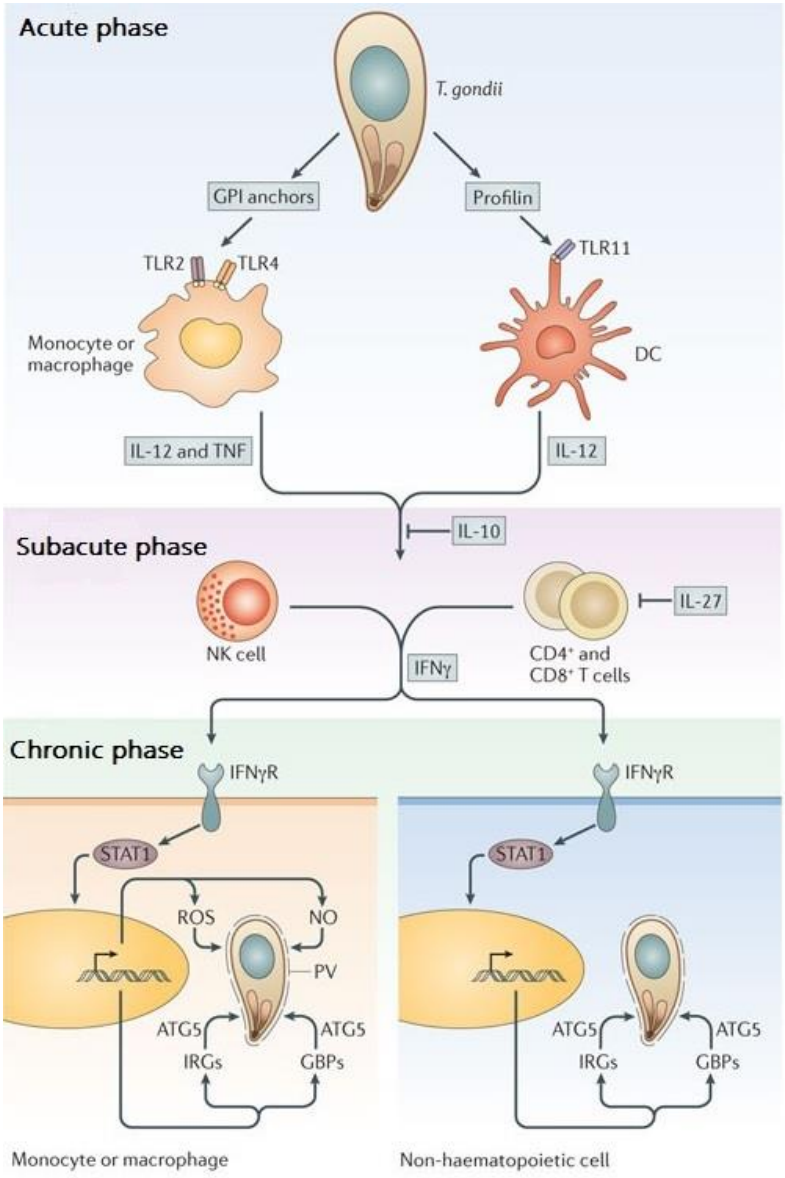


Figure 2.1 : Major steps in the pathogenesis of *T. gondii* infection and the involved cell populations (adapted from: Yarovinsky, 2014).

2.2 Acute and sub acute phase of the infection

Acute *T. gondii* infection is initiated fairly immediate after the natural or experimental oral ingestion of any of the developmental stages of the parasite. It lasts on average less than ten days and is characterized by the presence of the fast multiplying tachyzoites within the enterocytes, which burst open and release a new generation of

daughter cells. This allows the dissemination of this stage of *T. gondii* towards all the other organs, with the preference of the central nervous system, heart and skeletal muscles (Filisetti and Candolfi, 2004).

In the early stage of the infection robust Th1-biased innate and cellular immune reactions are initiated (Figure 2.1). However, the first defense line against the pathogenic microorganisms includes the cells of the innate immune system, primed by the contact with the disseminated parasites or by the intracellular infection.

Different activation pathways play here a predominant role and several cell populations are involved (Miller et al., 2009; Andrade et al., 2013; Gazzinelli et al., 2014; Sturge and Yarovinsky, 2014). The main mechanisms rely on the identification of the pathogen-associated molecular patterns (PAMP's) displayed by the microorganisms (Murphy et al., 2008; Momeni et al., 2014). These PAMP's are characterized by the highly conserved expression on exclusively microbial cells, essential for the pathogen's survival (Gazzinelli and Denkers, 2006). The corresponding scavenger receptors at the host's side are the pathogen recognition receptors (PRR's), which are expressed on DC's, macrophages and neutrophils. By binding the parasitic ligand with the compatible PRR the pro-inflammatory immune response is initiated, intended to protect the host from the parasite's invasion. The most important PRR's are the Toll-like receptors (TLR's), more specifically TLR11 and 12 in mice; further TLR7, 8 and 9 in humans and TLR7 and 9 in other mammals like pigs (Figure 2.1) (Miller et al., 2009; Andrade et al., 2013; Koblansky et al., 2013; Gazzinelli et al., 2014). Several parasitic antigens have been associated with the certain TLRs, for instance, tachyzoites-derived surface antigens (SAG's), SAG-related sequences (SRSs) and SAG-unrelated surface antigens (SUSA's), activate TLR2, TLR4 and TLR11 (Debierre-Grockiego et al., 2007). Additionally, TLR11 and TLR12 are the host cell receptor in mice for *T. gondii* actin-binding protein profilin (Plattner et al., 2008; Yarovinsky et al., 2008; Miller et al., 2009; Denkers, 2010; Kucera et al., 2010; Skillman et al., 2012; Koblansky et al., 2013; Raetz et al., 2013). Another antigen, heat shock protein 70 (TgHSP70), acts via stimulation of TLR2 and TLR4, activating B cells and DC's (Fang et al., 2008; Kikumura et al., 2010; Ge et al., 2014). The

parasite's protein profilin is recognized by interferon regulatory factor 8 (IRF8) positive DC's in mice; on the contrary, human and porcine DC's and monocytes are activated by the recognition of the parasite's ssRNA and DNA via TLR7 and TLR9, respectively (Uneishi et al., 2012; Andrade et al., 2013).

It is striking that different parasitic antigens seem to be simultaneously identified by various TLR's; consequently, their synergistic action leads to stimulation of the corresponding immune cells, which contribute to a more optimal host's defense (Debierre-Grockiego et al., 2007). Despite the differences in the antigen recognition, the activation mechanisms remain the same: TLR's are known to possess an adaptor protein, the myeloid differentiation primary-response protein factor 88 (MyD88) (Miller et al., 2009), which is stimulated after uptake of the parasite or parasite's antigens. The MyD88 signalling pathway subsequently activates other signalling molecules such as, the nuclear-factor-kappaB (NFκB) or the mitogen-activated protein kinase (MAPK) (Murphy et al., 2008; Ge et al., 2014). Both the NFκB and MAPK are essential for the production of the pro-inflammatory cytokines IL-2 and IL-12. These cytokines act in synergy with the tumor necrosis factor-α (TNF-α) produced by macrophages, resulting together in the stimulation of NK's to secrete another cytokine, interferon gamma (IFN-γ) (Murphy et al., 2008; Ge et al., 2014). In parallel to these events, the extensive IL-12 production by DC's upon the uptake of profilin leads to the activation of NK's cells, which are responsible for the IFN-γ release and subsequent stimulation of the macrophages and granulocytes (Sturge and Yarovinsky, 2014).

As already described, multiple cell populations of the innate and acquired immunity play a role during the early stages of toxoplasmosis.

Dendritic cells (DC's)

Immature DC's reside normally in peripheral tissues, more specifically in the lamina propria of the intestine. Upon activation by the recognition of *T. gondii* antigens, for instance profilin via the TLRs or chemokine CCR5 ligand by the corresponding receptor,

DC's migrate into the inflammatory and maturation sites in lymphoid tissues (Miller et al., 2009). During the maturation DC's present an increased expression of the Major Histocompatibility Complex (MHC) molecules, which upregulates the proliferation of naive T-cells. When activated, DC's stimulate further this cells subset by the releases of cytokines, among which IL-12 (Miller et al., 2009). The latter produced predominantly by DC's in the acute *T. gondii* infection, facilitates the NK's activation, and induction of a Th1 immune response (Filisetti and Candolfi, 2004; Miller et al., 2009; Denker, 2010). Further, mature DC's secrete IFN-γ and act as Antigen-Presenting Cells (APC's), activating naive $CD4^+$ T-lymphocytes. The stimulated NK's and Th1 cells also react with IFN-γ, which contributes to persistent DC's activation (Murphy et al., 2008; Miller et al., 2009; Cohen and Denkers, 2014).

Macrophages

The primary role of the macrophages is the detection of the tachyzoites via the PRR's and the subsequent cytotoxicity by phagocytosis. The cells are attracted to the inflammation site by the local secretion of cytokines and chemokines, as the result of the multiplication of the parasite in the enterocytes. Macrophages are activated by IFN-γ (auto-secreted and by NK's and T cells); TNF-α (auto-secreted) and IL-2 (produced by $CD4^+$ T cells) (Filisetti and Candolfi, 2004; Miller et al., 2009).

The inhibition of the parasite's replication is accomplished by production of reactive oxygen intermediates (ROI) and nitric oxide (NO), which together inhibit essential mitochondrial and nuclear enzymes, and by nutrient deprivation mechanisms such as enzymatic tryptophan degradation (Miller et al., 2009; Zhang et al., 2013). Finally, macrophages develop autophagolysosomes, which break down the parasitic parts ingested via phagocytosis, by the action of the proteolytic enzymes. Macrophages, as APC's, display *T. gondii* antigens to $CD4^+$ T lymphocytes, and, hence, trigger T cell activation (Sibley et al., 1992; Filisetti en Candolfi, 2004; Kasper et al., 2004; Ling et al., 2006; Miller et al., 2009). On top of these activities, macrophages are crucial during the

dissemination stage of the parasites by the early IL-12 production, which contributes to the NK's activation by the DC's (Miller et al., 2009).

Natural Killer Cells (NK's)

NK's are the leucocytes belonging to the innate immune system, which circulate in the blood stream or residue in the lymph nodes. They become activated in response to cytokines derived from themselves or other cell populations: IFN-γ (NK's), IL-12 (DC's, granulocytes, macrophages), TNF-α (macrophages) and IL-2 ($CD4^+$ T cells). NK cells also possess CCR5 chemokine receptor (Filisetti and Candolfi, 2004; Murphy et al., 2008; Gigley, 2016).

Activated NK cells are the major source of IFN-γ production, which has as direct effect an increased MHC II expression and an increased parasite recognition and uptake by the phagocytic cells, proliferation of $CD4^+$ T cells and subsequently their differentiation into inflammatory Th1 cells in the very early phase of the immune response against *T. gondii* (Miller et al., 2009; Murphy et al., 2008). The NK's do not possess any direct cytotoxic activity towards the parasite (Denkers and Sher, 1993; Cohen and Denkers, 2014; Sturge and Yarovinsky, 2014).

Granulocytes

The main cell population among the granulocytes, which are actively involved in *T. gondii* infection are the neutrophils. Shortly after infection of the enterocytes, $IL\text{-}12^+$ neutrophils are attracted to the infection site by the locally increased production of cytokines and chemokines. Neutrophils have the capacity to phagocyte the parasite and to defeat it by the release of toxic compounds from the granules, by the ROI and NO production, and, finally, by the synthesis and secretion of the pro-inflammatory cytokines such as TNF-α, CCR5 ligands and IL-12 to attract other immune cells (Denkers et al., 2004; Filisetti and Candolfi, 2004; Bennouna et al., 2006; Miller et al., 2009). Neutrophil depletion affects not only the mentioned cytokines but has also an indirect

effect on IFN-γ expression and lower counts of activated NK-cells, $CD4^+$ and $CD8^+$ T-lymphocytes (Bliss et al., 2001; Kasper et al, 2004; Miller et al., 2009).

T-lymphocytes

The $CD4^+$ and $CD8^+$ T-lymphocytes are the central populations of the adaptive cellular host resistance against acute toxoplasmosis. The cytokines produced by earlier activated cells will determine the polarisation of the $CD4^+$ T-helper cells. Consequently, IL-12, IFN-γ and TNF-α produced by DC's, macrophages, NK's and neutrophils will stimulate the activation of Th-1 cells. On the contrary, IL-4 will tip the differentiation towards the Th-2 population. The $CD4^+$ Th-1 cells start shortly with IL-2 and IFN-γ secretion to pass on the activated state to other cells of the adaptive system.

Three signals are required for $CD8^+$T cells activation, namely the antigen presentation via MHC class I, in combination with a co-stimulatory signal from B7 (on an APC) and CD28 (on a T-cell) complex, and of the TNFR/TNF superfamily of proteins. Finally, the presence of IL-2 and IFN-γ produced by Th1 $CD4^+$ T cells contributes as well to the cytotoxic activity of $CD8^+$ cells (Filisetti and Candolfi, 2004; Murphy et al., 2008).

Activated $CD8^+$ T cells produce more IFN-γ, creating a feedback loop, and differentiate into effectors T cells. Upon differentiation, they express their cytotoxic activity and cytokines secretion against tachyzoites or cells infected intracellularly with *T. gondii* (Gigley et al., 2011). Additionally, they also produce TNF-α, which induces secretion of IFN-γ by NK cells (Dimier-Poisson, 2003; Filisetti and Candolfi, 2004). By the cytotoxic action of the $CD8^+$, viable tachyzoites may possibly be released from infected cells and disseminate systemically or infect the surrounding cells. Nevertheless, the innate immune system remains active and mobilized to capture the parasite.

After primary infection, T cells ($CD4^+$ and $CD8^+$) transform to memory cells that protects the host against re-infection via the rupture of existing tissue cysts, or by recurrent alimentary infections (Denkers, 1999; Filisetti and Candolfi, 2004; Suzuki et al., 2012; Sturge en Yarovinsky, 2014).

B-lymphocytes

Although it has been sporadically reported that humoral immune response alone does not play an important role in the protection against *T. gondii* (Lindberg and Frenkel, 1977; Frenkel and Taylor, 1982), more recent studies have proven the opposite (Kang et al., 2000; Johnson and Sayles, 2002; Filisetti and Candolfi, 2004; Santana et al., 2012; Zhang et al., 2013).

The produced target specific immunoglobulins act on the extracellular tachyzoites, released after bursting of the infected cells, by facilitating the lysis, opsonisation and phagocytosis of the parasites in the presence of the complement system (Kang et al., 2000; Filisetti and Candolfi, 2004).

The several classes of the specific immunoglobulins appear on different time-points following the inoculation. The IgM class appears in serum at the end of the first week post infection. It activates the complement system, which leads to opsonisation and cell lysis. The IgM detection has been very useful for serological diagnosis, as described in Chapter 1 (Filisetti and Candolfi, 2004; Garweg et al., 2011). The limits of IgM detection are due to its time-dependent appearance and its low affinity for the parasite, which is compensated by its high avidity (as they possess 10 antigen-binding sites) (Murphy et al., 2008).

The IgG class appears during the subacute and/or chronic stages of *T. gondii* infection and mostly remains detectable at a moderate level for the lifetime of the host. The IgG's protect from the homologous re-infection (as long as the strains from the primary and the secondary infection belong to the same genotype) and are also able to cross the placental barrier in both humans and animals (Chucri et al., 2010). Their main targets are the surface antigens of the parasite, but the IgG's are also involved in the antibody-dependent cytotoxicity or opsonisation (Filisetti and Candolfi, 2004; Santana et al., 2012; Zhang et al., 2013).

The IgA class is released to secretions such as saliva, tears, milk, mucous but they are a predominant isotype of the immunoglobulin in the intestinal and respiratory tract

(Murphy et al., 2008). A significant IgA concentration can be temporarily measured in the intestines at the initial site of *T. gondii* infection (Igarashi et al., 2010). When transiting towards a subacute phase, high titers of IgA can be measured in serum of animals and humans, displaying clinical toxoplasmosis (Filisetti and Candolfi, 2004; Saadatnia et al., 2011; Amin et al., 2012).

The B cells are an important immune component of the chronic infection phase, since they contribute greatly to controlling the infection on long term. B-cell-deficient mice, which survived the acute infection, succumbed one month later due to the impaired antibody production (Kang et al., 2000). These findings demonstrate that neither the adaptive humoral nor cellular immunity alone is sufficient to combat the parasite but the combined effect of the different immune components is the most effective in defeating chronic toxoplasmosis.

2.3 Chronic phase of the infection

As highlighted in the previous chapter, the production of high levels of IFN-γ by different cell populations triggers the conversion of tachyzoites into bradyzoites and prevents cysts rupture, generating the chronic infection. The main feature of this phase of the infection is the presence of hundreds of bradyzoites, enclosed in tissue cysts and disseminated into different organs (Filisetti and Candolfi, 2004). Tissue cysts are the stage of the parasite which can persist during the host lifetime and are highly prevalent in brain, heart, eyes, internal organs such as lungs or liver and skeletal muscles of intermediate hosts (Afonso et al., 2012; Batz et al., 2012).

The presence (and persistence) of the *T. gondii* cysts in the peripheral tissues is maintained by the synergistic action of $CD4^+$ and $CD8^+$ T lymphocytes. The $CD4^+$ T cells stimulate predominantly a Th-1 response by producing IFN-γ, which is of pivotal importance for keeping the parasite in the latent form by driving the conversion of the rapidly multiplying tachyzoites towards the slow multiplying bradyzoite stage, and by suppressing their reversion to tachyzoites (Miller et al., 2009). However, $CD4^+$ play also

a critical role in the protection against the chronic infection or a reinfection by the induction of the antigen-specific antibodies production (Johnson and Sayles, 2002).

Although the intracellular bradyzoites are enclosed within the tissue, the latent $CD8^+$ T cell cytotoxic activity may be still involved. In the immunocompetent intermediate hosts the bradyzoites remain dormant for the rest of the host's life, while in the immunocompromised individuals a reactivation of the latent tissue cysts may occur, leading to severe and even lethal course of toxoplasmosis (Denkers, 2010; Zhang et al., 2013) (see also Chapter 1, paragraph 1.3.3 Clinical symptoms).

T. gondii is considered a successful parasite, which not only relies on the rapid host invasion, in order to avoid different components of the immune system such as phagocytes, complement and antibodies; in addition, its survival strategy relies on preservation of the latent stage of the parasite within the tissue cysts and inhibition of the stage conversion towards the more vulnerable tachyzoites by the following mechanisms:

1) expression of the homologous epitopes with the host's cells, or the molecular mimicry, by which the parasite may persist within the tissues without any immune response from the host;
2) forming of PV, consisting of elements from the parasite and host's cell;
3) immunosuppression in the chronic phase by the synergistic effect of the IL-10 stimulation and IL-12 inhibition;
4) induction of the dormant stage of the cells, by which it does not undergo apoptosis; in that way the tissue cysts may persist for the entire lifespan of the host without losing any activity or virulence (Denkers and Sher, 1993; Birner et al., 2000; Filisetti and Candolfi, 2004; Aliberti, 2005; Miller et al., 2009; Esch and Petersen, 2013; Yarovinsky, 2014).

However, the continuous IFN-γ production induces several modes of action aiming the parasite's elimination (Figure 2.2), such as:

expression of the inhibitory protein indoleamine 2,3-dioxygenase (IDO), which depletes tryptophan or an essential amino acid for the growth and replication of *T. gondii:*

1) production of the nitric oxide (NO) by the increased activity of the inducible NO-synthase (iNOS), which inhibits the mitochondrial and nuclear enzymes and prevents the parasite's replication; it appears that NO is highly toxic for the tachyzoites, while nearly harmless for the bradyzoites, protected by the cyst wall; another action of NO is the depletion of another essential amino acid or arginine, decreasing the multiplication rate;
2) expression of the effector proteins immunity related GTP-ases (IRG's) and guanylate-binding proteins (GBP's); there are 21 IRG's known in mice and just a single one in humans, while there are 12 GBP's described in mice and 7 in humans; these molecules contribute together to destruction of the PV's membrane, and, hence, lead to exposure of the parasite to endolysosomal enzymes, followed by the enzymatic degradation;
3) increased activity of the phagocytes for the elimination of the free parasites (Denkers and Sher, 1993; Birner et al., 2000; Filisetti and Candolfi, 2004; Aliberti, 2005; Miller et al., 2009; Esch and Petersen, 201; Yarovinsky, 2014).

It is noteworthy to mention that the excess of IFN-γ on short and long term might lead to pathologic changes in the infected tissues. More specifically, the uncontrolled cytokine production triggered by the presence of the cysts, is directly associated with the high NO levels released by, among others, macrophages. The NO is highly toxic under every circumstances for the target parasitic cells, but it may have a major impact on the surrounding healthy tissues.

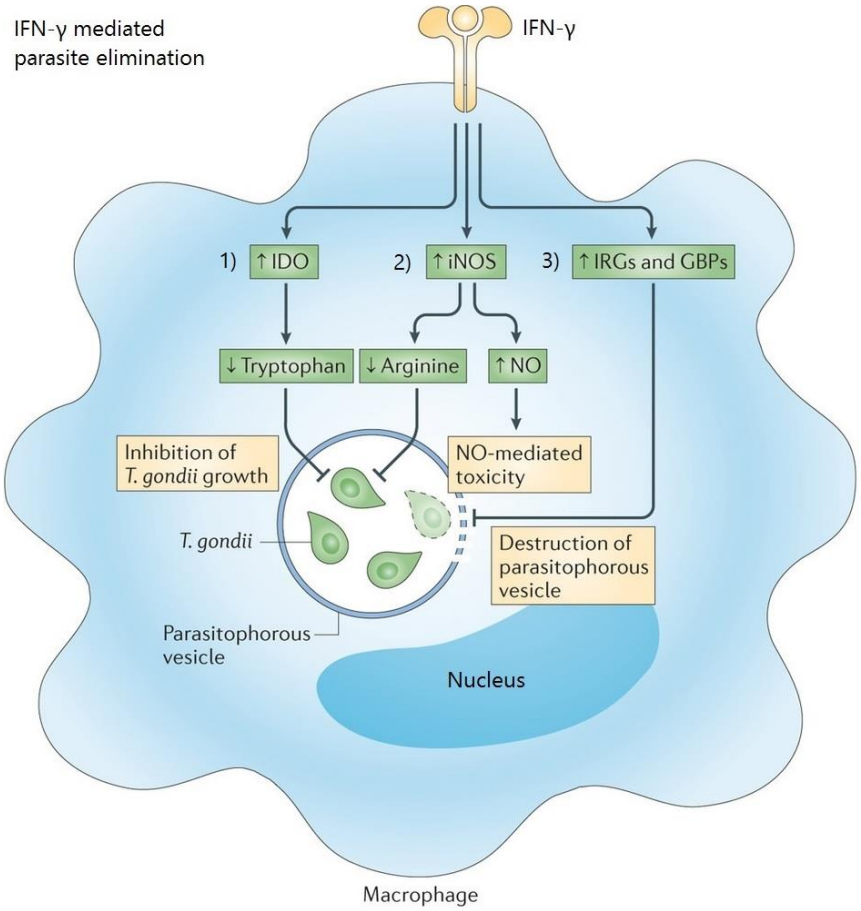


Figure 2.2 : The mechanisms of IFN-γ mediated elimination of the parasite from the infected cells (adapted from: Yarovinsky, 2014).

Therefore, a well-balanced inhibitory mechanism is required to temper the excessive IFN-γ production to moderate levels such as the release of anti-inflammatory IL-10 (Miller et al., 2009; Rijkers et al., 2009). The mode of action of IL-10 mainly relies on the immunomodulation of the APC's, or on inhibition of cytokine production and cytotoxic activity of the T cells. When intentionally deprived from IL-10, animals suffering from a chronic *T. gondii* infection develop a severe inflammation that can lead to necrotic sites in the intestine and liver (Filisetti en Candolfi, 2004; Aliberti, 2005; Miller et al., 2009).

Chapter 3 Pig as a model for an intermediate host of *T. gondii*

3.1 Prevalence of *T. gondii* in domestic pig

As mentioned in chapter 1, the pig is, among other domestic and wild mammals, a common intermediate host for *T. gondii*. At the same time, the parasite can cause human infection via the consumption of raw, undercooked or cured porcine meat products (Cook et al, 2000; Gauss et al., 2005; Klun et al., 2006; Pastiu et al., 2013; Vitale et al., 2014). Therefore, pork is indicated as one of the major meat sources associated with human foodborne *T. gondii* infection worldwide (Havelaar et al., 2015; Scallan et al., 2015; Opsteegh et al., 2016b).

Although the exact prevalence of this food pathogen in consumption meat is difficult to establish, the subsequent infection rate of humans has been estimated as an average of 300 consumers per 1 infected animal (Fehlhaber et al., 2002; Ortega-Pacheco et al., 2013). In addition, nearly all tissues from a pig are used directly for consumption or processed in potentially hazardous meat products without freezing or cooking, increasing in that way the chance of the transmission of the disease (Tenter et al., 2000). In comparison with other meat sources such as beef, horsemeat or poultry, pork seems to be more likely infected with *T. gondii* due to the high susceptibility of swine to the parasite (Hill and Dubey, 2013).

The global incidence of porcine infection per continent and country is shown in Table 3.1, taking into account the origin and the age of the animals, the farm management and the serologic assay applied (Dubey, 2009b; Dubey, 2010; Guo et al., 2015b). According to the report from the European Food Safety Agency (2012) describing the number of the positive animals between 2008 and 2010, the highest proportion of samples positive in PCR or serology for *T. gondii* across all countries was reported for sheep and goats. However, the clinical manifestation of toxoplasmosis is particularly obvious in these two species due to abortion, so it is also more likely to be confirmed, compared with other animal species, in which the more subtle signs of infection (particularly in the acute phase of the disease) may be missed. Referring to the same report, the prevalence of *T. gondii* specific antibodies in porcine serum samples collected in all the EU-states was 2.2% (EFSA report, 2012).

In general, the seroprevalence of *T. gondii* dropped significantly during the last decades in Europe and the USA. Corresponding to that, a low (0.0%-0.4%) to moderate (36%) prevalence was estimated on conventional pig farms in European countries (Kijlstra et al., 2004; Bartova et al., 2011; Guo et al., 2015b). In the USA a higher (25%) prevalence was detected in the free-range animals than in the outdoor- (6.8%) or indoor-ranged finishers (2.8%), (Dubey et al., 2008c; Hill et al., 2008; Gebreyes et al., 2008). In most industrialized countries the prevalence is not higher than 5% (Robert-Gangneux and Dardé, 2012). For Belgium, official data are missing, but based on a recent study 3.3% and 4% seropositive animals were detected in Flanders and 53.5% and 73.3% in Walloon, as determined with GRA7 and TLA ELISA, respectively (Jennes et al., 2017, submitted). In the Netherlands a spectacular drop in prevalence was seen over the period of 20 years for the finishers (from 54% to 1.9%, van Knapen, 1989) and for the seropositive sows (30.9% to 5.6%, van der Giessen et al., 2007). Nevertheless, the low prevalence in USA cannot prevent the high transmission rate to humans, as even as low as 1 % is equal to 1 million *T. gondii* seropositive animals, infecting potentially 300 million humans (Jones and Dubey, 2012).

To date, there is no obligatory screening on national level or notification system in the EU-states to detect and sample by molecular methods the seropositive animals on farm level or in slaughterhouses.

These observations and the possible reasons of the decreased prevalence are further discussed in detail (see 3.2).

Table 3.1 : Seroprevalence of *T. gondii* in domestic pigs (adapted from Guo et al., 2015b)

Continent	Country	Origin	# Animals	Prevalence (%)	Assay and titer	Reference
Europe	Romania	Backyard	2,564	30.50	IFAT, 1/32	Pastiu et al., 2013
		Sows	371	12.40		
		Finishers	660	0.00		
	Ireland	Finishers	137	4.70	LAT, 1/64	Halova et al., 2013
	Latvia	Finishers	269	0.40	In-house ELISA	Deksne and Kirjusina, 2013
		Free-range	543	6.20		
	Portugal	Farm	254	9.80	MAT, 1/20	Lopes et al., 2013
	Slovakia	Slaughter	923	2.16	ELISA kit[1]	Turcekowa et al., 2013
		Sows	47	4.26		
	Czech Republic	Slaughter	551	36.00	ELISA kit[2]	Bartova and Sedlak, 2011
	France	Slaughter	49	2.00	ELISA kit[3]	Roqueplo et al., 2011
	Italy	Indoor raised	960	16.10	IFAT, 1/16	Veronesi et al., 2011
		Slaughter	2160	16.30	ELISA kit[1]	Villari et al., 2009
	Serbia	Slaughter	488	9.20	MAT, 1/25	Klun et al., 2011
	Switzerland	Finishers	50	14.00	ELISA kit[3]	Berger-Schoch et al., 2011
		Adult	120	3.60		
		Free range	100	13.00		
	Spain	Finishers	1570	9.70	MAT, 1/25	Garcia-Bocanegra et al., 2010
		Sows	1400	24.20		

	Germany	Finishers	4999	4.10	In-house ELISA	de Buhr et al., 2008
		Sows	2041	16.50	IFAT, 1/16	Damriyasa et al., 2004
	Poland	Slaughter	106	26.40	MAT, 1/40	Sroka et al., 2008
	The Netherlands	Organic	406	10.9	In-house ELISA	Kijlstra et al., 2008
		Indoor raised	265	0.40		van der Giessen et al., 2007
		Organic	402	2.70		
		Free range	178	5.60		
		Organic	2796	3.00	ELISA-kit[3]	Meerburg et al., 2006
		Indoor raised	621	0.00	LAT, 1/64	Kijlstra et al., 2004
		Free range	635	4.70		
		Organic	660	1.20		
North-America	Mexico	Backyard	337	17.20	MAT, 1/25	Alvarado-Esquivel et al., 2012
		Farm	188	0.50		
	Canada	Finisher	6048	0.74	ELISA kit[4]	Poljak et al., 2008
	USA	Indoor raised	6238	2.60	ELISA kit[4]	Hill et al., 2010
		Outdoor raised	324	6.80	In-house ELISA	Gebreyes et al., 2008
		Indoor raised	292	1.10		
		Free range	48	25.00	ELISA kit[4]	Dubey et al., 2008b
		Slaughter	152	16.40	Western blot	Saavedra et al., 2004
South-America	Brazil	Farm	143	25.50	In-house ELISA	de Sousa et al., 2014
		Slaughter	190	19.50	IFAT, 1/64	Cademartori et al., 2014
		Indoor raised	27	11.50	In-house ELISA	Luciano et al., 2011

		Free range	34	20.60		
		Farm	606	13.40	MAT, 1/25	Piassa et al., 2010
	Chile	Slaughter	340	8.80	ELISA kit[4]	Munoz-Zanzi et al., 2012
	Panama	Indoor raised	290	32.10	IFAT, 1/20	Correa et al., 2008
	Argentina	Sows	230	37.80	MAT, 1/25	Venturini et al., 2004
	Peru	Slaughter	137	27.70	Western blot	Saavedra et al., 2004
Asia	Nepal	Slaughter	742	11.70	In-house ELISA	Devleesschauwer et al., 2013
	China	Finishers	1014	4.60	IHAT, 1/64	Chang et al., 2013
		Farm	2,277	29.60	In-house ELISA	Du et al., 2012
		Slaughter	1164	12.00	IHAT, 1/64	Wu et al., 2012
		Finishers	3558	24.50	In-house ELISA	Tao et al., 2011
		Mixed farm	813	53.40	ELISA kit[5]	Yu et al., 2011
		Sows	605	14.40	IHAT	Huang et al., 2010
	Malaysia	Sows	100	0.00	IFAT, 1/200	Chandrawathani et al., 2008
	Taiwan	Slaughter	395	10.10	LAT, 1/32	Tsai et al., 2007
	Vietnam	Finishers	325	23.00	MAT 1/25	Huong et al., 2007
		Sows	207	32.30		
		Free range	70	35.70		

[1] Institut Pourquier, Montpellier, France; [2] ID Screen Toxoplasmosis Indirect, ID-Vet, Grabels, France; [3] p30-ELISA; [4] SafePath Laboratories, Carlsbad, CA; [5] Haitai Biological Pharmaceuticals Co., Ltd., Zhuhai, People's Republic of China. IFAT: indirect fluorescence antibody test; IHAT: indirect haemagglutination test; LAT: latex agglutination assay; MAT: modified agglutination assay.

3.2 Risk factors associated with porcine toxoplasmosis

Several factors can potentially modify the risk of *T. gondii* infection in pigs, such as the presence of cats on farms, rodent control, age of the animals, size and type of the herd, outdoor access, the carcass disposal, and feeding of unprocessed animal products such as goat whey to pigs (van der Giessen et al., 2007; Dubey, 2009b; Villari et al., 2009; García-Bocanegra et al., 2010a, b; Hill et al., 2010; Meerburg et al., 2012).

3.2.1 Risk factors

The cat is responsible for the direct transmission of the infection to farm animals such as pigs by entering the stables and shedding the oocysts in the animal facilities, or by contaminating the near environment of the stables, and indirect spreading by animals entering the stables like dogs or birds. The free access to the stables for people, without the application of strict hygienic measures (e. g. disinfecting foot bath or protective footwear and clothes for the exclusive use in the animal facilities), can also contribute to the dissemination of the oocysts within the herd or the transmission to the stables from the contaminated environment (Lehmann et al., 2003; Hill et al., 2008; Hill et al., 2010). The lack of these measures is especially important if domesticated or feral cats live in the close neighbourhood of the animal facilities.

The second factor, namely the insufficient rodent control in the stables, drives the persistence of the parasite on farm level in two ways: the rodents serve as a constant reservoir of *T. gondii* for the cats, maintaining the infection risk within the herd; additionally, the rodents can directly be involved in the infection transmission by the predation or accidental ingestion of the mice by pigs (Lehmann et al., 2003; Kijlstra et al., 2008).

Age as a risk factor contributes to an increased seroprevalence in older animals, due to a longer exposure to the pathogen from the potentially hazardous environment.

Although the titer of the colostral antibodies in piglets declines by 120 days of age, irrespectively from the source of the infection in sows (natural versus experimental), maternal immunoglobulins do not provide a full protection. Consequently, post-weaning pigs are more susceptible to the infection than older animals (Garcia-Bocanegra et al., 2010). An age-dependent increase in seroprevalence in pig farms has been described in numerous studies, with a higher incidence in adult pigs (19.5%) than in young animals (10.9%), (Alvorado-Esquivel et al., 2011), or in breeding sows than in finishers: 30.9% versus 1.9% in the Netherlands (van Knapen et al., 1995) and 24.2% versus 9.7% in Spain (Garcia-Bocanegra et al., 2010).

The age of the animals at the slaughter has also an important implication for the transmission of the disease towards human consumers and the epidemiology of human toxoplasmosis. Indeed, the younger the animals, the more chance that the meat will be consumed fresh and unprocessed, while the meat derived from older animals such as sows, will undergo processing to end up in different pork products, which is harmful for the parasite and thus safer for the consumer in terms of parasitic load (Dubey, 2009b).

One of the major factors contributing to the incidence of the porcine infection is the size and the management type of the herd. The on-farm prevalence showed a reverse correlation with the size: small herds showed a higher rate of seropositive animals (4.1%) than medium (1.9%) or large (0.6%) herds (Zimmerman et al., 1990; Hill et al., 2006). The reason would be the higher exposure per animal in smaller farms due to the lower density of the pigs.

Even more critical for the risk of porcine toxoplasmosis is the management type of the farm. The recently observed decrease in seroprevalence of the infection in pig population might be due to the implementation of the modern management system in porcine herds, with a visible shift from housing of a smaller number of animals in less strictly confined establishments or outdoor, to large scale facilities with a high output and a fast turn-over, characterized by all-in-all-out or farrow-to-finish models

(Davies et al., 1998; Hill et al., 2010; Guo et al., 2015b). The intensive pig production expressed by the increasing number and size of porcine herds is driven by the high consumption of pork in the developed countries in Europe and the USA. In Belgium for instance, the total population of pigs comprises 6.5 million animals, housed in 5000 conventional farms, ranging between on average 700 to 1300 pigs, as estimated by the National Institute of the Calculations, Federal Public Service Economy, (Actualization of the Industrial Study on Pork, 2015). Consequently, nearly 12 million of animals are slaughtered each year for consumption (35 kg/year/inhabitant) or export in comparison with other countries. The majority of the in Belgium slaughtered pigs are exported within EU (56% of the carcasses to Germany, 18% to Poland and 6% to UK). The export of the fresh meat products covers 86% the export within EU countries: to Germany (36%), The Netherlands (11%), Italy (10%), France (10%) and UK (5.5%). Finally, the processed meat products are mainly sold to The Netherlands (34%), UK (14%), France (14%) and Germany (13%). On the contrary, only 3% of the carcasses processed in Belgium is imported (mainly from France (70%), UK (10%) and Germany (5%)), while almost 25% of the fresh meat comes from Germany (25%), The Netherlands (18%), Italy (17%), France (17%) and Spain (15%). Further, nearly 50% of the processed pork products sold in Belgium is imported: from Germany (35%), France (31%), The Netherlands (16%), Italy (8%) and Spain (5%). Summarizing, the porcine meat on the Belgian market does not originate from Belgium only and, hence, implies the necessity of a common approach with EU to maintain the same food safety standards.

Summarizing, in the modern large-scale herds, as the great majority of the Belgian farms, the risk of *T. gondii* infection, and, thus, the prevalence of seropositive animals, can be substantially reduced by the use of the strict confinement housing with restrictive biosecurity regulations (Tenter et al., 2000; Gebreyes et al., 2008; Hill et al., 2010; Wang et al., 2012; Guo et al., 2015b).

However, in the last years a new tendency in animal husbandry deserves the attention, namely the animal-friendly herds, housing organic or free-range animals, providing

daily a permanent or a temporary outdoor access to the animals. The term 'organic' refers to the quality and safety of the porcine products, with constraints about the chemical compounds originating from the feed or drug treatment, while 'free-range' stands for the life-quality of the animals during the production round. Hence, organic pigs are mainly reared outdoor, receive organic feed, are provided with an animal friendly living space, the piglets are weaned at a later age than 3-4 weeks as on the intensive farms, and undergo a restrictive use of antibiotics. The free-range pigs differ from the regular pigs by the outdoor access and straw bedding, but are fed with a standard porcine feed and may receive drug treatment, if necessary, without losing a label as in case of the organic pigs (Kijlstra et al., 2004).

In both types of farming, pigs are continuously exposed to the parasite by the contact with contaminated soil or ground water and can easily transmit the infection further in the food chain (Kijlstra et al., 2004; Hill et al., 2010; Dubey et al., 2012b). As estimated by others, the statistical chance for the detection of *T. gondii*-specific antibodies is approximately 2-times higher in the free-range than in the organic farms, or 8-times and 16-times higher in the organic and free-range farms, respectively, than in the conventional herds (van der Giessen et al., 2007).

As indicated above, an appropriate rodent control is of significant importance for the reduction of the risk for porcine infection; in addition, a proper carcass disposal of pigs seems to be equally essential, since both measures are intended to avoid the ingestion of formerly infected tissues (Lehmann et al., 2003; Hill et al., 2010). The cases of cannibalism by the accidental access to dead animals, especially when animal tissues are buried or composted, are considerably common (van der Giessen et al., 2007; Dubey, 2009b; Villari et al., 2009; García-Bocanegra et al., 2010a, b; Hill et al., 2010). Similarly, providing drinking water of unknown quality to the pigs, possibly contaminated with oocysts, and feeding of raw animal products such as goat whey is also a potential risk, if made from unprocessed milk containing tachyzoites from a

recently infected animal undergoing the dissemination phase (Meerburg et al., 2012; Gebremedhin et al., 2014; Boughattas, 2015a, 2015b; Cisak et al., 2017).

Finally, the prevalence of porcine toxoplasmosis may be influenced by the climate and geographical factors, e. g. altitude, temperature, and humidity, since they have a direct impact on the survival of *T. gondii* oocysts in the environment. As mentioned in Chapter 1, the oocysts remain infectious for a long period of time in a humid and cold environment, as long as they are not dried out or frozen. Hence, the prevalence of toxoplasmosis in pigs is higher in the mountains (32.1%) than in semi-desert areas in Mexico (14%) (Alvarado-Esquivel et al., 2011), or in the coastal and northern regions of the USA (2.9-3.2%) than in the south continental part of the country (Hill et al., NAHMS report, 2013).

3.2.2 Preventive measures

The preventive measures in porcine farming are focused on the elimination of the risk factors listed above. As mentioned earlier, the decline in infection prevalence in pigs may be attributed mainly to the implementation of the modern management systems on large scale, but also to some other improvements in farm management, which support further reduction of the infection rate. Therefore, they include: 1) farm management with confinement of the animals, 2) general hygienic measures for people entering the facilities 3) the controlled access to the stable for other animals like cats, dogs, birds or others, 4) rodent control, 5) feed and water supply.

The strict confinement housing, with no outdoor access of the animals and the all-in-all-out or farrow-to-finisher model, contributes to the greatest extent to the decreased prevalence of *T. gondii* infection in the pig population. The restrictive biosecurity regulations defining the access to the animal facilities for the visitors and animals, combined with the disinfection of the footwear, clothes and the surfaces on the one

hand, along with an efficient rodent control on the other hand, prevent transmission of the infection within the facility.

In contrast with the free-range farming, an important preventive measure taken in the modern porcine herds is providing disinfected water and feed to the animals, instead of the regular feed or scavenging opportunities with the outdoor access, surface water of unknown composition and sterility, and possibly contaminated straw bedding. Likewise, the animal friendly organic farms cannot circumvent the potential risk of the infection when the free access is provided to the soil, grass or plants, ground water or highly qualitative feed, as the cats and small rodents can enter such facilities (Jones and Dubey, 2012; Robert-Gangneux and Dardé, 2012).

Finally, an appropriate carcass disposal inhibits cannibalism and accidental ingestion of the tissue cysts or tachyzoites.

It is worth mentioning that these precautionary measures not only reduce the prevalence of porcine *T. gondii* infection, but also indirectly prevent the transmission of the infection towards humans (see also Chapter 1).

To date, there is no successful vaccine candidate on the market to control the infection incidence in pigs. The only registered attenuated vaccine (Ovilis® by MSD Animal Health, New Zealand) is available for sheep and goats, and prevents temporarily from the high rate of abortion, which normally occurs upon *T. gondii* infection in these species. The vaccine-induced protection is essential due to the high infection prevalence in grazing livestock, and because of the high economic losses in case of ovine or caprine congenital toxoplasmosis. However, due to the severe hazards for the operator of the vaccine and the potential reversion of the attenuated strain to the virulent wild type strain, this vaccine is limited in use to several countries only (Montoya and Liesenfeld, 2004; Buxton et al., 2007; Dubey et al., 2010).

One of the missing elements in the prevention of infection transmission by porcine meat is the regulatory screening of the animals prior to or at the arrival in the slaughterhouse. Since a seropositive animal is most likely hosting multiple tissue cysts,

the serologic screening could be used to prevent that tissues from chronically infected animals would enter the food chain without steps necessary to kill the parasites and as such disseminate the disease.

3.3 Pathogenesis and clinical symptoms in a porcine host

3.3.1 Pathogenesis

Pig as intermediate host can be infected with different stages of the parasite: oocysts, tissue cysts or, in rare cases, via tachyzoites. The most common infection route, by which pigs become infected with *T. gondii*, is via ingestion of oocysts. This is in particular notable for free-ranging or organic pigs, having access to a most likely contaminated environment. Depending on the stringency of the on-farm hygienic measures, inside-raised pigs can also be exposed to cats, which shed millions of oocysts, subsequently contaminating drinking water, stable bedding or animal feed.

Another transmission mode for pigs is predation of intermediate hosts such as mice or rats, occasionally present in the stables. Unprocessed animal products such as whey made from raw milk has been described to serve as the source of infection, when fed to the pigs or consumed by humans. Occasionally and mainly in non-EU countries, pigs can also be exposed to tissues containing cysts with bradyzoites by the intended feeding with meat scraps or accidentally by scavenging porcine tissues, if the carcasses or tissues like placentas are not optimally removed or locked away (Meerburg et al., 2012; Gebremedhin et al., 2014; Boughattas, 2015a, 2015b; Cisak et al., 2017).

Finally, transplacental transmission is very rare in pigs but possible to be induced in experimental conditions but, when so, the parasite causes mortality in neonatal pigs (Dubey and Urban, 1990; Dubey, 2009b; Pastiu et al., 2013).

Upon infection, the parasite rapidly undergoes a multiplication step in the small intestines and subsequently disseminates to peripheral tissues to convert into a latent stage or bradyzoites (see also Chapter 1).

3.3.2 Clinical symptoms

In general, *T. gondii* leads to a subclinical infection in pigs, but there are very few cases documented, describing most striking clinical signs. The symptoms include anorexia, fever, dyspnoea, limb weakness or death (Jungersen et al., 1999; Dubey, 2010). Systemic toxoplasmosis in pigs has been very rarely noticed (Klein et al., 2010). The infection has been documented in prenatal (Chang et al., 1990), neonatal (Haritani et al., 1988; Kumagai et al., 1988; Giraldi et al., 1996; Venturini et al., 1999; Thiptara et al., 2006) and weaned pigs (Okamoto et al., 1989; Weissenböck et al., 1993; Liao et al., 2006; Kim et al., 2009).

Prenatal outbreaks of *T. gondii* among pregnant sows result often in abortion. Most likely, systemic tachyzoites' dissemination leads to degenerative changes in the placental and fetal tissues, and was found the causative event of the abortion in a described case (Chang et al., 1990). The first incidences of neonatal toxoplasmosis in pigs were described during several outbreaks in Japan (Haritani et al.,1988; Kumagai et al. 1988; Okamoto et al, 1989). Stillborn piglets showed pneumonia and hepatic necrosis, while newborn piglets displayed an abnormal motoric coordination and encephalitis, pneumonia and necrotic lymphadenitis when autopsied upon their death at two weeks of age.

Depending on the genotype and the virulence of the strain, the clinical and pathological signs can vary in affected animals. Strikingly, even the piglets from the same litter may show a different degree of the clinical symptoms; presumably in function of the extent of parasite transmission per animal during the gestation. In a well-documented case in Brazil aborted foetusses, stillborn piglets and piglets born without clinical signs were reported. Moreover, the parasite was isolated from brains, hearts, lungs, livers, retinas and spleens from diseased and clinically healthy piglets (Giraldi et al., 1996).

Two more cases of stillborn piglets but with contrasting clinical signs in the surviving piglets were described in Argentina (Venturini et al., 1999) and Thailand (Thiptara et al., 2006), respectively. In Argentina, stillborn and live piglets were born from a seropositive sow; however, the infection could only be demonstrated by the presence of antibodies in fetal fluids but there were no clinical signs. In Thailand on the contrary, tachyzoites were identified on smears from lungs of the stillborn piglets, while surviving piglets were suffering from a haemorrhagic diarrhoea and dyspnoea. On the same farm also animals from different age groups (neonatal and growing piglets, adult sows and a boar) without clinical signs showed anti-*T. gondii* antibodies (Thiptara et al., 2006).

Nevertheless, clinical signs due to porcine toxoplasmosis have also been described in other age groups than piglets and this by other sources of infection than the transplacental transmission (Okamoto et al., 1989). Similarly, feed borne toxoplasmosis in Austria showed the typical set of the symptoms (Weissenböck and Dubey, 1993). Fatal toxoplasmosis in two adult sows was also documented (Liao et al., 2006). A more recent *T. gondii* manifestation in adult sows occurred in Korea (Kim et al., 2009), where clinical signs included fever, anorexia, neurological symptoms and abortions.

In analogy with the natural course of infections in piglets or sows, also experimental infections have been performed. Inoculation of pigs with oocysts of the SSI-119 strain can induce fever, anorexia or loss of appetite and a decline in the general condition (Wingstrand et al., 1997). A clear age effect and the corresponding severity of the clinical signs was described upon inoculation of sows in gestation or piglets with RH-strain tachyzoites at different ages: a congenital infection had the greatest impact on the foetus, while neonatal inoculation of piglets resulted in fewer and less pronounced clinical signs, whereas young adult animals frequently experienced subclinical infections (Work et al. 1970).

3.4 Acute and chronic immune responses induced by natural and experimental infections in pigs

Immune responses associated with toxoplasmosis in pigs have been studied in the experimentally induced infection models, due to the constraint that the natural course of the infection is mainly subclinical (Dubey et al., 1995b). Few studies have determined the immunological mechanisms associated with the acute and/or chronic stage of the disease in pigs, as described more in detail further (Dubey, 1998c; Solano Aguilar et al., 2001; Dawson et al., 2004; Kringel et al., 2004; Dawson et al., 2005; Garcia et al., 2005; Jongert et al., 2008; Verhelst et al., 2011; Burrells et al., 2015; Verhelst et al., 2015).

3.4.1 Natural infections

As described in Chapter 1, in most cases *T. gondii* infection proceeds asymptomatic in pigs, and the animals only occasionally develop clinical signs. Therefore, the naturally infected animals can be distinguished via anti-*T. gondii* IgM and/or IgG antibody detection by the use of one or a combination of several serological assays. Since each test has its own characteristics, namely, method of cut-off value determination and subsequently, sensitivity and specificity, the confirmation of the acute or chronic phase of the infection is not straightforward. Additionally, the presence of parasite-specific antibodies is not necessarily indicative for a recent exposure, but may also indicate a former exposure of the host to the parasite, since even IgM's can persist for a long time or rise again in concentration upon re-infection (Kortbeek, 1999; van der Giessen et al., 2003; Rorman et al., 2006; Petersen, 2007; Dubey et al., 2010; Mancianti et al., 2010).
It would be recommended to combine and match the results of different tests to obtain the highest certainty about the infection status within the animal. However, only few studies combined different techniques (Chang et al., 1990; Damriyasa et al., 2005; Cavalcante et al., 2006; Kijlstra et al., 2008). The majority of the research groups applied a single technique (see Table 3.1).

Efforts have been made to isolate viable parasites or to detect the parasitic DNA by PCR from naturally infected animals. Based on the DNA isolated from the slaughtered animals, the majority of the strains circulating within the porcine population in North America belongs to the genotypes II and III or has a mixed allele composition, while in Europe genotype II seems to be the most predominant clonal type (Lehmann et al., 2003; de Sousa et al., 2006). In South America, on the contrary, genotype I or mixed types are prevalent (Belfort-Neto et al., 2007).

3.4.2 Experimental infections

Infection models under controlled and experimentally induced circumstances have shown a comparable pattern, as far as the immune response is concerned.

First of all, inoculation of pigs with any infectious stage of *T. gondii* (oocysts, tissue cysts or tachyzoites) leads to development of antibodies against stage-specific parasitic antigens. The time-interval between the inoculation and the first detection of the immunoglobulins or cytokines varies depending on the virulence of the used strain, the infectious stage and the applied dose. Since the maternal antibodies do not persist for a long period of time (maximal 120 days), the weaned pigs develop actively own IgM and IgG immunoglobulins, which remain present at high concentration and partially or fully protect from a homologous challenge (Dubey et al., 1986; Lind et al., 1997; Dawson et al., 2005; Garcia et al., 2005, Jongert et al., 2008).

Secondly, a significant Th1-immune response is observed as an increase of IFN-γ production after inoculation with particular *T. gondii* strains at a well-defined inoculation dose (Solano Aguilar et al., 2001; Dawson et al., 2004; Dawson et al., 2005; Verhelst et al., 2015). This increase was positively correlated with the time-interval from the inoculation time point until a high-level plateau concentration was achieved. The produced cytokine was detected in the infected animals in serum samples, in the supernatant from cultured PBMCs, and also as IFN-γ mRNA expression in PBMCs and

intestinal lymphoid tissues. The IFN-γ production associated with experimental toxoplasmosis in pigs was the result of the activation of the cells of the innate and acquired immunity. The responses were investigated *in vitro* by determining the cytokine profile until 14 (Dawson et al., 2005), 40 (Solano Aguilar et al., 2001) or 56 days post infection (dpi) (Verhelst et al., 2011).

Next to IFN-γ, also other cytokines are involved in the immune response, such IL-12 or TNF-α, as described earlier (see Chapter 2). Infection with the oocysts of the VEG-strain induced a Th-1 immune response, with production of IL-15 and TNF-α, determined as gene expression on mRNA level (Dawson et al., 2005). In another study, upon inoculation with the same infectious stage of the latter strain, a more pronounced Th-2 profile with IL-10 and IL-12 was detected after the acute phase of the infection, dominated by IFN-γ production (Solano Aguilar et al., 2001).

During the acute phase of the infection the immune responses are directed against the disseminating tachyzoites, while throughout the chronic toxoplasmosis they also target the latent cysts with bradyzoites in the variety of the tissues. It is well described that the parasite can persist within the intermediate host for a life span and can be found in all internal organs and muscles (Dubey et al., 1998c; Black and Boothroyd, 2000; van der Giessen et al., 2003; Montoya and Liesenfeld, 2004). Referring to the pigs as the intermediate host, viable *T. gondii* was recovered from porcine brains, hearts, tongues, diaphragms, livers and kidneys. Additionally, all edible commercial cuts of meat tested also positive, representing thereby a potential risk for consumers (Dubey, 1988, Opsteegh et al., 2016b). Nevertheless, it is a subject of discussion and study whether the host can clear tissues during the chronic infection phase. There is scientific evidence that the number of the cysts containing parasites can gradually decrease in porcine tissues, with a decline in its viability, as tested by bioassay (Verhelst et al., 2011).

Summarizing, the induction of both a humoral and cellular immune response is necessary to control the infection in pigs. Although *T. gondii* as an intracellular parasite cannot be combatted and completely cleared from tissues, the elevated antibody and

cytokine production lead to a protective immunity, as evidenced by recovery from experimental infection with viable parasites (Dubey et al., 1991; Dubey et al., 1994; Dubey et al., 1998c; Solano Aguilar et al., 2001; Dawson et al., 2005). However, a delicate balance between the protective mechanisms and the cytokines' toxicity should be maintained (see Chapter 2).

Finally, a partial protection can be obtained in vaccination studies and challenge experiments, but there is still need to improve the current knowledge (Kringel et al., 2004; Garcia et al., 2005; Jongert et al., 2008, Verhelst et al., 2011; Burrells et al., 2015). One of the goals used as a success rate parameter is the reduction of the parasitic load, obtained in the vaccinated and challenged group and determined by bioassay and qPCR on murine and porcine tissues. By achieving this, not only porcine toxoplasmosis is likely to be reduced in prevalence, or in severity of clinical signs, if applied in other species such as small ruminants; correspondingly, human *T. gondii* infection would lose one of the important natural sources.

Part II

Aims of the study

T. gondii is considered an underestimated pathogen, infecting one third to half of the world human population and causing a severe disease in vulnerable groups, such as newborns and immunocompromised patients (Tenter et al., 2000; Gamarra et al., 2008; Halonen and Weiss, 2009; Shapiro et al., 2010; Robert-Gangneux and Dardé, 2012, Flegr et al., 2014). The estimations of the congenital and acquired toxoplasmosis burden revealed between one and two million healthy life years lost each year (Torgerson and Mastroiacovo, 2013; Torgerson et al., 2015). Since the majority (30-63%) of foodborne human toxoplasmosis cases is due to consumption of infected meat (Cook et al., 2000), knowledge of the *T. gondii* distribution among meat-producing animals is of pivotal importance for the implementation of efficient prevention strategies and, consequently, for the reduction of the potential exposure to the pathogen (Kijlstra and Jongert, 2008; Robert-Gangneux and Dardé, 2012). However, evidence is still missing about the natural course of infection in livestock and the parameters indicating the parasite's dissemination. The potential risk for humans upon consumption of infected tissues is, hence, inevitable.

As indicated earlier in the introduction, domestic (or wild) pigs not only maintain *T. gondii* infection, as intermediate hosts, but can also serve as an important source of this zoonosis for humans (Lehmann et al., 2003; Kijlstra et al., 2008; Meerburg et al., 2012). Consequently, *T. gondii* infection in pigs can potentially affect multiple consumers due to distribution and processing of the fresh porcine edible tissues into the food chain. Despite the recent European initiatives to thoroughly investigate and document the presence of toxoplasmosis in a multispecies study, the complete information on the prevalence, parasite burden, the infectious capacity of the edible tissues and immunological parameters to identify the infection is still not available for pigs (EFSA report, 2012; Opsteegh et al., 2016b). For instance, the prevalence of *T. gondii* infection in pigs and the associated risk factors within the Belgian herds are not yet defined. Importantly, the intensive pork production in Belgium is characterized by a high consumption rate per inhabitant on the one hand, and a significant contribution

to the export of the produced meat within and outside of the EU on the other hand. It would be highly recommended to estimate the risk of foodborne *T. gondii* transmission via consumption of porcine meat products, and, on a long-term, to eradicate the infection in livestock both in Belgium, as in other countries. As a starting point, defining and analysing the immunological parameters associated with the progress of the infection in the porcine host could help with the identification of the circumstances, which potentially contribute to a reduction of the parasite burden in edible tissues. Further, identifying the parasite's antigens inducing a strong immune response, could lead to the development of a vaccine.

In order to address the above listed gaps and gain more insights into the host-pathogen interaction of porcine toxoplasmosis in the acute and chronic phase of the infection, the following aims were formulated:

1) Estimation of the true prevalence of *T. gondii* infection in Belgian swine and first steps towards identification of the risk factors (Chapter 4).
2) Evaluation of the strain and dose effects in a single-strain experimental infection in pigs on:
    a. The immune response (Chapter 5, 6 and 7).
    b. The parasite burden and viability in tissues (Chapter 5, 6 and 7).
3) The effect of reinfection with a heterologous strain on the parasite burden and the immune response (Chapter 6).
4) Identification of the more potent immunogenic fractions within Total Lysate Antigen (TLA) of tachyzoites based upon *in vitro* IFN-γ induction (Chapter 7).

Part III

Experimental part

Chapter 4 True prevalence of anti-*Toxoplasma gondii* antibodies on Belgian pig farms

Adapted from:

Jennes, M., Devleesschauwer, B., De Craeye, S., Praet, N., Verhelst, D., Czaplicki, G., Vanrobaeys, M., Dierick, K., Dorny, P. and Cox, E. True prevalence of anti-*Toxoplasma gondii* antibodies on Belgian pig farms. Veterinary Parasitology (under review).

4.1 Abstract

Toxoplasma gondii remains one of the most significant foodborne zoonoses in developed countries. Pork can serve as the source of human *T. gondii* infection, however, no data are available on the prevalence of the parasite in the porcine population in Belgium. We performed a serological analysis of 2263 serum samples from 251 conventional herds with intensive management system in Belgium. Recombinant (GRA7) or native (Total Lysate Antigen—TLA) antigens of *T. gondii* were used in ELISA to determine the seroprevalence of porcine *T. gondii* infection. Both tests showed a significantly higher total, between-herd and within-herd apparent prevalence of anti-*T. gondii* IgG in Wallonia than in Flanders ($P < 0.001$). Additionally, a Bayesian model was developed to estimate true within-herd and between-herd prevalence based on the results of both diagnostic tests. The total true prevalence across all herds reached 65% (95% uncertainty interval (UI) 52%–76%) in Wallonia, compared to 1% (95% UI 0%–2%) in Flanders. Likewise, the true between-herd prevalence reached 73% (95% UI 59%–85%) in Wallonia, versus 4% (95% UI 1%–10%) in Flanders. The true within-herd prevalence of infected farms was 89% (95% UI 82%–96%) in Wallonia and 33% (95% UI 15%–55%) in Flanders. Together with the estimation of the true prevalence, the assay characteristics for the applied tests were calculated. The sensitivity of both the TLA- and GRA7-ELISA was lower in Flanders than in Wallonia, however without any significance. On the contrary, the specificities of the assays were significantly higher in Flanders, in particular for the GRA7-ELISA (91% vs 61%). This study is the first providing data on porcine toxoplasmosis seroprevalence in Belgium, and demonstrates a high burden in the Walloon Region.

4.2 Introduction

Toxoplasma gondii is an intracellular pathogen of all warm-blooded mammalian species, infecting wildlife, livestock and humans. With more than 10 million new clinical

infections each year, toxoplasmosis is one of the most common foodborne parasitic diseases worldwide. Congenital and acquired foodborne toxoplasmosis are estimated to result in between one and two million healthy life years lost each year (Torgerson et al., 2015).

Domestic pigs can serve as the intermediate hosts for the parasite, and, simultaneously, as the source of the infection for humans even in the modern and intensive swine production systems (Lehmann et al., 2003; Kijlstra et al., 2008; Meerburg et al., 2012). Several factors seem to be of pivotal significance to influence the risk of the infection in pigs, such as the presence of cats on farms, increasing age of the animals due to a longer exposure, small size of the herd, free-range or backyard pigs rather than the strict confinement housing with restrictive biosecurity regulations, poor rodent control, and cannibalism by access to infected tissues by inappropriate carcass disposal (van der Giessen et al., 2007; Dubey, 2009b; Villari et al., 2009; García-Bocanegra et al., 2010a, b; Hill et al., 2010). Consequently, raw, undercooked or cured pork is considered to be an important source of human infection (Cook et al, 2000; Guo et al., 2015a; Opsteegh et al., 2016b). Additionally, no serological or molecular screening technique is mandatory in the slaughterhouses in Europe. Therefore, application of standard hygienic procedures on farm level, including the prevention of contact with the intermediate or final host, are required to eradicate or reduce the transmission of foodborne toxoplasmosis to humans (Hill et al., 2010). A reliable serological surveillance applied on farm or slaughterhouse level could be an efficient tool in the early detection and prevention of this zoonosis.

The seroprevalence of *T. gondii* infection in pigs has been estimated in numerous studies. For European countries, in the last decade, a low (0.0%-0.4%) to moderate (36%) prevalence was estimated on conventional pig farms (Kijlstra et al., 2004; Bartova et al., 2011; Guo et al., 2015b).

To the authors' knowledge, no data have been published on the seroprevalence of *T. gondii* in pigs on Belgian farms. The total Belgian pig population comprises 6.5 million

animals, including 400,000 breeding sows, 200,000 fattening pigs above 110 kg of weight, 4.3 million fattening pigs under 110 kg of weight and 1.6 million piglets (Actualisation of the Industrial Study on Pork, National Institute of the Calculations, Federal Public Service Economy, 2015). Consequently, nearly 1 million animals are slaughtered per month for the export and the use on the national market, due to an excessive consumption rate per inhabitant (35 kg/year), in comparison with other countries. The animals are raised in approximately 5000 conventional registered pork farms, located mainly (93%) in Flanders, and particularly in the provinces of West Flanders, East Flanders and Antwerp (Actualisation of the Industrial Study on Pork, National Institute of the Calculations, Federal Public Service Economy, 2015). In the past years the number of herds decreased with 50% but the total amount of pigs produced in Belgium reduced only with 12% due to an increased average herd size. Consequently, these changes led to a rise in animals' concentration per farm from approximately 700 to 1300 pigs, exceeding 2000 animals in 20% of Flemish farms. In the Walloon Region, on the other hand, only 7% of the national pig farms are located, uniformly distributed across the different provinces. There the pig production is characterized by size-limited herds, raising free-range pigs or providing an outdoor access to the animals. According to recent data, there are only 14 registered organic (or biologic) farms in Flanders, housing approximately 2500 pigs, while in Wallonia there are 6800 pigs in 50 herds in this category. Organic pig raising requires an outdoor access, biologic pig feed, animal friendly housing conditions, delayed weaning and restricted use of the medical treatment, as regulated by the European Union Council (No 834/2007 and No 2092/91). In addition to the certified organic pig farms in Wallonia, there are herds, providing an unlimited outdoor access to the animals. These farms are, however, not classified as such and, therefore, assumed to raise free-range pigs in conventional conditions (Actualisation of the Industrial Study on Pork, National Institute of the Calculations, Federal Public Service Economy, 2015).

To study the prevalence of porcine toxoplasmosis in Belgium we conducted a serological survey on porcine serum samples collected as part of a preventive Aujeszky-screening from 10 provinces across Flanders and the Walloon Region.

Whereas isolation of *T. gondii* by bioassay using laboratory animals is considered the gold standard for demonstrating parasite infection, the Sabin-Feldman Dye Test is considered the gold standard to establish anti-*Toxoplasma*antibodies in humans (Dubey, 2010). However, this assay does not demonstrate the same accuracy in animals such as swine and has the disadvantage that live parasites are used as the antigen. In animals several serological assays have been described to detect anti-*T. gondii* antibodies, such as the modified agglutination test (MAT), latex agglutination test (LAT), indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) (Dubey, 2010).

Because no commercial ELISA for porcine serum samples was available at the time of the data collection, we used two in-house ELISAs, based on recombinant (GRA7) and native (Total Lysate Antigen - TLA) *T. gondii* antigens, respectively. None of these serological assays could be considered a gold standard, possibly resulting in false positive or false negative results, limiting their accuracy and yielding only an apparent prevalence estimate (Speybroeck et al. 2013). To account for the imperfectness of the serological assays used, and because the tissues of the sampled animals were not available for testing in bioassay, true prevalence was estimated in a Bayesian framework. In this approach, prior external information, e.g. based on expert knowledge, serves as a probabilistic restriction to make an assumption on the diagnostic sensitivity (i.e. conditional probability that an individual/sample having a disease will be correctly identified as such) and specificity of the assays (Berkvens et al, 2006). In addition to calculating the apparent percentage of *T. gondii* positive animals, we therefore developed a Bayesian model to estimate true total, within-herd and between-herd prevalence, adapted from covariance-based true prevalence estimation model proposed by Branscum et al. (2004).

4.3 Material and methods

4.3.1. Sampling design

In total, 2263 serum samples were collected between 2009 and 2013 from finisher pigs at 4 to 6 months of age, corresponding to 110 kg of weight, as part of a preventive Aujeszky-screening on Belgian pig farms. Since no earlier data were reported on the prevalence of *T. gondii* in Belgium in conventional farms, the samples available from the Aujeszky screening were used for this preliminary study in Flanders; in the further stage it was completed with the data set from Wallonia. The sample size per herd was based on a power calculation for Aujeszky virus prevalence and was set at 12 per herd, according to the formula: 1- (1-prevalence)^n, where (1-prevalence): negative samples, 1-negative samples : positive samples, n : number of samples tested. However, in several cases less or more samples were collected and included in the screening. Samples were drawn from the jugular vein using 21G needles and a vacutainer system (BD, Erembodegem, Belgium) by the local veterinarian. The serum samples obtained after centrifugation (2500 x g, 10 min at 4°C) were stored at -20°C prior to analysis in the Laboratory for Immunology, Faculty of Veterinary Medicine, Ghent University.

The serum samples originated from 151 Flemish (1773 samples) and 100 Walloon farms (490 samples). Samples from one additional Walloon farm had been collected but were unsuitable for testing due to excessive haemolysis and, therefore, excluded from the study.

Farms participating in the study were randomly selected. No prior information was available on their serological status in reference to *T. gondii* nor their location or herd size, in order to avoid selection bias in the experimental setup. The Flemish farms originated from the five following provinces, according to a decreasing number of herds tested, roughly reflecting the density of pig farming in the Flanders region: West Flanders (88), East Flanders (39), Antwerp (14), Limburg (7) and Flemish Brabant (3). The number of serum samples per farm ranged from 2 to 17 (median n = 12). The Walloon

farms were selected in order to ensure a uniform geographical distribution among the study area. In that way, all five provinces (Liège [25], Luxembourg [21], Hainaut [19], Walloon Brabant [18] and Namur [17]) were represented with an approximately equal numbers of farms per province and a standardized (n = 5) number of samples per farm. With the exclusion of the samples where haemolysis was too excessive, the final number of tested samples per Walloon farm ranged between 2 and 5 (median n = 4.5).

4.3.2 Serological assays

Preparation of rGRA7 antigen

Recombinant dense granule protein 7 (GRA7) was used in the serological assay to detect *T. gondii*-specific IgG antibodies, since it has be proven to be a reliable marker of an active infection, being expressed by all *T. gondii* stages (Jacobs et al., 1999; Verhelst et al., 2015). The antigen was prepared as previously described (Jongert et al., 2007).

Preparation of total lysate antigens

Total lysate antigens (TLA) consist of parasitic crude proteins, extracted from viable tachyzoites of the *T. gondii* RH-strain as previously described (Vercammen et al., 2000; Jongert et al., 2007). In brief, tachyzoites were purified by differential centrifugation and filtration steps, followed by sonication under cooling conditions. Finally, the purified antigens were aliquoted and stored for further use at -20°C. All procedures were approved by the Ethical Committee of the National Reference Laboratory for Toxoplasmosis, Scientific Institute for Public Health, Brussels, Belgium (approval number 20140704-01).

rGRA7 and TLA ELISA

The rGRA7 and TLA antigens were coated to the wells of a Nunc Polysorp™ ELISA plate (Life Technologies, Merelbeke, Belgium) at a concentration of 10 µg/ml in bicarbonate buffer (pH = 9.4) for 2 h at 37°C. The coating and washing steps were followed by overnight blocking at 4°C with 0.2% Tween®-80 in PBS, and 1 h incubation at 37°C with the serum samples 1/50 diluted in dilution buffer (0.05% Tween®-20 in PBS). On each plate, control serum samples from 3 seronegative animals (with no anti-*T. gondii* IgM or IgG) and from 1 seropositive animal (with anti-*T. gondii* IgG) were included in duplicate and at the same dilution. The seropositivity status of these control samples was determined earlier by indirect immunofluorescence assay (IFA) in the National Reference Laboratory for Toxoplasmosis. Subsequently, the plates were incubated for 1 h at 37°C with goat anti-pig IgG-H+L HRP conjugate (Bethyl Laboratories Inc., Montgomery, Texas, USA), at a 1/10000 dilution, and developed by adding 2, 2'-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS) (Hoffman-La Roche, Basel, Switzerland) as substrate-chromogen solution after 30 min of incubation at 37°C. In between steps wells were washed with PBS 0.2% Tween®20. Absorbance was read at 405 nm and the obtained data were managed with Microsoft Excel software. Diluted serum samples were considered positive if they exceeded the plate-specific cut-off value, calculated as the mean OD405 of the negative controls plus three times their standard deviation. Across plates, the cut-off value ranged between 0.115 – 0.305 for GRA7 and between 0.117 – 0.430 for TLA. All assays were performed by the same operator in the same laboratory facility, using the same lots of reagents and devices. Additionally, an external validation of the assays was performed on 100 porcine samples with an unknown serological status but the same approximate age (pigs at the end of the fattening period) in combination with IFA.

4.3.3 Statistical analysis

Apparent within-herd and between-herd prevalence

Apparent average within-herd prevalence was calculated by averaging herd level prevalence across herds. Apparent overall and between-herd prevalence, with corresponding 95% exact confidence intervals (CI) were calculated using the 'propCI' function in the prevalence package for R 3.3.1 (Devleesschauwer et al. 2015; R Core Team 2016). Differences in apparent within-herd prevalence between the Flemish and Walloon regions were analyzed using logistic regression, while differences in apparent overall and between-herd prevalence were analyzed using Pearson's Chi-squared test.

True within-herd and between-herd prevalence

We adapted the model by Branscum et al. (2004) to obtain true within-herd and between-herd prevalence based on the combined results of the two tests.

In each region, $\boldsymbol{n}_k = n_1, n_2, \ldots, n_K$ animals were randomly sampled from K farms and subjected to two serological assays. The apparent test results for each herd k are given by a vector $\boldsymbol{x}_k = (x_{11,k}, x_{10,k}, x_{01,k}, x_{00,k})$, with $x_{11,k}$ the number of animals from herd k testing positive on both tests, $x_{10,k}$ the number of animals from herd k testing positive on the first test but negative on the second, and so on. The different vectors $\boldsymbol{x}_k = \boldsymbol{x}_1, \boldsymbol{x}_2, \ldots, \boldsymbol{x}_K$ were assumed to be independent and distributed according to a multinomial distribution:

$$\boldsymbol{x}_k \sim \text{multinomial}(n_k, \boldsymbol{AP}_k)$$

with $\boldsymbol{AP}_k = (AP_{11,k}, AP_{10,k}, AP_{01,k}, AP_{00,k})$ the vector of apparent prevalences corresponding to each possible combination of test results. In line with Branscum et al. (2004), we defined the apparent prevalences in terms of the true within-herd prevalence π_k, the test sensitivities (SE_1, SE_2), the test specificities (SP_1, SP_2), and the covariances between the two tests for infected and non-infected animals (a, b):

$$AP_{11,k} = \pi_k[SE_1SE_2 + a] + (1 - \pi_k)[(1 - SP_1)(1 - SP_2) + b]$$
$$AP_{10,k} = \pi_k[SE_1(1 - SE_2) - a] + (1 - \pi_k)[(1 - SP_1)SP_2 - b]$$
$$AP_{01,k} = \pi_k[(1 - SE_1)SE_2 - a] + (1 - \pi_k)[SP_1(1 - SP_2) - b]$$
$$AP_{00,k} = \pi_k[(1 - SE_1)(1 - SE_2) + a] + (1 - \pi_k)[SP_1SP_2 + b]$$

We modelled the true prevalence π_k within each of the K sampled herds as a mixture of point mass at zero and a Beta distributed random variable μ, reflecting the true within-herd prevalence of infected herds:

$$\pi_k = 0, \qquad \text{with probability } 1 - \tau$$
$$\pi_k = \mu \sim \text{Beta}(a_\mu, b_\mu), \qquad \text{with probability } \tau$$

with τ reflecting the true between-herd prevalence (i.e., the true prevalence of infected herds). The product of μ and τ then reflects the true within-herd prevalence across all herds.

Table 4.1 presents the external information used on the test sensitivities and specificities. We fitted Beta distributions to these constraints using function 'betaExpert' in the prevalence package for R 3.3.1 (Devleesschauwer et al. 2015; R Core Team 2016). The prior information was based on the application of the serological assays in the literature, where the recombinant and native antigens were prepared corresponding to the protocols applied in this study, and served as a serodiagnostic tool for the diagnosis of toxoplasmosis in humans and livestock (Lind et al., 1997; Jacobs et al., 1999; Beghetto et al., 2006; Shapaan et al., 2008; Holec-Gąsior et al., 2011; Terkawi et al., 2013). We assumed independent $\text{Beta}(1,1)$ priors for μ and τ. For the covariances between two tests, we assumed $\text{Uniform}(-0.25,0.25)$ priors, i.e., uninformative priors spanning the natural limits of the covariance parameters.

Table 4.1 : Prior information on test sensitivities and specificities for the estimation of true herd prevalence.

Test	Sensitivity		Specificity	
	information	*distribution*	*information*	*distribution*
T1: TLA	>0.80	$\mathrm{Beta}(43,5.6)$	>0.90	$\mathrm{Beta}(100,6.2)$
T2: GRA7	0.78–0.91	$\mathrm{Beta}(119,23)$	>0.85	$\mathrm{Beta}(63,5.9)$

Models for the true within-herd and between-herd prevalence were implemented independently for Flanders and Wallonia. For each model, we simulated two chains of 20,000 iterations, of which the first 10,000 were discarded as burn-in. Convergence of the models was assessed by calculating the multivariate potential scale reduction factor (Brooks and Gelman, 1998) and by assessing trace and density plots. The models were implemented in JAGS 4.2.0 (Plummer 2003), using the rjags package in R 3.3.1 (Plummer 2014; R Core Team 2016). The applied JAGS code is available in Appendix A.

4.4 Results

4.4.1 Apparent prevalence

Appendix B provides the apparent test results per herd in both regions. A farm was considered positive from a single positive sample in any of the two serological tests. Table 4.2 shows the total, between-herd, and average within-herd apparent prevalence in both regions, for the two diagnostic tests. Both tests showed apparent prevalence estimates to be significantly lower in Flanders than in Wallonia ($P < 0.001$). In both regions, more samples tested positive with TLA-ELISA than with GRA7-ELISA. This inter-assay difference was most pronounced in the Walloon region, where it reached significant levels.

Table 4.2 : Total, between-herd, and average within-herd apparent prevalence (AP) of porcine toxoplasmosis per region.

	TLA		**GRA7**		**Comparison**
	*pos/tested**	*AP (95%CI)*	*pos/tested**	*AP (95%CI)*	*P-value*
Flanders					
Total	72/1773	0.04 (0.03–0.05)	59/1773	0.03 (0.03–0.04)	0.285
Between-herd	42/151	0.28 (0.21–0.36)	38/151	0.25 (0.18–0.33)	0.696
Within-herd	—	0.04 (0.00–0.25)	—	0.03 (0.00–0.23)	0.349
Wallonia					
Total	359/490	0.73 (0.69–0.77)	262/490	0.53 (0.49–0.58)	<0.001
Between-herd	93/100	0.93 (0.86–0.97)	81/100	0.81 (0.72–0.88)	0.021
Within-herd	—	0.73 (0.00–1.00)	—	0.54 (0.00–1.00)	<0.001

* positives in ELISA/number of tested serum samples

When considering the joint test results, only 1.0% (0.6%–1.5%; 17/1773) of the animals sampled in Flanders were found positive in both serological tests, while 41% (37%–46%; 201/490) of the animals sampled in the Walloon region yielded a double positive result. On the other hand, 6.4% (5.3%–7.7%; 114/1773) of the animals sampled in Flanders were found positive in at least one serological test, versus 86% (82%–89%; 420/490) of the animals sampled in the Walloon region. At the herd level, 8.6% (4.7%–14%; 13/151) of Flemish herds were classified as positive in both serological tests, versus 71% (61%–80%; 71/100) of Walloon herds. Likewise, 42% (34%–51%; 64/151) of Flemish herds were classified as positive based on the results of at least one serological test, versus 100% of Walloon herds. In line with the low regional prevalence in Flanders, more than half of the positive farms only showed one positive animal, while the remaining positive farms showed two to seven positive animals on at least one test. On more than half of the positive farms from the Walloon region, on the other hand, all sampled animals tested positive on at least one test.

Table 4.3 shows the between-herd apparent prevalence of porcine *T. gondii* infection in the Flemish and Walloon provinces. In Flanders, the apparent herd level prevalence ranged from 0% to 38% based on TLA-ELISA, versus 8% to 50% based on GRA7-ELISA. East Flanders was the Flemish province with the highest apparent herd level prevalence (38%) based on TLA-ELISA, while the same province yielded the lowest apparent herd level prevalence in GRA7-ELISA (8%). In the Walloon Region, the apparent herd level prevalence ranged from 89% to 95% based on TLA-ELISA, versus 67% to 100% based on GRA7-ELISA. TLA-ELISA yielded a higher apparent herd level prevalence than GRA7-ELISA in all Walloon provinces except for Hainaut.

Table 4.3 : Between-herd apparent prevalence (AP) of porcine toxoplasmosis per province.

	TLA		**GRA7**	
	*pos/tested**	*AP (95%CI)*	*Pos/tested**	*AP (95%CI)*
Flanders				
Antwerp	3/14	0.21 (0.05–0.51)	7/14	0.50 (0.23–0.77)
East Flanders	15/39	0.38 (0.23–0.55)	3/39	0.08 (0.02–0.21)
Flemish Brabant	0/3	0.00 (0.00–0.71)	1/3	0.33 (0.01–0.91)
Limburg	2/7	0.29 (0.04–0.71)	1/7	0.14 (0.00–0.58)
West Flanders	22/88	0.25 (0.16–0.35)	26/88	0.30 (0.20–0.40)
Walloon Region				
Hainaut	17/19	0.89 (0.67–0.99)	19/19	1.00 (0.82–1.00)
Liège	23/25	0.92 (0.74–0.99)	20/25	0.80 (0.59–0.93)
Luxembourg	20/21	0.95 (0.76–1.00)	15/21	0.71 (0.48–0.89)
Namur	16/17	0.94 (0.71–1.00)	15/17	0.88 (0.64–0.99)
Walloon Brabant	17/18	0.94 (0.73–1.00)	12/18	0.67 (0.41–0.87)

* positives in ELISA/number of tested serum samples

4.4.2 True prevalence

For both regions, convergence was achieved. Model estimates are listed in Table 4.4. In Flanders, the true between-herd prevalence was 4% (1%–10%), while the average true within-herd prevalence reached 33% (15%–55%). In Wallonia, on the other hand, both parameters were significantly higher, with a true between-herd prevalence of 73% (59%–85%) and an average true within-herd prevalence of 89% (82%–96%). In both regions, and in Flanders in particular, there tended to be a negative correlation between pairwise test results for infected animals.

Table 4.4 : Model estimates (mean and 95% uncertainty interval).

Variable	Flanders	Wallonia	P-value
True between-herd prevalence (τ)	0.04 (0.01–0.10)	0.73 (0.59–0.85)	<0.001
True within-herd prevalence of infected herds (μ)	0.33 (0.15–0.55)	0.89 (0.82–0.96)	<0.001
True within-herd prevalence across all herds ($\tau * \mu$)	0.01 (0.00–0.02)	0.65 (0.52–0.76)	<0.001
Sensitivity, TLA-ELISA (SE_1)	0.87 (0.76–0.95)	0.92 (0.86–0.97)	0.161
Sensitivity, GRA7-ELISA (SE_2)	0.83 (0.77–0.89)	0.86 (0.81–0.90)	0.038
Specificity, TLA-ELISA (SP_1)	0.96 (0.95–0.97)	0.90 (0.85–0.95)	<0.001
Specificity, GRA7-ELISA (SP_2)	0.97 (0.96–0.98)	0.61 (0.52–0.69)	<0.001
Covariance between both tests for infected animals (a)	-0.16 (-0.25–-0.01)	-0.03 (-0.07–0.01)	0.093
Covariance between both tests for non-infected animals (b)	0.01 (0.00–0.01)	0.01 (-0.01–0.04)	0.011

Interestingly, the specificity of both tests was estimated to be significantly lower in the Walloon region than in Flanders. This was particularly the case for the specificity of the GRA7-ELISA, which reached an estimated value of only 61% (52%–69%) in the Walloon region, compared with an estimated value of 97% (96%–98%) in Flanders.

4.5 Discussion

As the consumption of raw or undercooked wildlife and livestock meat containing *T. gondii* tissue cysts is one of the main sources of human infection with the parasite, together with the accidental ingestion of sporulated oocysts (Cook et al, 2000; Opsteegh et al., 2016b), the assessment of the prevalence of porcine toxoplasmosis is of pivotal importance for public health in countries, such as Belgium, where cultural feeding habits include regular consumption of pork or derived products. Since, to date, no former research has been performed to estimate the infection rate of *T. gondii* of conventionally raised Belgian pigs, we conducted a study to determine both the apparent and true prevalence in herds distributed in the entire country.

In the present study, a GRA-7-specific as well as a TLA-specific antibody ELISA were used to determine the apparent prevalence of toxoplasmosis among swine in Flanders and Wallonia, and to estimate the true prevalence in a Bayesian framework. The outcomes of this research indicate a significant difference in infection levels between both regions. When comparing the results of the apparent prevalence, significant differences were observed for the total, between-herd, and within-herd prevalence estimates. These discrepancies can be explained by the different farm management practices. Indeed, the farms in the Flemish region tend to be very intensive and high output-orientated, while the pig farms in the Walloon region are in general characterized by housing of a smaller number of animals in less strictly confined establishments or outdoor. As mentioned earlier, according to the Belgian governmental agricultural service, there are 50 registered organic farms in Wallonia, compared to 15 Flemish farms, raising biologic pigs (data from 2015). However, there are possibly even more free-ranging pigs in Wallonia, since providing the outdoor access does not require a registration of the farm in the national database and obtaining an EU-label; consequently, these animal facilities could be included in our study as conventional farms likewise in Flanders. It is well described that free-ranging or organic pigs are exposed to a higher risk of acquired *T. gondii* infection than indoor

animals (Hill et al., 2010; Dubey et al., 2012b), therefore, the average prevalence of porcine toxoplasmosis in the Walloon region may even be higher than currently estimated. Based on preliminary results from an ongoing study, an apparent seroprevalence of 91% (1804 out of 1983) was found among fattening pigs in Wallonia when tested with the TLA-ELISA. Additionally, molecular detection of the parasitic DNA by PCR in hearts of 92 Walloon pigs from organic farms collected in the slaughterhouse, showed a positive result in 14 samples (15%). In contrast, serum samples of 233 slaughter-age pigs from conventional Flemish herds gave a negative or a non-consistent positive result in serological assays (IFA, TLA- and rGRA7 ELISA); in none of 16 collected hearts the parasite could be detected by PCR (personal communication: Algaba, 2017).

There is a need to improve the currently available serological assays, mostly used in large scales studies, in order to improve their sensitivity and specificity. Inconsistencies in the test results could be much affected by the various systemic (e.g. sampling strategy), technical (e.g., assay procedure, antigen used) or biological factors associated with infection such as, genetic strain, dose, viability of the pathogen, or simply by the diversity of the hosts and their immune efficiency (Greiner and Gardner, 2000).

Looking at the test accuracy with the use of one particular antigen such as GRA7 or SAG1 indirect ELISA applied on serum samples, satisfactory sensitivity and specificity could be achieved (Jacobs et al., 1999; Beghetto et al., 2006; Pardini et al., 2012; Basso et al., 2013; Terkawi et al., 2013). High concentrations of these recombinant antigens can be relatively easily produced for use in serological assays. Additionally, the advantages of the recombinant antigenic proteins above the parasite-derived include the well-defined, precise and homogenised composition of the product, the possibility of defining the phase of the infection, and, eventually, reduction in time- and labour-consumption in the production process (Holec-Gąsior, 2011).

The GRA-family proteins are however, not exclusively expressed in *T. gondii* but they are shared by closely related parasites from the Sarcocystidae family such as

Hammondia spp., *Neospora* spp., *Sarcocystis* spp. and *Besnoitia besnoiti*. Although the well-established final and intermediate hosts for these parasites do not include pigs, it is inevitable that the omnivorous foraging behaviour of these animals could result in accidental exposure of the (oo)cysts and subsequently, to a predicted cross-reactivity in serological assays (Gondim et al., 2017). This may explain why in our study the estimated GRA-7 ELISA specificity was much lower in Wallonia than in Flanders, since there are more free-range, and organic pig herds in Wallonia.

The isolation of antigens obtained from viable tachyzoites requires, conversely, tissue culture or mice inoculation. The latter is not favourable for the animal welfare, since the inoculated mice develop an acute infection with, among others, peritonitis and have to be sacrificed to collect the ascites containing tachyzoites. Additionally, the manipulation of the highly virulent strain is associated with biohazard for the operator and high mortality of the host and, thus, an excessive production cost. Moreover, the *T. gondii* lysate may contain residues from culturing or host cell's material and its composition can vary significantly between laboratories or production rounds, what makes the assay difficult to standardize and evaluate (Liu et al., 2015).

Despite that, the combined use of multiple parasitic antigens at a time in TLA, instead of single recombinant protein such as GRA7, should provide more certainty about the seroprevalence status or at least increase the chance of polyclonal antibodies detection by the higher number of available epitopes for the antibody binding (Basso et al., 2013; Bokken et al., 2015).

Indeed, in this study the TLA-ELISA detected more frequently *T. gondii*-specific antibodies on both animal and farm level, irrespective of the geographical distribution in Belgium. Strikingly, individual samples could yield contradictory results when comparing both types of antigens, as multiple TLA-positive serum samples were detected in GRA7-negative farms, and conversely, in TLA-negative herds, some animals had antibodies against GRA7. This could be explained by different stages of the ongoing infection or genetic diversity of the circulating strains in Belgian pig

population. Alternatively, however, this may also have resulted from false positive test results on one or the other test.

It is well established that the apparent prevalence values could unwillingly include a number of false positive and negative results, altering the true outcome. For this reason, a more exact estimation of the prevalence should be applied to compensate for this imperfectness, generated by the use of prior estimates and statistical modelling, such as Bayesian approach (Bokken et al., 2015).

One of the constraints of the study was a limited number of sampled farms from Wallonia, together with a smaller number of samples per farm. Nonetheless, the obtained results showed a consistently high prevalence across and within herds in the Walloon region, supporting the validity of our results, and, as explained earlier, in correlation with recently obtained unpublished results, showing a very high seroprevalence in Walloon pigs.

Since the detection methods still should be improved in terms of sensitivity, in meanwhile efforts should be made to focus on prevention of toxoplasmosis in swine herds. Rodent control is of pivotal importance to reduce the risk of *T. gondii* infection in pigs, together with the proper disposal of carcasses, since both measures are intended to avoid the ingestion of formerly infected tissues (Lehmann et al., 2003; Hill et al., 2010). More factors can, however, contribute to an increased risk of infection in pigs, such as the presence of the cats on farms and the free access to the stables, an increasing age of the animals, a small herd size, free-range or backyard pig raising rather than strict confinement housing, source of water, and feeding of unprocessed animal products such as goat whey to the pigs (Meerburg et al., 2012). The age of the animals at the slaughter has important implications for the transmission of the disease and the epidemiology of human toxoplasmosis. Indeed, conventionally raised pigs are slaughtered at 5-6 months of age (corresponding to the weight of approximately 110 kg), and their meat is mainly intended for fresh products. Therefore, the younger the animals, the more chance that the meat will access the market unprocessed and fresh.

Opposite to that, the lower quality carcasses from multiparous sows or reproduction boars, will undergo processing, which is harmful for the parasite and, thus, safer for the consumer in terms of parasitic load (Dubey, 2009b).

In conclusion, in the present study we could demonstrate that both GRA7 and TLA-ELISA showed a significantly higher apparent total, within-herd and between-herd prevalence of anti-*T. gondii* IgG antibodies in Wallonia than in Flanders ($P < 0.001$). We also applied conventional and Bayesian approaches to estimate total, within-herd and between-herd prevalence of *T. gondii* in Flanders and Wallonia. The use of the TLA-ELISA for the detection of *T. gondii*-specific antibodies resulted in a higher rate of positive samples on both animal and farm level. The specificity of both serological assays differed significantly between Flanders and Wallonia. These observations underscore the advantages and the constraints of the here applied serological tests, but also the value of statistical models, which take the limitations of the diverse assays into account as shown in our study.

4.6 Acknowledgements

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Appendix A. JAGS code for estimating true within-herd and between-herd prevalence.

```
model {
  prob_se[1] <- SE[1] * SE[2] + a[1]
  prob_se[2] <- SE[1] * (1 - SE[2]) - a[1]
  prob_se[3] <- (1 - SE[1]) * SE[2] - a[1]
  prob_se[4] <- (1 - SE[1]) * (1 - SE[2]) + a[1]
  prob_sp[4] <- SP[1] * SP[2] + b[1]
  prob_sp[3] <- SP[1] * (1 - SP[2]) - b[1]
  prob_sp[2] <- (1 - SP[1]) * SP[2] - b[1]
  prob_sp[1] <- (1 - SP[1]) * (1 - SP[2]) + b[1]
  for (i in 1:4) {
    constraint3[i] <- step(prob_se[i])
    O3[i] ~ dbern(constraint3[i])
    constraint4[i] <- step(prob_se[i] - 1)
    O4[i] ~ dbern(constraint4[i])
    constraint5[i] <- step(prob_sp[i])
    O5[i] ~ dbern(constraint5[i])
    constraint6[i] <- step(prob_sp[i] - 1)
    O6[i] ~ dbern(constraint6[i])
  }
  for (j in 1:N) {
    x[j, 1:4] ~ dmulti(AP[j, 1:4], n[j])
    for (i in 1:4) {
      AP[j, i] <- TP[j] * prob_se[i] + (1 - TP[j]) * prob_sp[i]
        constraint1[j, i] <- step(AP[j, i])
      O1[j, i] ~ dbern(constraint1[j, i])
      constraint2[j, i] <- step(AP[j, i] - 1)
      O2[j, i] ~ dbern(constraint2[j, i])
    }
```

```
    tp.between[j] ~ dbern(tau)
    tp.within[j] <- mu
    TP[j] <- tp.between[j] * tp.within[j]
  }
  tau ~ dbeta(1, 1)
  mu ~ dbeta(1, 1)
  pi <- tau * mu
  SE[1] ~ dbeta(42.57316, 5.61924)   # TLA
  SP[1] ~ dbeta(99.69833, 6.194649)  # TLA
  SE[2] ~ dbeta(119.0083, 22.97782)  # GRA7
  SP[2] ~ dbeta(62.55002, 5.918684)  # GRA7
  a[1] ~ dunif(-0.25, 0.25)
  b[1] ~ dunif(-0.25, 0.25)
}
```

Appendix B. The apparent prevalence per individual herd in Flanders (upper section) and Wallonia (lower section); T1: TLA, T2: GRA7.

FARM	PROVINCE IN FLANDERS	T1+,T2+	T1+,T2-	T1-,T2+	T1-,T2-
1	Limburg	0	1	0	11
2	West Flanders	0	0	0	12
3	East Flanders	0	2	0	10
4	East Flanders	0	2	0	10
5	West Flanders	0	0	0	17
6	West Flanders	0	0	0	12
7	East Flanders	0	1	0	9
8	West Flanders	0	0	1	11
9	West Flanders	0	0	0	12
10	West Flanders	0	0	1	11
11	West Flanders	0	0	0	2
12	West Flanders	1	0	0	13
13	East Flanders	0	0	0	3
14	East Flanders	0	0	0	8
15	West Flanders	0	1	0	11
16	West Flanders	0	0	0	12
17	West Flanders	0	0	0	11
18	East Flanders	0	0	0	12
19	West Flanders	0	3	0	9
20	West Flanders	1	0	0	11
21	West Flanders	0	0	0	12
22	East Flanders	0	0	0	15
23	West Flanders	0	1	0	12
24	West Flanders	0	1	0	9
25	West Flanders	0	0	0	12
26	West Flanders	0	0	0	12
27	Limburg	0	0	1	11
28	West Flanders	0	0	0	13
29	East Flanders	0	0	0	11
30	West Flanders	0	0	0	12
31	East Flanders	0	1	0	12
32	East Flanders	0	1	0	7
33	East Flanders	0	0	0	12
34	Flemish Brabant	0	0	0	11
35	East Flanders	0	0	0	13
36	West Flanders	1	3	0	5
37	East Flanders	0	3	0	9
38	East Flanders	0	0	0	12
39	East Flanders	0	1	0	10
40	East Flanders	0	0	0	12

41	West Flanders	0	3	0	9
42	West Flanders	0	2	0	12
43	East Flanders	0	1	0	11
44	East Flanders	0	3	0	9
45	East Flanders	0	3	0	9
46	East Flanders	0	1	1	10
47	East Flanders	0	0	0	12
48	East Flanders	0	2	0	10
49	East Flanders	0	0	0	10
50	East Flanders	1	1	0	10
51	East Flanders	0	1	0	9
52	Antwerp	0	0	0	12
53	East Flanders	0	0	0	12
54	East Flanders	0	0	0	12
55	East Flanders	0	0	1	11
56	West Flanders	0	0	0	11
57	West Flanders	0	0	0	13
58	West Flanders	0	0	0	12
59	West Flanders	0	0	1	11
60	West Flanders	0	0	2	10
61	West Flanders	0	0	1	11
62	West Flanders	0	1	1	10
63	West Flanders	0	0	0	12
64	West Flanders	0	0	0	13
65	West Flanders	0	0	0	12
66	West Flanders	0	1	0	11
67	West Flanders	0	0	0	12
68	West Flanders	0	0	0	12
69	West Flanders	0	0	1	11
70	West Flanders	0	0	2	10
71	West Flanders	0	1	0	11
72	West Flanders	0	0	0	12
73	West Flanders	0	0	0	12
74	West Flanders	0	0	1	11
75	East Flanders	0	0	0	12
76	West Flanders	1	0	0	11
77	East Flanders	0	0	0	12
78	West Flanders	0	0	1	11
79	West Flanders	0	0	0	12
80	West Flanders	0	0	0	12
81	West Flanders	0	0	0	12
82	West Flanders	0	0	0	12
83	West Flanders	0	0	0	12
84	West Flanders	0	0	0	12

85	West Flanders	0	0	0	12
86	West Flanders	0	1	0	12
87	West Flanders	0	0	0	12
88	West Flanders	0	0	1	10
89	East Flanders	0	0	0	13
90	West Flanders	0	0	0	12
91	West Flanders	0	0	2	8
92	East Flanders	0	0	0	10
93	East Flanders	0	0	0	12
94	West Flanders	0	0	0	12
95	West Flanders	1	0	0	11
96	West Flanders	0	0	0	12
97	West Flanders	4	0	3	5
98	West Flanders	0	0	2	10
99	West Flanders	0	1	1	10
100	West Flanders	1	0	1	11
101	West Flanders	0	0	0	10
102	West Flanders	0	0	0	9
103	West Flanders	0	0	4	8
104	West Flanders	0	0	0	14
105	Flemish Brabant	0	0	1	11
106	Antwerp	0	0	0	12
107	Antwerp	0	0	0	12
108	Antwerp	0	0	0	12
109	Antwerp	0	0	3	7
110	Antwerp	0	0	2	10
111	Antwerp	0	0	4	8
112	Antwerp	1	0	0	11
113	East Flanders	0	0	0	12
114	Antwerp	0	0	1	11
115	West Flanders	0	0	0	12
116	Antwerp	1	2	0	9
117	East Flanders	0	1	0	11
118	West Flanders	1	0	0	9
119	West Flanders	0	0	0	5
120	West Flanders	0	0	1	11
121	Limburg	0	0	0	12
122	Limburg	0	0	0	12
123	West Flanders	0	0	0	14
124	West Flanders	0	2	0	10
125	West Flanders	0	0	0	12
126	Antwerp	2	3	0	7
127	West Flanders	0	0	0	12
128	West Flanders	0	0	0	12

129	Flemish Brabant	0	0	0	12
130	West Flanders	0	0	0	13
131	Antwerp	0	0	0	11
132	East Flanders	0	0	0	12
133	West Flanders	1	0	0	11
134	West Flanders	0	0	0	12
135	East Flanders	0	0	0	12
136	Antwerp	0	0	0	12
137	Limburg	0	0	0	12
138	West Flanders	0	0	0	12
139	West Flanders	0	1	0	11
140	West Flanders	0	0	0	12
141	Limburg	0	3	0	9
142	West Flanders	0	0	0	12
143	Antwerp	0	0	0	12
144	West Flanders	0	0	1	11
145	East Flanders	0	0	0	12
146	East Flanders	0	0	0	12
147	West Flanders	0	0	0	12
148	West Flanders	0	0	0	12
149	Limburg	0	0	0	12
150	West Flanders	0	0	0	12
151	West Flanders	0	0	0	12

FARM	PROVINCE IN WALLONIA	T1+,T2+	T1+,T2-	T1-,T2+	T1-,T2-
1	Namur	5	0	0	0
2	Luxembourg	4	0	1	0
3	Walloon Brabant	2	2	0	0
4	Liège	4	0	1	0
5	Namur	2	0	1	0
6	Luxembourg	4	1	0	0
7	Walloon Brabant	0	0	4	1
8	Liège	1	2	0	2
9	Hainaut	5	0	0	0
10	Hainaut	3	1	1	0
11	Walloon Brabant	1	0	2	2
12	Luxembourg	0	0	4	1
13	Namur	0	0	5	0
14	Luxembourg	1	0	4	0
15	Liège	0	0	4	1
16	Namur	0	1	2	2
17	Walloon Brabant	2	0	3	0
18	Namur	2	0	2	1

19	Liège	5	0	0	0
20	Liège	2	3	0	0
21	Hainaut	3	2	0	0
22	Hainaut	4	1	0	0
23	Liège	5	0	0	0
24	Luxembourg	4	1	0	0
25	Liège	1	4	0	0
26	Walloon Brabant	2	2	0	1
27	Namur	3	0	1	0
28	Walloon Brabant	5	0	0	0
29	Luxembourg	1	0	1	0
30	Walloon Brabant	4	0	0	0
31	Liège	3	1	1	0
32	Luxembourg	4	1	0	0
33	Namur	4	0	0	1
34	Walloon Brabant	5	0	0	0
35	Luxembourg	5	0	0	0
36	Namur	5	0	0	0
37	Namur	4	1	0	0
38	Luxembourg	4	0	1	0
39	Liège	0	0	3	2
40	Liège	4	0	1	0
41	Namur	3	1	1	0
42	Luxembourg	5	0	0	0
43	Hainaut	4	0	1	0
44	Hainaut	4	0	1	0
45	Hainaut	1	0	3	1
46	Hainaut	0	1	1	3
47	Hainaut	0	0	3	2
48	Hainaut	0	0	5	0
49	Walloon Brabant	2	0	2	1
50	Walloon Brabant	0	5	0	0
51	Liège	0	5	0	0
52	Walloon Brabant	2	3	0	0
53	Luxembourg	0	5	0	0
54	Liège	0	3	0	2
55	Liège	1	2	0	2
56	Walloon Brabant	2	1	0	2
57	Hainaut	1	1	0	3
58	Hainaut	2	1	0	2
59	Hainaut	1	4	0	0
60	Hainaut	3	1	0	1
61	Hainaut	0	1	1	3
62	Luxembourg	0	4	0	1

63	Namur	5	0	0	0
64	Liège	0	3	0	1
65	Namur	0	3	0	2
66	Luxembourg	0	3	0	2
67	Liège	1	4	0	0
68	Liège	0	5	0	0
69	Liège	1	3	0	1
70	Liège	1	1	0	3
71	Liège	4	1	0	0
72	Liège	1	4	0	0
73	Liège	5	0	0	0
74	Liège	3	2	0	0
75	Liège	1	3	0	1
76	Liège	1	3	0	1
77	Hainaut	3	2	0	0
78	Hainaut	3	2	0	0
79	Hainaut	1	2	0	2
80	Hainaut	2	3	0	0
81	Walloon Brabant	0	5	0	0
82	Walloon Brabant	1	4	0	0
83	Walloon Brabant	0	2	0	3
84	Walloon Brabant	0	2	0	2
85	Walloon Brabant	0	3	0	2
86	Walloon Brabant	0	1	0	4
87	Liège	0	4	0	1
88	Luxembourg	2	2	0	1
89	Luxembourg	0	5	0	0
90	Luxembourg	0	2	0	3
91	Luxembourg	0	2	0	3
92	Luxembourg	3	2	0	0
93	Luxembourg	1	3	1	0
94	Luxembourg	3	2	0	0
95	Luxembourg	2	2	0	1
96	Namur	5	0	0	0
97	Namur	0	5	0	0
98	Namur	4	1	0	0
99	Namur	1	4	0	0
100	Namur	3	2	0	0

Chapter 5 Immune responses and tissue distribution of *Toxoplasma gondii* in pigs experimentally infected with the IPB-Gangji strain

Adapted from:

Verhelst, D., De Craeye, S., Jennes, M., Dorny, P., Goddeeris, B., Cox, E. (2015). Interferon gamma expression and infectivity of *Toxoplasma* infected tissues from experimentally infected sheep in comparison with pigs. Veterinary Parasitology 207, 7-16. doi: 10.1016/j.vetpar.2014.11.014.

5.1 Abstract

Livestock animals are a potential risk for transmission of toxoplasmosis to humans. In particular, pork is considered an important source of infection, since it is often consumed undercooked, and processed in many meat-derived food products. In the current study, IFN-γ (type 1 response), IL-4 (type 2 response) and IL-10 mRNA (anti-inflammatory response) expression by blood mononuclear cells, and the serum antibody responses against *Toxoplasma gondii* total lysate antigen, recombinant GRA1, rGRA7, rMIC3 and rEC2, a chimeric antigen composed of MIC2, MIC3 and SAG1, were studied in pigs two months upon an oral *T. gondii* strain IPB-Gangji inoculation. Additionally, the parasite distribution in heart, brain and two skeletal muscles was evaluated. Surprisingly, from the five antigens included to study the humoral response, only rGRA7-specific antibodies could be demonstrated. The cytokine expression was exclusively elevated for IFN-γ, whereas it remained beyond the detection level for IL-4 and IL-10. Although the viable parasite and its DNA could be demonstrated in all the porcine samples tissues by both bio-assay and qPCR, the results indicate that heart could be an important target tissue to demonstrate the presence of *T. gondii* infection in pigs.

5.2 Introduction

T. gondii, an obligate intracellular protozoan parasite, is the cause of the most common parasitic zoonosis worldwide (Tenter et al., 2000). By estimation, at least one third of the world population has been infected and shows detectable levels of anti-*T. gondii* specific antibodies (Denkers and Gazzinelli, 1998; Montoya and Liesenfeld, 2004). Livestock animals are a potential risk for transmission of *T. gondii* infection to humans. Consumption of raw or undercooked meat has been regarded as a major route of infection in many countries (Cook et al., 2000; Kijlstra and Jongert, 2008). Although the seroprevalence in pigs is very low in European countries (Kijlstra et al., 2004; Guo et al.,

2015b), pork still remains an important source of foodborne toxoplasmosis. The reason for this statement is derived from the fact that pork is often consumed undercooked or processed into different products, reaching even a broader range of potential consumers.

Following oral ingestion of oocysts or tissue cysts, the parasite can establish a chronic infection in animals and humans. There, the parasite transforms into tachyzoites, which invade the small intestine and rapidly replicate in the epithelial cells. Simultaneously, tachyzoites successfully spread to other gut associated tissues. This is accompanied with the induction of cellular and humoral immune responses. The involvement of both the innate and adaptive immune system has been broadly described (Miller et al., 2009; Gazzinelli et al., 2014; Sturge and Yarovinsky, 2014). Briefly, cellular responses are initially associated with local and systemic IFN-γ production by Natural Killer cells, immediately followed by the release of IL-2 by T-lymphocytes and IL-12 by dendritic cells (DC's). In the subacute and chronic phase of the infection the IFN-γ production is taken over by the $CD4^+$ and $CD8^+$ T-lymphocytes. These T-cell responses have a dual function: they are necessary for the host to control the infection and survive from acute toxoplasmosis, and as the result the tachyzoites undergo a stage conversion towards the bradyzoites, which successfully escape the immune system and persists in a cyst form in the host tissues (Denkers and Gazzinelli, 1998). On average, tissue cysts appear 7–10 days (d) post infection in visceral organs such as lungs, liver and kidneys, and predominantly in the central nervous system and heart and skeletal muscle tissues, where they persist for the entire host's life (Black and Boothroyd, 2000).

Since the parasite becomes undetectable in most intestinal and systemic lymphoid tissues around 3 weeks post infection (wpi), Verhelst et al., (2014) postulated that this is the result of clearance of the parasite from these tissues by an immune response. Additionally, previous study has shown that the majority of the skeletal muscles shows a decreased viability of the cysts, and a diminished amount of the parasitic DNA 6

months post infection (mpi), as compared to a group of animals sampled at 6 wpi (Verhelst et al., 2011).

The objectives of the present study were to determine the distribution of the parasite in different tissues at 6 wpi, and to assess the status of the adaptive immune response in the subacute phase at 2–6 wpi, which is shortly after the parasite left the intestinal tissues. Furthermore, we focused on the cytokine production profile and interaction with different antigens to obtain information on the optimal antigen target(s) for serological diagnosis of the infection in pigs.

5.3 Material and methods

5.3.1. *T. gondii* strains

T. gondii IPB-Gangji (IPB-G) and RH strains are routinely maintained in the National Reference Laboratory for Toxoplasmosis (Scientific Institute for Public Health, Brussels, Belgium) by passage in Swiss female mice, as approved by the Ethical Committee (nr 20140704-01) and conform the European legislation (2010/63/EU). The strain IPB-G, originally isolated from the placenta of a congenitally infected baby and was named after its family name, belongs to a mixed I-II type and shows a high virulence in mice (Vercammen et al., 1998; Ajzenberg et al., 2002). The tissue cysts of *T. gondii* were harvested from the brains of chronically infected Swiss mice and counted under a contrast-phase microscope. The brain tissue homogenates were diluted in PBS at a concentration of 300 cysts/ml and were used as inoculum for the infection experiments in pigs.

The RH is a highly virulent type I strain, causing acute toxoplasmosis in Swiss mice. The strain is maintained by passage every 3-5 days by the collection of the ascites containing tachyzoites from the intraperitoneal cavity. *T. gondii* total lysate antigen (TLA) from tachyzoites of the RH-strain was prepared as previously described (Jongert et al., 2007). Briefly, the tachyzoites suspension in PBS was subsequently washed,

centrifuged and filtrated through a 5 µm syringe filter (MilleX®SV, Merck KGaA, Darmstadt, Germany), followed by lysis via alternating sonication with cooling cycles using an Ultrasonic disintegrator (MSE, Leicester, United Kingdom). The protein content of the lysate was determined with the bicinchoninic acid (BCA) reaction (Thermo Scientific Pierce BCA protein Assay Kit, Erembodegem, Belgium) and the TLA aliquots were stored at -20°C until further use.

5.3.2. Animals

Six indoor-born Belgian Landrace pigs were weaned at 21 d and housed in isolation units (Biosafety permit nr: AMV/11062013/SBB219.2013/0145) at the Faculty of Veterinary Medicine, Ghent University, Belgium. The animals were *T. gondii*-specific IgM and IgG negative, as determined by IFA. Five animals were orally inoculated at 4 weeks of age with 3000 tissue cysts of the IPB-G strain. The remaining pig served as negative control and was given orally a brain homogenate from a non-infected mouse. All pigs were bled weekly from infection till euthanasia at 6 wpi. Euthanasia was performed by intravenous injection of an overdose sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). Animal experimentation was performed with the prior approval of the Animal Ethical Committee of the faculties Veterinary Medicine and Bioscience Engineering at Ghent University (EC2007/103).

5.3.3 Detection of parasites by bio-assay and real-time quantitative PCR (qPCR)

In order to quantify and compare the presence of the parasite in the tissues of the infected animals, the number of bradyzoites was determined by real-time quantitative PCR (qPCR) in brain, heart, M. gastrocnemius and M. longissimus dorsi. After euthanasia of the pigs, 100 g samples of each tissue were collected from each animal for detection of parasites by bioassay and quantification by qPCR. Hereto, the tissues were homogenized in 15 ml 0.9% NaCl, and the suspensions were incubated 1–2 h in

a 250 ml acidic pepsin solution (0.8 g l−1pepsin and 7 ml l−1HCl) at 37°C. Thereafter the suspensions were filtered, centrifuged at 1180 × g during 20 min, and the pellets were resuspended in 5 ml PBS with 0.04% gentamicin. A small aliquot (200 µl) of the pellet was collected for the qPCR, while the rest was used for the bioassay. For the latter, 5 mice were intraperitoneally inoculated with 1 ml tissue suspension to evaluate the infectivity of the tissues. Lungs and brains of mice that died from acute toxoplasmosis were examined for *T. gondii* cysts by phase-contrast microscopy and for the parasitic DNA by qPCR (Verhelst et al., 2011). From the surviving mice, serum was collected at day 45 pi for *T. gondii*-specific antibody detection with the IFA. Their brain tissues were sampled and processed for demonstrating *T. gondii* infection by qPCR as described by Kijlstra et al. (2008) and Rosenberg et al. (2009). For qPCR, DNA was extracted with the QIAamp DNAMini kit (Qiagen GMBH, Hilden, Germany) from 85 µl of tissue suspension, according to manufacturer's instructions. As a reference for the quantification of *T. gondii* parasites, two standard lines of 10-fold dilutions were used: one with a counted number of RH-strain tachyzoites, and one with a counted number of cultured pig kidney cells (SK-6) as described previously (Rosenberg et al., 2009). DNA was tested by duplex Taqman-based qPCR on a BioRad iCycler (Biorad, Hercules, CA) using the *T. gondii* repeat element (AF146527) as the first target. The second target was based on the ribosomal 18S rDNA of the host cells. The reaction was performed as described by Kijlstra et al. (2008).

5.3.4 Indirect immunofluorescence assay

The presence of IgM and IgG antibodies against *T. gondii* in sera of the weaned piglets was evaluated by the IFA. Fifty microliter of a 1/50 in PBS diluted serum sample was applied for 30 min at 37°C on a slide coated with formalin-treated tachyzoites from the RH-strain (Toxo-Spot IF, Bio-Mérieux, Marcy-l'Etoile, France). Subsequently, the slides were washed with PBS and incubated for 30 min at 37°C with 30 µl of 1/50 in PBS-

Evans Blue diluted fluorescein isothiocyanate (FITC) conjugated anti-pig IgM or anti-pig IgG (KPL, MD, USA). After washing with PBS and distilled water, and drying at 37°C, the slides were read with a fluorescence microscope (Carl Zeiss, Germany). The cut-off read-out of the fluorescence test was established with *T. gondii* seronegative and seropositive porcine reference sera at 1/50 dilution. For detection of seroconversion in the mouse bio-assay, sera from these mice were tested at a 1/25 dilution, and a secondary Alexa 488 anti-mouse IgG antibody (Invitrogen, Merelbeke, Belgium) (1/500) was used as the conjugate.

5.3.5 Purification of recombinant antigens

Recombinant GRA1, rGRA7 and rEC2 were purified as described previously (Bivas-Benita et al., 2003; Jongert et al., 2007 and 2008). The $mic_{3234–307}$ fragment was amplified from pcEC2 with the forward primer: 5′ GCGCGGATCCCTCCCCCAGGATGCCATT 3′and the reverse primer: 5′ GCGCGGATCCAGGACTGGATGTCATGCC 3′. The amplicon was purified with the PCR purification kit (QIAGEN GmbH, Hilden, Germany) and digested overnight with the enzymes BamHI and HindIII. Further, it was ligated into pQE80 expression vector (QIAGEN GmbH, Hilden, Germany). A clone was identified by colony PCR using the same primers, and sequencing confirmed the presence of $mic_{3234–307}$. Additionally, the expression of $rMIC_{3234–307}$ was also confirmed by SDS-PAGE and Western blot with serum from infected mice. The his-tagged $rMIC_{3234–307}$ was produced at large scale and purified according to a protocol described previously by Bivas-Benita et al. (2003).

5.3.6 Antibody ELISA

Simultaneously, the total antigen-specific IgM and IgG antibodies were detected via ELISA. Hereto, Nunc Polysorb™ immunoplates (Life Technologies, Ghent, Belgium)

were coated for 2 h at 37°C with TLA, rGRA1, rGRA7, rEC2 or MIC3 at a concentration of 10 µg ml/ml coating bicarbonate buffer (pH 9.7).

In subsequent steps, plates were blocked overnight at 4°C in PBS supplemented with 0.2% Tween®80; further, they were incubated for 1 h at 37°C with porcine serum diluted 1/50 in PBS and for 1 h at 37°C with HRP-conjugated rabbit anti-porcine gamma heavy chain antibodies (IgG; 1/1000) (Serotec, Belgium). Between each step, plates were thoroughly washed with PBS 0.2% Tween®20. Finally, a substrate *o*-phenylenediamine dihydrochloride tablet (Sigma Fast; Sigma) in H_2O_2 solution was added. The reaction was stopped by addition of 2 N sulfuric acid ($H_2SO_{4)}$, and the absorbance was read at 450 nm in aniMARK Microplate reader (Biorad, Nazareth, Belgium). Positive and negative control sera were included on each plate. The cut-off value was calculated from the pre-immune sera (day 0) at a 1/50 dilution, with as cut-off value the mean of the optical density OD450 + 3 × standard deviation (SD). The so obtained cut-off values were as follows: TLA: 0.095; rGRA1: 0.123; rGRA7: 0.146; $rMIC_{3234–307}$: 0.136; rEC2: 0.07.

5.3.7 Real-time quantitative PCR for porcine cytokines

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Nycomed, Brussels, Belgium). Porcine lymphocytes were counted and resuspended at a concentration of 5×10^6 cells/ml, and kept at -80°C in RLT buffer (Qiagen, Nazareth, Belgium) for later isolation of cytokine mRNA. mRNA was extracted using an RNeasy kit (Qiagen GMBH, Hilden, Germany). Reverse transcription into total cDNA was performed with the iScript kit (Biorad, Nazareth, Belgium), following the manufacturer's protocol. The obtained single-stranded cDNA was diluted 100 times for amplification in qPCR. The qPCRs were set up in 96-well optical microtitre plates with 25 µl mixture of iQ SYBR Green Supermix (Bio-Rad,

Hercules, CA). The oligonucleotide primers used for the detection of IL-4, IL-10, IFN-γ, and the three housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase or

Table 5.1 : Oligonucleotide primers used in cytokine qPCR.

Target	Sequence
IL-10	F: CCTGGGTTGCCAAGCCTT
	R: GCTTTGTAGACACCCCTCTCTT
IL-4	F: CTGGTCTGCTTACTGGYATGTA
	R: CTGTCAAGTCCGCYCAGGA
IFN-γ	F: GAGCCAAATTGTCTCCTTCTACTT
	R: CTGACTTCTCTTCCGCTTTCT
GAPDH	F: CCATCACTGCCACCCAGAA
	R: CAGGGATGACCTTGCCCA
B-actin	F: GGCATCCTGACCCTCAAGTA
	R: GCCTCGGTCAGCAGCA
r18S	F: GTTGATTAAGTCCCTGCCCTTT
	R: GATAGTCAAGTTCGACCGTCTT

GAPDH, β-actin and 18S rDNA) are presented in Table 5.1. Each sample of cDNA was tested in duplicate, and non-template reactions were included in the runs as an internal control. For each sample, the target gene was amplified in parallel with the three housekeeping control genes in separate wells. Amplification conditions were identical for all genes: a first activating cycle of the Taq polymerase of 95°C for 2 min, followed by 45 cycles of 2 steps: 95°C for 15 sec and 60°C for 30 sec. qPCR data were analyzed using a mathematical model described by Vandesompele et al. (2002), based on the qPCR efficiencies and the mean threshold value (Ct) deviation between the sample and control group. The normalization was done relative to the geometric average of GADPH, r18S rDNA and β-actin genes, and data is represented as the normalized

cytokine gene expression compared to the geometric mean number of the housekeeping genes.

5.3.8 Statistical analysis

Statistical analysis of the parasite load, and antibody and cytokine response was performed using a one-way ANOVA Friedman test (GraphPad Prism). A *p*-value <0.05 was considered statistically significant.

5.4 Results

5.4.1 Isolation of parasites by bio-assay and detection of bradyzoites by qPCR.

The viable parasites were isolated by bio-assay from all tested tissues from all the inoculated animals at the time point 6 wpi, except from the M. gastrocnemius of one pig (Table 5.2). These results were confirmed by qPCR on the heart tissue, but detection of parasite DNA seems to be less sensitive than bio-assay, since 1, 2 and 2 out of 5 animals were found negative for brain, M. gastrocnemius and M. longissimus dorsi, respectively.

Table 5.2 : Infectivity of porcine tissues, determined by bio-assay and detection of bradyzoites by qPCR.

Tissues	brain	heart	M. gastrocnemius	M. longissimus dorsi
Assay (positives/tested)				
bio-assay	5/5	5/5	4/5	5/5
qPCR	4/5	5/5	3/5	3/5

* positives/number of tested tissues

5.4.2 Quantification of the parasite load in tissues at euthanasia

In the euthanized pigs at 6 wpi the parasite load was determined in the positive animals in the diminishing order as follows: M. longissimus dorsi: 112.7 ± 79.9, brain: 80.8 ± 51.3 and heart 64.1 ± 36.0 bradyzoites per 10^8 porcine cells (Figure 5.1). The parasites were clearly less prominent in the M. gastrocnemius, with the parasite load of only 12.0 ± 6.2 bradyzoites per 10^8 cells. The negative control animals remained *T. gondii*-free in all tissues (data not shown).

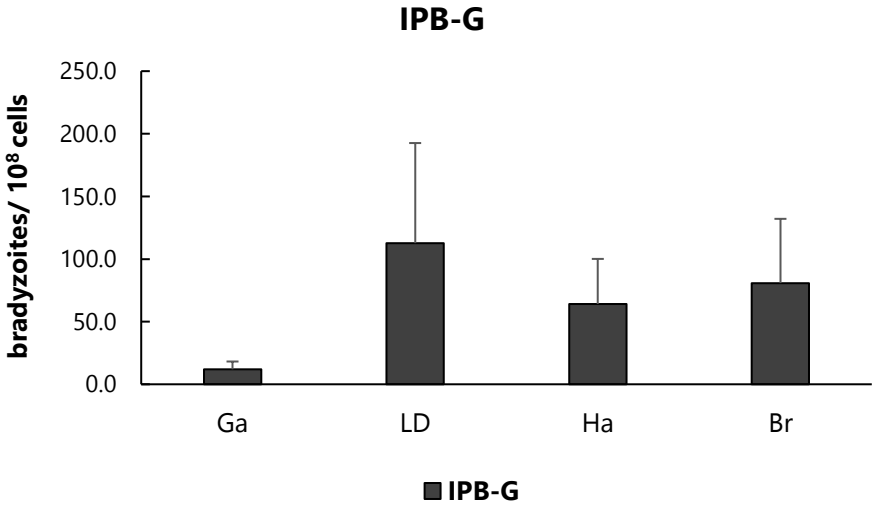

Figure 5.1 : Bradyzoite load in tissues of pigs orally infected with *T. gondii* IPB-G strain, determined by qPCR. The number of bradyzoites per 10^8 porcine cells is presented in the following tissues: brain (Br), heart (Ha), M. gastrocnemius (Ga), M. longissimus dorsi (LD).

5.4.3 Humoral response after infection

In IFA, all infected pigs were found seropositive from 2 wpi for both IgG and IgM. Then a gradual decline in IgM positive animals was observed (Table 5.3). The IgM disappeared by week 6 pi, while the IgG still clearly persisted in all infected animals.

Table 5.3 : Detection of *T. gondii* specific IgM and IgG by IFA in pigs experimentally infected with the IPB-G strain.

Weeks pi (wpi)	0	1	2	3	4	5	6
IgM	0/5	nd	5/5	4/5	3/5	2/5	0/5
IgG	0/5	nd	5/5	3/3	4/5	5/5	5/5

nd: not determined

By indirect antigen ELISA, the profile of the humoral responses against the different recombinant antigens and TLA were followed (Figure 5.2). The IgG against rGRA7 was detected in three animals at 2 wpi. This initially moderate antibody response gradually increased so that all animals seroconverted against rGRA7 at 4 wpi. The rGRA7-specific OD value showed a moderate decline at 5 wpi, followed by a significant increase at the next time point. For the other antigens either no (TLA and rEC2) or a temporary early but very weak response was observed (in 2 out of 5 at 2 wpi for $rMIC_{3234–307}$; in 1 out of 5 pigs at 2 wpi for rGRA1). In general, at 6 wpi, a tendency for an increase in the antibody production against the majority of the antigens (rGRA7, TLA, $rMIC_{3234–307}$) could be seen, except for rGRA1, even though values mostly remained below the cut-off.

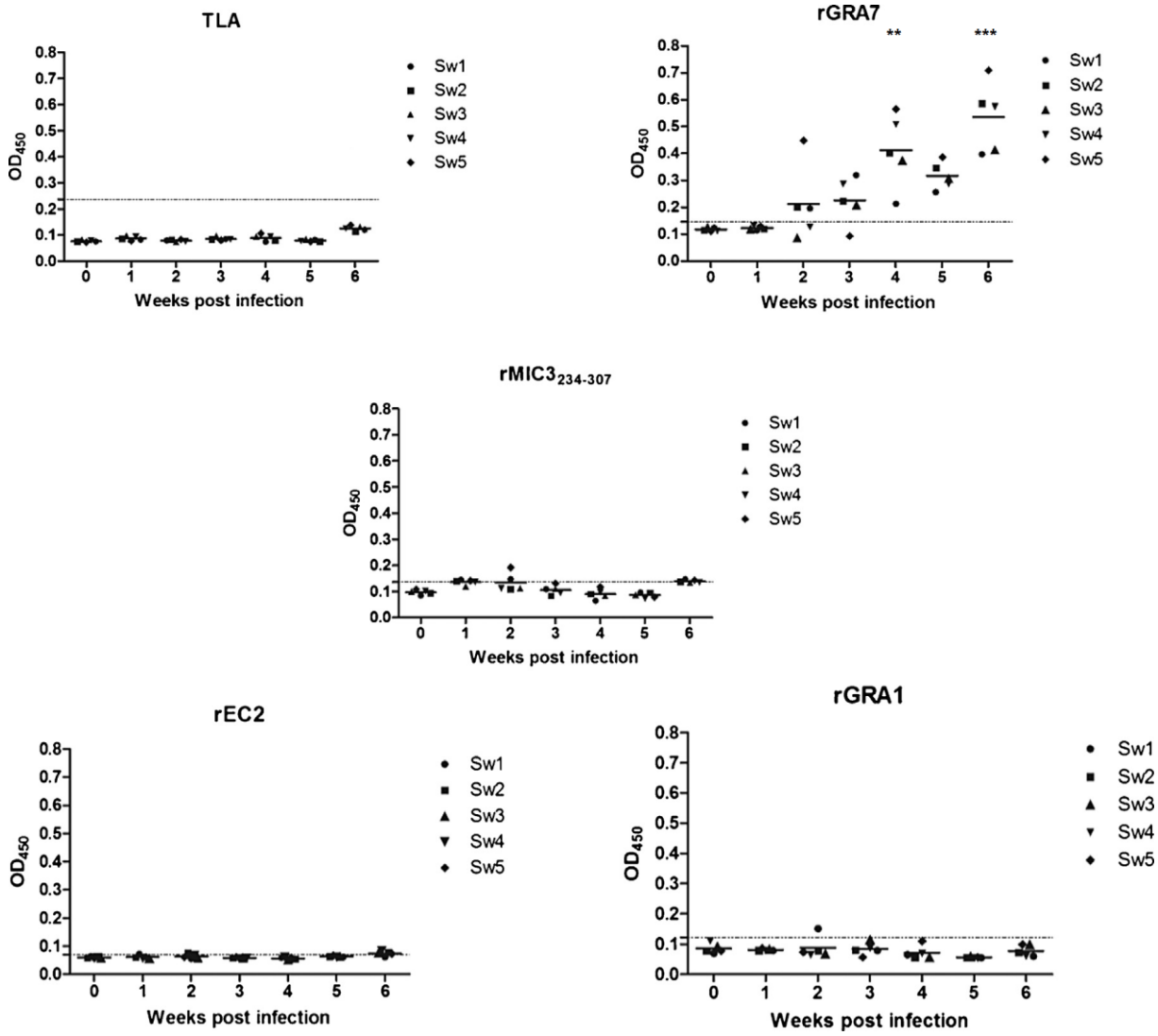

Figure 5.2 : Kinetics of the antibody responses against TLA, rGRA1, rGRA7, rEC2 and $rMIC3_{234–307}$ presented as OD-values for each experimentally infected pig with *T. gondii* IPB-G strain. The solid lines present the mean OD-value at each time point. The dashed lines indicate the cut-off values defined with the average + 3x SD of the pre-immune sera. ** and *** present significance differences at $P<0.01$ and $P<0.001$, respectively, in comparison with the pre-immune sera.

A control pig infected orally with *T. gondii* negative brain tissue did not become seropositive in IFA or the ELISAs at any time (data not shown).

5.4.4 Kinetics of cytokine responses in peripheral blood mononuclear cells

In order to evaluate whether a *T. gondii* infection could increase IFN-γ, IL-10 and/or IL-4 mRNA expression levels in the blood, the PBMCs were isolated on a weekly basis until 5 wpi. Relative normalized IFN-γ, IL-10 and IL-4 mRNA levels were determined by qPCR. The cytokine levels of the control animal remained stable during the whole study period and served as a reference.

Based on the obtained results, IFN-γ expression clearly increased in a time-dependent manner starting from 2 wpi. The altitude of this response and the number of responding animals gradually increased from 1 out of 5 IFA-seropositive animals at 2 wpi to 4 out of 5 seropositive animals at 4 wpi. Thereafter, the IFN-γ mRNA expression declined in most animals but remained above the initial level at 2 wpi in 4 of 5 animals. Opposite to IFN-γ, no gradual increase in IL-10 and IL-4 mRNA levels occurred, however, the expression of both cytokines was already higher at the first time point 2 wpi than for IFN-γ. Overall, the IL-10 mRNA levels tended to decrease in amount and in number of animals, after a temporary elevation in 2-3 out of 5 animals at 3 wpi. Interleukine-4, on the other hand, showed a rather stable expression, when comparing the group averages per time point, but a significant higher amount of cytokine mRNA was measured in 1 out of 5 animals at all time points.

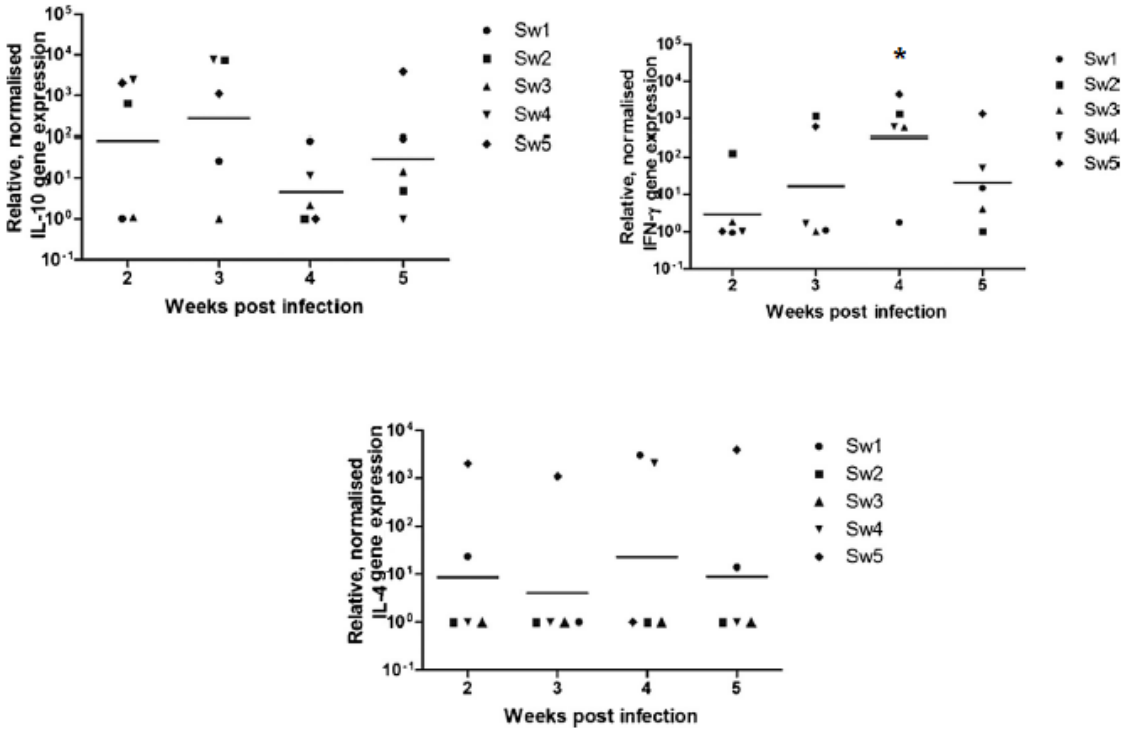

Figure 5.3 : Relative normalized IL-10, IFN-gamma and IL-4 cytokine expression by PBMCs from pigs orally infected with *T. gondii* IPB-G are presented in scatter dot plots. * presents significant differences at $P<0.05$ in comparison with values at 2 wpi.

5.5 Discussion

Antibodies to *T. gondii* have been found in nearly all mammal and avian species worldwide (Dubey, 2010). The infection rate in humans can be as high as >80%, depending on the geographical location, and is strictly associated with the prevalence of this parasite in domestic animals and livestock. Corresponding to that, different species can serve as the source of the foodborne infection for humans, among which pigs (Guo et al., 2015a and 2015b). Although a low to moderate (0.0 - 36%) prevalence was estimated on conventional pig farms in Europe and USA, the exact transmission rate is difficult to estimate (Kijlstra et al., 2004; Bartova et al., 2011; Guo et al., 2015a). Consequently, a vaccine that could prevent infection in livestock would not only have

a high economical value for some species such as small ruminants, but it could also diminish the risk of the infection in humans, reducing the short- and long- term effects for human health.

Therefore, we studied previously different immune parameters in 5-week-old seronegative pigs upon infection with 3000 tissue cysts of *T. gondii* IPB-G strain, and the corresponding infectivity and parasite load in different edible tissues (Verhelst et al., 2011). One group of five animals was maintained for 6 weeks and the second group of three pigs for 6 months. All pigs were sampled weekly until the euthanasia at 6 wpi or 6 mpi, respectively. The cytokine mRNA expression in the blood, and antibody responses against recombinant *T. gondii* GRA1, GRA7, MIC3 proteins and a chimeric antigen EC2 encoding MIC2–MIC3–SAG1 were investigated. We observed that starting from 14 dpi an IFN-γ response was detected in the blood, with a comparable kinetic profile as the GRA7 antibody response. The latter occurred in both groups of infected animals and seemed to be indicative for the presence of the viable parasite in some tissues at euthanasia 6 mpi, as demonstrated by bio-assay. Strikingly, two of the sampled skeletal muscle tissues (M. gastrocnemius and M. longissimus dorsi) were found negative of infectious parasites 6 mpi, while the parasite was still detectable in brain and heart. This apparent clearance of the infection in several porcine tissues in a chronic infection model could change the risk assessment of meat borne toxoplasmosis for human health. The diminishing presence of the tachyzoite stage and the gradual appearance of the bradyzoite stage in tissue cysts at the same time, when the host's immunity appears to build up, were already described by Denkers and Gazzinelli (1998). Based on their findings and the newly obtained results, we hypothesize that the changes in the number and/or distribution of the parasite in the tissues are correlated with certain immune parameters within the host. Therefore, the aims of this experiment included the study of the parasite's dissemination in systemic tissues upon oral ingestion of *T. gondii* cysts. Further, the investigation of the immune responses such as, expression of the cytokine mRNA and antibody production against different

antigens in relation to bradyzoites' load and viability, would elucidate more in detail the mechanisms of the host's defense against acute and chronic *T. gondii* infection.

It was previously described that the parasite load is not evenly distributed across tissues (Denkers and Gazzinelli, 1998; Dubey et al., 1998c). Bradyzoites were predominantly found in the central nervous system and heart but – depending on the host – also other tissues such as liver, diaphragm and skeletal muscles were infected (Denkers and Gazzinelli, 1998; Dubey et al., 1998c). In the current study the parasites were detected by bioassay and qPCR in the heart, brain, M. gastrocnemius and M. longissimus dorsi of the majority of the animals. The average parasite load was the lowest in M. gastrocnemius, followed by heart, brain and M. longissimus dorsi. Heart was consistently positive in all 5 infected animals in qPCR and in bioassay. Therefore, this tissue seems to be the most relevant to demonstrate the parasite's presence in this phase of the infection.

Referring to the humoral immune response, rGRA7 was the only of the 5 antigen preparations, against which antibodies occurred in all animals and which persisted till the end of the study. This is in line with previous findings in pigs (Verhelst et al., 2011). It is known that both humoral and cellular immune responses contribute to the host's immune defense against *T. gondii*. Sustaining strong T helper 1 (Th1)-mediated immunity, characterized by the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells, is crucial in preventing the emergence of and disease by *T. gondii* (Gazzinelli et al., 1993; Denkers, 1999). The production of IL-4 by Th2 lymphocytes, in addition to innate cells such as mast cells, eosinophils, basophils or macrophages, is associated with the progression of the infection and with a reactivation of latent disease (Hope et al., 2005; Wynn, 2015). Interleukin-10, expressed mainly by the cells of the innate rather than adaptive immune system, among which Th2 T cells and $CD4^+CD25^+FoxP3^+$ Treg cells, has been shown to downregulate acute inflammatory responses against this intracellular parasite, and to inhibit the IFN-γ production by $CD8^+$ lymphocytes (Appelberg et al.,1992; Denkers, 1999, Saraiva and O'garra, 2010). In our study, looking at changes in IFN-γ mRNA

expression in PBMCs isolated directly from blood, a clear increase was seen at 2 wpi, which remained present until the last sampling 6 wpi. In contrast, the IL-10 and IL-4 mRNA expression did not show a consistent increase in all animals. This is in line with the Th1 type of response already described in sheep (Innes et al., 1995) and mice (Roberts et al., 1996).

In conclusion, the results in this study demonstrate that the parasite is present in brain, heart and skeletal muscles of pigs at 6 wpi. The parasite was most consistently demonstrated in heart tissue with bioassay and qPCR, however, a skeletal tissue showed a lower parasite load. Previously our lab described a reduction in load in skeletal muscles during the chronic phase of infection and suggested that this might be due to a possible clearance mechanism (Verhelst et al., 2011). Interestingly, the antibody response could only be observed against rGRA7 and not towards other recombinant antigens included in the testing. Nevertheless, all animals showed a clear IFN-γ response starting from 14 dpi, which might contribute to the overall reduction in the parasite load and the viability of *T. gondii*. As a future perspective, it will be interesting to determine if this Th1-type response persists in the animals in a chronic infection model, and to investigate the strain and dose effect on the same parameters as described here.

5.6 Acknowledgements

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Chapter 6 Strain- and dose-dependent reduction of *Toxoplasma gondii* burden in pigs is associated with Interferon-gamma production by $CD8^+$ lymphocytes in heterologous challenge model

Adapted from:

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 (2017). Frontiers in Cellular and Infection Microbiology. doi: 10.3389/fcimb.2017.00232.

6.1 Abstract

Toxoplasma gondii is a worldwide prevalent parasite of humans and animals. The global infection burden exceeds yearly one million disability-adjusted life years (DALY's) in infected individuals. Therefore, effective preventive measures should be taken to decrease the risk of infection in humans. Although human toxoplasmosis is predominantly foodborne by ingestion of tissue cysts in meat from domestic animals such as pigs, the incidence risk is difficult to estimate due to the lack of screening of animals for infection and insights in location and persistence of the parasite in the tissues. Hence, experimental infections in pigs can provide more information on the risk for zoonosis based on the parasite burden in hazardous meat products intended for human consumption and on the immune responses induced by infection. In the present study, homo- and heterologous infection experiments with two distinct *T. gondii* strains (IPB-LR and IPB-Gangji) were performed. The humoral and cellular immune responses, the presence of viable parasites and the parasite load in edible meat samples were evaluated. In homologous infection experiments the parasite persistence was clearly strain-dependent and inversely correlated with the infection dose. The results strongly indicate a change in the amount of parasite DNA and viable cysts in porcine tissues over time. Heterologous challenge infections demonstrated that IPB-G strain could considerably reduce the parasite burden in the subsequent IPB-LR infection. A strong, however, not protective humoral response was observed against GRA7 and TLA antigens upon inoculation with both strains. The *in vitro* IFN-γ production by TLA-stimulated PBMCs was correlated with the infection dose and predominantly brought about by $CD3^{+}CD4^{-}CD8\alpha^{bright}$ T-lymphocytes. The described adaptive cellular and humoral immune responses in pigs are in line with the induced or natural infections in mice and humans. Previous studies underscored the heterogeneity of *T. gondii* strains and the corresponding virulence factors. These findings suggest the potential of the IPB-G strain to elicit a partially protective immune response and to reduce the parasite burden upon a challenge infection. The IPB-G

strain could be used as a promising tool in limiting the number of viable parasites in edible tissues and, hence, in lowering the risk for human toxoplasmosis.

6.2 Introduction

Toxoplasmosis is a parasitic infection caused by the intracellular protozoa *Toxoplasma gondii*. This parasite has a complex lifecycle and affects its definitive host as well as various intermediate hosts, among which domestic and wild animals and humans (Dubey, 2010).

Several infection routes have been described for the different hosts of *T. gondii*. In humans, foodborne toxoplasmosis mainly results from the consumption of raw or undercooked meat from infected animals, like domestic pigs. The global prevalence of this parasite includes one third of the human population and as such represents one of the most common parasitic zoonosis worldwide (Tenter et al., 2000; Ajzenberg et al., 2002; Aspinall et al., 2002; Bosch- Driessen et al., 2002; Kijlstra and Jongert, 2008; Innes, 2010; Robert-Gagneux and Dardé, 2012; Torgerson and Mastroiacovo, 2013). Consequently, infection in human has a severe short- and long-term impact, ranging from congenital or adult toxoplasmosis in healthy individuals to *T. gondii*-induced encephalitis in immune-compromised patients. Therefore, numerous preventive measures are recommended in terms of meat processing or preparation, in an attempt to decrease the global infection burden in the human population. Especially, pork is often consumed undercooked and is processed in many other meat products, reaching on average 300 consumers per pig (Fehlhaber et al., 2002; Belluco et al., 2016). The data on the prevalence of *T. gondii* infection in pigs are not uniform and may vary per country or the farm management. Nevertheless, the estimated average prevalence in the pig population seems to be very low in European countries (2.2%) and the USA (2.7%), presumably due to a shift from small and less strictly confined to large scale facilities, implementing all-in-all-out or farrow-to-finish models (Hill et al., 2008; EFSA report, 2012; Guo et al., 2015b). However, the recent rise of organic or free-range

farming in order to improve animal welfare seems to contribute to an increase in infection rate in pig livestock and, as such, to the incidence of foodborne human toxoplasmosis (Kijlstra et al., 2004; Dubey et al., 2012b; EFSA report, 2012). The risk for humans to become infected by consumption of undercooked or raw pork is also not clear. The knowledge of the parasite persistence in edible tissues of naturally infected pigs is limited, as are the role of strain or dose in the parasite survival in the host.

Nevertheless, several experimental data in pigs reported reduction in parasite burden in infected and subsequently heterologous challenged pigs, in which the choice of the strain had an important effect on the viability of the parasite (Solano Aguilar et al., 2001; Dawson et al., 2004; Kringel et al., 2004; Dawson et al., 2005, Garcia et al., 2005; Verhelst et al., 2011; Verhelst et al., 2015). Despite the active role the different components of the host's immune system play in the early stage of *T. gondii* infection, it remains a subject of discussion and ongoing research, whether the intermediate host can clear the tissues from the cysts on long term. It is noteworthy, however, that several studies in pigs notified reduced or undetectable counts of the parasite DNA in multiple porcine tissues, and a decline in viability of the cysts, as tested by bioassay in mice (Jongert et al., 2008; Verhelst et al., 2011; Burrells et al., 2015; Verhelst et al., 2015). Taking into account the lack of an obligatory screening of pigs or pork meat to prevent transmission to humans, knowledge on the pig as an intermediate host for *T. gondii*, and in particular strategies to reduce the amount of viable parasites in tissues, may contribute to diminishing the risk of zoonosis by consumption of porcine meat (EFSA, 2007; EFSA 2012; Opsteegh et al., 2016b). In light of these data, the aim of this study was to confirm differences between T. *gondii* strains in persistence of the parasite in tissues of experimentally infected pigs and to relate the dose and strain to the immune responses in the pigs upon a single infection or a heterologous challenge.

6.3 Material and methods

6.3.1. *T. gondii* strains

Two *T. gondii* strains were used for the experimental infections: the IPB-Gangji (IPB-G) strain and the IPB-LR strain. The first one was isolated from the placenta of a patient with congenital toxoplasmosis and is highly virulent in mice. It produces a large number of tissue cysts and has an atypical mixed type I and type II genotype (Ajzenberg et al., 2002). The latter, with the code name referring to the pig farm where it was initially isolated, belongs to genotype II, which is less pathogenic and commonly present in the European pig population (Dubey, 2009b; Dubey et al., 2012b). Both strains are maintained at the National Reference Laboratory for Toxoplasmosis (Scientific Institute for Public Health, Brussels, Belgium) by passage in Swiss female mice, since there is no alternative available to obtain a sufficient number of tissue cysts for the inoculation experiments than via bioassay, as approved by the Ethical Committee (nr 20140704-01) and conform the European legislation (2010/63/EU). Tissue cysts from both strains were isolated from homogenized brain tissue, counted by phase-contrast microscopy and suspended in 10 ml of sterile phosphate buffered saline (PBS) solution at the desired concentration (700 cysts for the low dose and 6000 for the high dose). The animals were inoculated within 8 hours after cysts isolation. The inoculum for the negative control group was prepared identically from naive Swiss mice.

6.3.2 Animals and experimental design

Two-week-old Belgian Landrace piglets were tested for the presence of anti-*T. gondii* serum antibodies (IgM and IgG) with an indirect immunofluorescence assay (IFA) as described previously (Verhelst et al., 2015). For the infection experiments, 3-week-old newly weaned, seronegative piglets were selected and randomly assigned to 10 groups of 3 animals (Table 6.1). These groups were housed in isolation units (Biosafety permit nr: AMV/11062013/SBB219.2013/0145) at the Faculty of Veterinary Medicine, Ghent

University, Belgium. All experiments were approved by the Ethical Committee of the faculties Veterinary Medicine and Bioscience Engineering at Ghent University (EC 2009/149).

In a first experiment we aimed to study the effect of a low or high infection dose of two different *T. gondii* strains on the humoral and cellular immune responses and tissue cyst persistence until 120 days after inoculation (Table 6.1). In a second experiment we focused on the effect of a subsequent challenge with a heterologous strain at 60 dpi and the persistence of the parasite in the tissues at 120 dpi (Table 6.1). In study 3 we compared kinetics of the IFN-γ producing porcine T cell subsets following infection with high doses of the IPB-G or the IPB-LR strain until 98 dpi (Table 6.1). In each experiment the peripheral blood monomorphonuclear cells (PBMCs) were sampled at regular intervals for the detection of cytokine mRNA by qPCR, and for the quantification of the IFN-γ producing T cell subsets, respectively. At euthanasia, PBMCs and lymphocytes from the peripheral lymph nodes and spleen were isolated for further *in vitro* assays, whereas heart, diaphragm, skeletal muscles and brain were collected to determine the parasite load as explained further. The experimental timeline presenting the collected samples and the sampling intervals is shown in Figure 6.1.

Table 6.1 : Experimental design: G: IPB-G strain; LR: IPB-LR strain. Groups: a single low or high dose of the IPB-G strain (G_{low} and G_{high}); a single low or high dose of the IPB-LR strain (LR_{low} and LR_{high}); a high dose of the IPB-G strain, followed 60 dpi by a high dose of the IPB-LR strain (G_{high}/LR_{high}); a high dose of the IPB-G strain ($G_{high1/2t}$) 60 dpi; a high dose of the IPB-LR strain, followed 60 dpi by a high dose of the IPB-G strain (LR_{high}/G_{high}); a control group for both infected groups ($G+LR_{cltr}$).

Study n°	Strain	Dose of tissue cysts	Group	Number of animals	Duration (dpi)	Heterologous challenge (y/n)
1	G	700	G_{low}	3	120	no
	G	6000	G_{high}	3	120	no
	LR	700	LR_{low}	3	120	no
	LR	6000	LR_{high}	3	120	no
2	G	6000	$G_{high/}$ LR_{high}	3	120	yes (at 60 dpi)
	G	6000	$G_{high1/2t}$	3	60	no
	LR	6000	$LR_{high/}$ G_{high}	3	120	yes (at 60 dpi)
3	G	6000	G_{high}	3	98	no
	LR	6000	LR_{high}	3	98	no
Control	/	0	$G+LR_{cltr}$*	7	120	no

*IPB-Gangi and IPB-LR control group

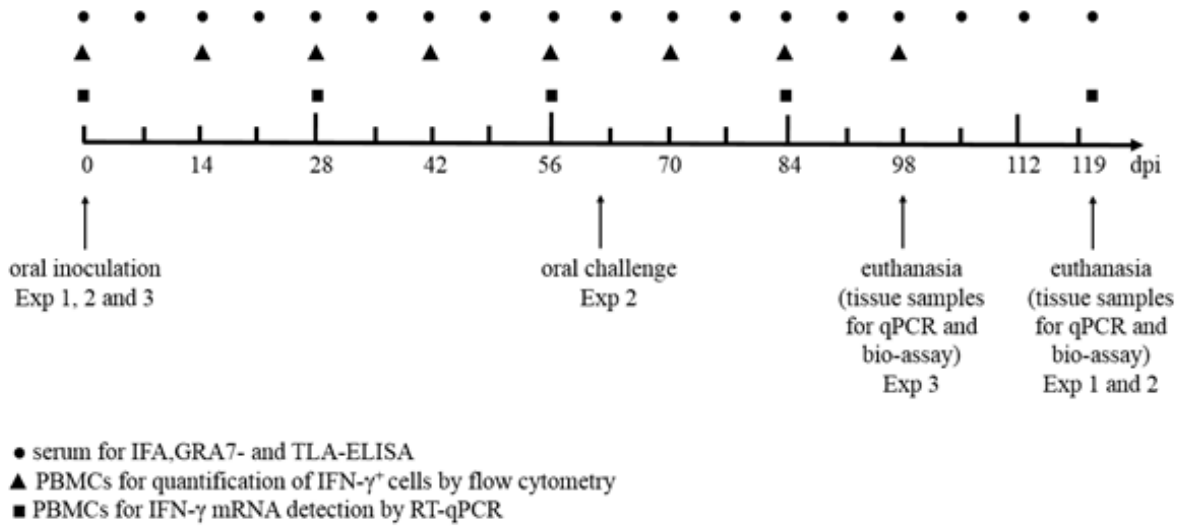


Figure 6.1 : The timeline of the experiments from the inoculation (day 0 dpi) until euthanasia (day 120 dpi). Exp: experiment; ● serum for IFA, GRA- and TLA-ELISA; ▲ PBMCs for the quantification of IFN-γ$^+$ T-lymphocytes by flow cytometry ■ PBMCs for IFN-γ mRNA detection by qPCR.

6.3.3 Humoral immune response

For each experiment the seroconversion was monitored during the first two weeks after inoculation (wpi) by daily and subsequently weekly blood collection from the vena jugularis until 120 days post infection (dpi).

Antibody ELISA's with recombinant GRA7 and native TLA antigens

As dense granule protein 7 (GRA7) is considered as a marker of an active infection, being expressed by all *T. gondii* stages, recombinant GRA7 is frequently used to demonstrate the immune response during acute and chronic toxoplasmosis in humans and animals (Jacobs et al., 1999). GRA7 was prepared as previously described (Jongert et al., 2007). Briefly, GRA7 was produced as a His-tagged fusion protein by *Escherichia coli* (*E. coli*) TOP 10 cells (Life Technologies, Ghent, Belgium) and purified under denaturing conditions (8 M urea, 0.1% SDS) using nickel-nitrilotriacetic acid (Ni-NTA)

chelate affinity column chromatography (Ni-NTA Superflow, Qiagen, Venlo, The Netherlands). GRA7 was then eluted from the Ni-NTA column using 250 mM imidazole and further purified by sequential dialysis steps reducing the urea and SDS concentration to 0.1 M and 0.01%, respectively.

T. gondii total lysate antigen (TLA) from tachyzoites of the RH-strain was prepared as previously described (Jongert et al., 2007) in the biosafety level 2 laboratory (Biosafety permit nr: 415240), as approved by the Ethical Committee (nr 20140704-01) at the National Reference Laboratory for Toxoplasmosis (Scientific Institute for Public Health, Brussels, Belgium). TLA-based assays show a high reactivity due to a broad range of antigens in the lysate, however, differences in the production method can affect the composition of the lysate (Gamble et al., 2005; Ferra et al., 2015). Concisely, tachyzoites were diluted with PBS and then purified by differential centrifugation and filtration through a 5 µm syringe filter (MilleX®SV, Merck KGaA, Darmstadt, Germany). The tachyzoite suspension was then lysed by alternating sonication with cooling cycles using an Ultrasonic disintegrator (MSE, Leicester, United Kingdom). To evaluate the protein content of the lysate, the bicinchoninic acid (BCA) reaction (Thermo Scientific Pierce BCA protein Assay Kit, Erembodegem, Belgium) was used. Finally, the TLA was aliquoted and stored at -20°C until further use.

Both TLA and GRA7 were used in indirect Enzyme-Linked Immunosorbent Assays (ELISA's) at 10 µg/ml to detect *T. gondii*-specific IgM and IgG antibodies in serum samples diluted 1/50 with the goat anti-pig IgM- and IgG-Horse Radish Peroxidase (HRP) conjugate (Bethyl Laboratories Inc., Montgomery, Texas, USA), respectively, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as substrate-chromogen solution (Verhelst et al., 2015). On each plate previously collected sera from one positive and three negative control animals as established by IgM and IgG immunofluorescence assay (IFA) were included and diluted 1/50 in dilution buffer (0.05 % Tween-20 in PBS). The absorbance was measured at 405 nm (TECAN Spectra Fluor, Tecan Group Ltd., Männedorf, Switzerland) and the obtained data were analysed in

Microsoft Excel. Serum samples from infected animals were considered positive when exceeding the cut-off value calculated using the formula: mean OD_{405} negative controls + 3 x its standard deviation (SD).

Immunofluorescence assay

The presence of IgM and IgG antibodies against *T. gondii* was also evaluated by IFA using slides coated with formalin-fixed tachyzoites from the *T. gondii* RH-strain (Toxo-Spot IF, Biomérieux, Marcy-l'Etoile, France). Briefly, serum samples, diluted 1/50 in PBS, were applied to the slides for 30 min at 37°C, followed by washing with PBS. After drying, a second incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-swine IgM(µ) or IgG (H+L) (KPL, Maryland, USA) antibody (diluted 1/25 in PBS with Evans Blue as counter dye) was performed for 30 min at 37°C. After washing, drying and mounting with PBS-buffered glycerol, the slides were observed by fluorescence microscopy (Carl Zeiss, Germany). The cut-off read-out was established with positive and negative reference sera at a 1/50 dilution.

6.3.4 Detection of the cellular immune response

PBMCs were isolated from 20 ml heparinized blood (LEO Pharma, Ballerup, Denmark) by density gradient centrifugation (800 × *g* at 18°C, 25 min) using Lymphoprep™ (Axis-Shield, Oslo, Norway) (Sonck et al., 2010). Subsequently, the cell pellets were resuspended in leukocyte medium (RPMI-1640 (GIBCO BRL, Life Technologies, Merelbeke, Belgium), supplemented with fetal calf serum (10%) (Greiner, Bio-One, Merelbeke Belgium), non-essential amino acids (100 mM) (Gibco), Na-pyruvate (100 µg/ml), L-glutamine (292 µg/ml) (Gibco), penicillin (100 IU/ml) (Gibco), streptomycin (100 µg/ml) (Gibco) and kanamycin (100 µg/ml) (Gibco)). The cells (10^6 cells/well) were cultured for 6 and 72 h upon stimulation with either TLA (10 µg/ml) as a heterologous challenge or the mitogen concanavalin A (ConA, Sigma-Aldrich, USA; 5 µg/ml) as a positive control.

Cytokine mRNA quantification by qPCR

After 6 h of incubation with TLA, ConA or medium, the cells were lysed by adding 350 µl of RLT-buffer (Qiagen) supplemented with 1 % β-mercaptoethanol (99%, Thermo Fisher Scientific, Aalst, Belgium) and stored at -80°C until RNA isolation. Total RNA extraction and conversion into cDNA was performed using the RNeasy kit (Qiagen) and the iScript kit (Biorad, Hercules, CA, USA), respectively. The purity of the RNA was assessed by an on-column DNase digestion step as recommended by the supplier. The amount of cytokine cDNA was then tested by quantitative polymerase chain reaction (qPCR). The qPCR reaction mix consisted of 12.5 µl iQ SYBR Green Supermix (Biorad), 0.5 µl of each primer set at a concentration of 20 µM, 1.5 µl PCR grade water and 10 µl of the 1/100 diluted cDNA. Interleukin (IL)-10, IL-12A, IL-17A and interferon-gamma (IFN-γ) cDNA was amplified with the primer sets presented in Table 6.2. In order to normalize the cytokine expression, β-actin, glyceraldehyde phosphate dehydrogenase (GAPDH) and the ribosomal 18S gene were used as reference genes (Table 6.2). Special care was taken to choose a set of primers on different exons or spanning exon-exon junctions to exclude the amplification of genomic DNA. The qPCR amplification protocol consisted of an initial denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 61°C for 20 s. After each run, a melt curve analysis was performed to confirm the presence of the correct amplicon and to exclude false positives due to the formation of primer dimers. The cDNA was tested in duplicate for each cytokine and the three reference genes (GADPH, β-actin, r18S), showing a stable expression. The mRNA expression in PBMCs was calculated with the CFX96 Manager™ Software v3.1 (Biorad), using a mathematical model (delta-delta Ct method). The mean value was determined for the target cytokines and normalized relative to the geometric mean of the reference genes (Verhelst et al., 2015).

Table 6.2 : List of primers for qPCR

Target	Sequence	Length amplicon (bp)
IL-10	F: CCTGGGTTGCCAAGCCTT R: GCTTTGTAGACACCCCTCTCTT	240
IL-12A	F: ACCAGCACAGTGGAGGC R: CGAATGAGAGTTGCCTGGCT	95
IL-17A	F: GGACAAGAACTTCCCTCAGCA R: CTCGTTGCGTTGGAGAGTC	124
IFN-γ	F: GAGCCAAATTGTCTCCTTCTACTT R: CTGACTTCTCTTCCGCTTTCT	262
GAPDH	F: CCATCACTGCCACCCAGAA R: CAGGGATGACCTTGCCCA	130
B-actin	F: GGCATCCTGACCCTCAAGTA R: GCCTCGGTCAGCAGCA	137
r18S	F: GTTGATTAAGTCCCTGCCCTTT R: GATAGTCAAGTTCGACCGTCTT	141

Flow cytometric detection of IFN-γ production

The flow cytometric detection of IFN-γ-producing proliferating lymphocytes was performed on cultured PBMCs 72 h after heterologous stimulation with TLA (10 µg/ml). First, the cell division marker Violet Proliferation Dye 450 (VPD450, BD Biosciences, Erembodegem, Belgium) was added to the isolated mononuclear cells, showing a diminishing fluorescence after each cell division. At the end of the incubation period, a protein transport inhibitor, Golgi Plug™, was added and the cells were fixed and permeabilized using the Cytofix/Cytoperm™ kit (both from BD Biosciences). Subsequently, cells were stained using murine monoclonal antibodies (mab) against

CD3 (IgG1, clone PPT3), CD4 (IgG2b, clone 72–14-4) and CD8 (IgG2a, clone 11/295/33) and anti-isotype-specific conjugates (goat anti-mouse IgG1-PerCP-Cy5.5 (Santa Cruz Biotechnology, Dallas, Texas, USA), goat anti-mouse IgG2b-FITC (Southernbiotech, Birmingham, Alabama, USA) and goat anti-mouse IgG2a-Alexa Fluor®647 (Invitrogen™, Merelbeke, Belgium). Finally, phycoeryrthrin (PE)-conjugated mAb against porcine IFN-γ (Mouse IgG1, BD Biosciences) was added to identify the lymphocyte subsets producing IFN-γ. A minimum of 10,000 events was recorded within the proliferating cell gate (Appendix 1). The IFN-γ secretion in the different lymphocyte subsets was determined and compared with the results of the isotype-matched control (Mouse IgG1-PE, Abcam, Cambridge, UK) using a FACSAria III and FACSDIVA™ software (both from BD). The gating strategy is included in the supplementary data (Appendix 1).

Animals were euthanized at 98 dpi and the splenocytes and lymphocytes from the peripheral lymph nodes (mediastinal, mesenteric and popliteal) were isolated as previously described (Verhelst et al., 2011). Subsequently, the cells were stimulated with the same antigens as the PBMCs for 6 h and 72 h, whereafter the same staining occurred for flow cytometric analysis as for the PBMCs.

6.3.5 Detection of the parasite: bioassay and qPCR

In experiments 1 and 2, all animals were euthanized at 120 dpi and the parasite load was determined in brain (Br), heart (He), spleen, diaphragm (Di) and skeletal muscles (M. gastrocnemius (Mg), Mm. intercostales (Ic), M. longissimus dorsi (Ld) and M. psoas major (Mp) by qPCR and a bioassay. For this, 100 g of each tissue was homogenized in 10 ml 0.85 % sodium chloride (NaCl) and digested with pepsin (0.8 g/l pepsin in 7 ml/l hydrogen chloride (HCl)) for 1 h for brain and 2 h for the other tissues, while stirring in a water bath at 37°C. The obtained suspension was filtered and centrifuged for 15 min at 1180 x *g*, the supernatant removed and the pellet resuspended in 10 ml PBS supplemented with 40 IU/ml gentamicin. For the bioassay, 1 ml of the tissue suspension

was inoculated intraperitoneally into 5 naive Swiss female mice. The mice were observed twice a day for the next 5 weeks and euthanized in respect to the human end points in case of acute toxoplasmosis associated with suffering or reduced welfare. The surviving mice were euthanized and tested serologically by immunofluorescence for the presence of *T. gondii* IgG antibodies. The parasite was demonstrated by qPCR in lungs and ascites of mice, which had to be euthanized for ethical reasons in case of acute toxoplasmosis. To determine the parasite load by qPCR DNA was extracted from the tissue suspensions with the QIAamp DNA Mini kit (Qiagen). A 10-fold serial dilution of *T. gondii* DNA prepared from RH-strain tachyzoite suspension containing 10^6 parasites per ml was used as a standard, with a detection limit of 2-4 tissue cysts per 100g of tissue. Real-Time PCR (RT-PCR) amplifying both the *T. gondii* repeat element (AF146527) and the ribosomal 18S rDNA of the host cells was performed as previously described (Rosenberg et al., 2009).

6.3.6 Statistics

The parasite-specific antibody and IFN-γ responses in different groups at different time points are presented as means ± SD. A one-way Analysis of Variance (ANOVA) was performed, followed by post hoc Bonferroni's and Dunnett's Multiple Comparison Tests for antibody production and cytokine response, respectively, to discriminate between infected and control groups (GraphPad Prism 5). A p-value <0.05 was considered statistically significant.

6.4 Results

6.4.1 Parasite burden and immune response after single inoculation with a low or a high infection dose of the IPB-G or IPB-LR strain

GRA-7 and TLA-specific antibody response

The GRA7-specific IgM antibodies appeared approximately at the same time in the low and the high dose group (10 and 9 dpi, respectively) upon inoculation with the IPB-G strain and declined gradually from 14 and 11 dpi, respectively, until 91 dpi (Figure 6.2A). In contrast, in the IPB-LR infected group a pronounced IgM production was detected 8 dpi in the low dose group and even a stronger response at 10 dpi in the high dose group (Figure 6.2B), but both declined to control levels around 12 dpi. GRA7-specific IgG antibodies were detected shortly after IgM, irrespective of the inoculation strain, and remained detectable until the end of the experiment (Figure 6.2C, 6.2D). Nevertheless, the high dose of the IPB-LR strain induced the highest levels of GRA7-specific IgG.

TLA-specific IgM occurred earlier than GRA7-specific IgM, namely 7 to 8 dpi in the G_{low} and G_{high} groups. In the latter, the IgM response remained present until the end of the experiment, slightly increasing in time, irrespective from the infection dose (Figure 6.3A). On the contrary, for the LR_{low} and LR_{high} groups the seroconversion to TLA-specific IgM was prominently present until 21 to 28 dpi, showing again the highest concentration in the high dose group (Figure 6.3B). The TLA-specific IgG antibodies appeared approximately 14 dpi in both dose groups inoculated with the IPB-G strain (Figure 6.3C), but already at 8 dpi in animals infected with the IPB-LR strain. There, the antibodies increased significantly starting from 28 dpi and remained elevated until 120 dpi (Figure 6.3D). In animals inoculated with the IPB-G strain no dose effect was neither seen for TLA-specific IgM nor for IgG production (Figure 6.3A and 6.3C), whereas the high dose induced a higher response for IgM and a similar response for IgG upon inoculation with the IPB-LR strain (Figure 6.3B and 6.3D).

The IFA results confirmed the seroconversion from *T. gondii* - negative towards IgM positive animals and the persistence of the IgG antibodies in each infection experiment (data not shown).

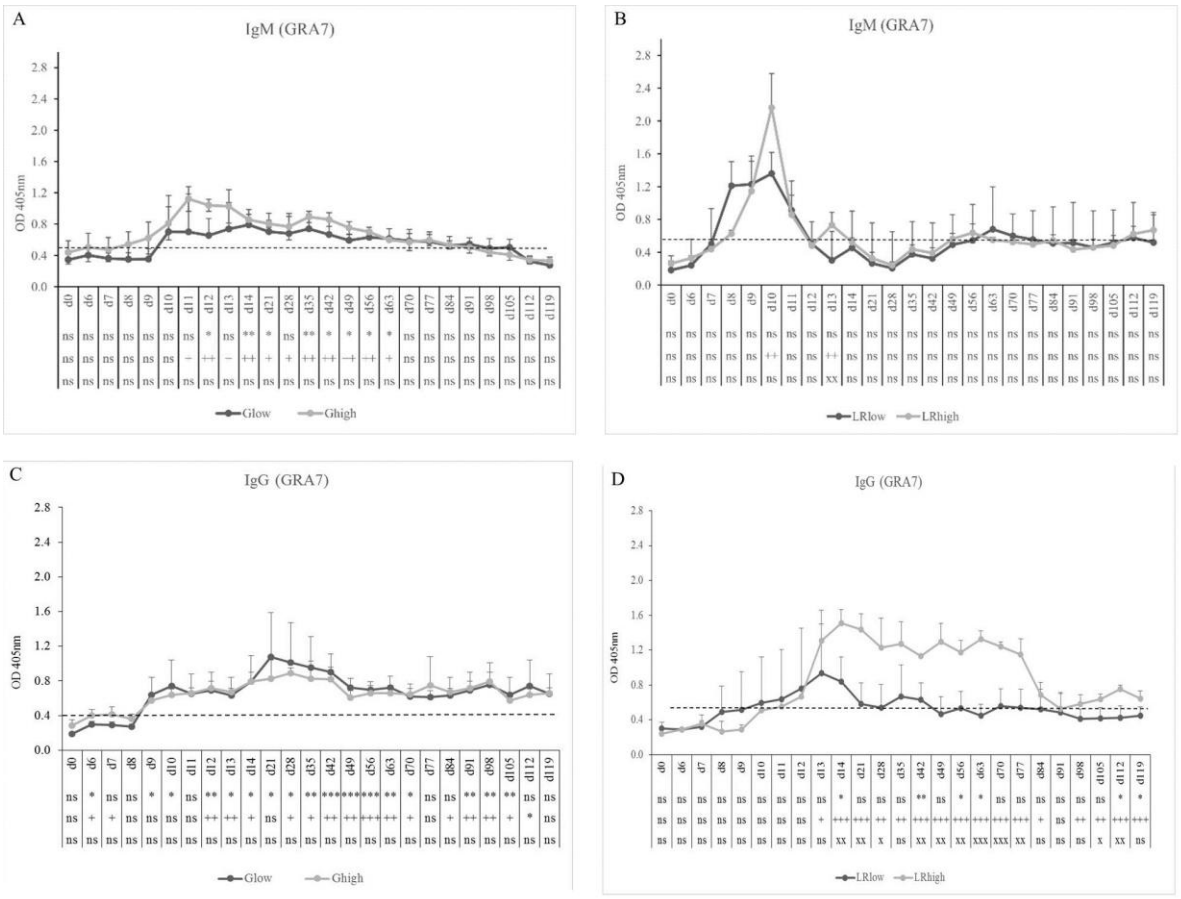


Figure 6.2 : GRA7-specific IgM and IgG responses after inoculation with IPB-G or IPB-LR *T. gondii* strain. IgM (A+B) and IgG (C+D) responses in animals inoculated with a low or a high dose of the IPB-G (A+C) or the IPB-LR (B+D) strain. Groups: G_{low}, G_{high}, LR_{low}, LR_{high}. The horizontal dashed line indicates the cut-off value based on the mean of the negative animals. The results represent a mean of the infected group ± SD; * (low dose versus controls) or + (high dose versus controls) or x (low dose versus high dose) : P < 0.05, ** or ++ or xx: P < 0.01; *** or ++ or xxx: P < 0.001; ns: not significant.

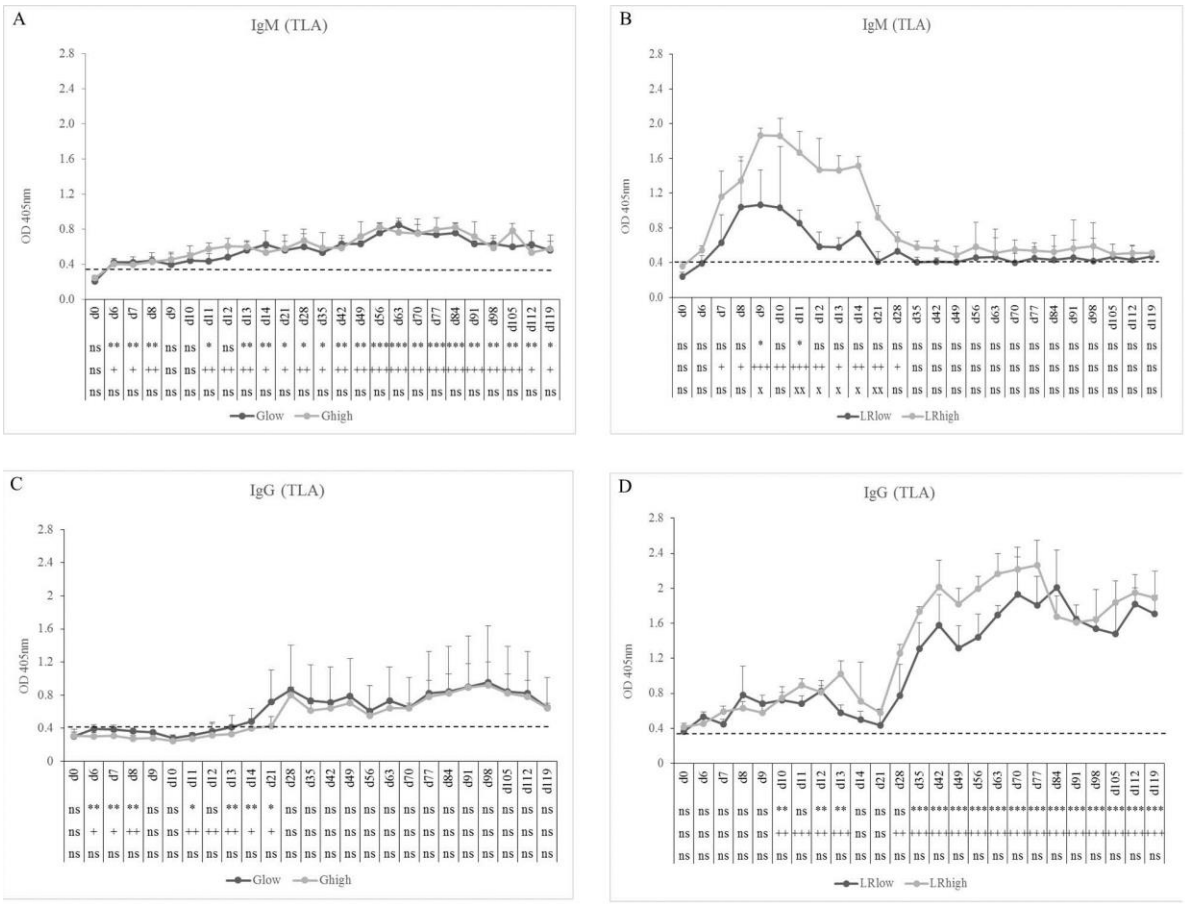


Figure 6.3 : TLA-specific IgM and IgG responses after inoculation with IPB-G or IPB-LR *T. gondii* strain. IgM (A+B) and IgG (C+D) responses in animals inoculated with a low or a high dose of the IPB-G (A+C) or the IPB-LR (B+D) strain. Groups: G_{low}, G_{high}, LR_{low}, LR_{high}. The horizontal dashed line indicates the cut-off value based on the mean of the negative animals. The results represent a mean of the infected group ± SD; * (low dose versus controls) or + (high dose versus controls) or x (low dose versus high dose) : P < 0.05, ** or ++ or xx: P < 0.01; *** or ++ or xxx: P < 0.001, ns: not significant.

TLA-specific IFN-γ mRNA responses in PMBCs

PBMCs were restimulated *in vitro* with TLA for 6 hours, where after IL-10, IL-12A, IL-17A and IFN-γ mRNA responses were determined. No detectable IL-10, IL-12 and IL-17A mRNA production was observed (data not shown) in any infected group,

irrespective of the strain or infection dose. However, a substantial increase in IFN-γ mRNA production was observed from one month post infection (mpi) onwards in the majority of the inoculated animals as compared to the control animals. This response was least pronounced in the animals infected with the low dose of the IPB-G (not significant, $p = 0.39$) (Figure 6.4A), followed by a significant ($p < 0.01$) and highly significant ($p < 0.001$) increase in the high dose of the IPB-G strain group (Figure 6.4B). In the low dose of the IPB-LR group we noticed a steady though not significant ($p = 0.18$) increase (Figure 6.4C). The highest IFN-γ production was observed in the animals infected with the high dose of the IPB-LR strain starting from 1 mpi ($p < 0.01$), which stayed high throughout the experiment, becoming highly significant ($p < 0.001$) at 2, 3 and 4 mpi (Figure 6.4D). No detectable IFN-γ level was detected in splenocytes from IPB-G or IPB-LR infected animals (data not shown).

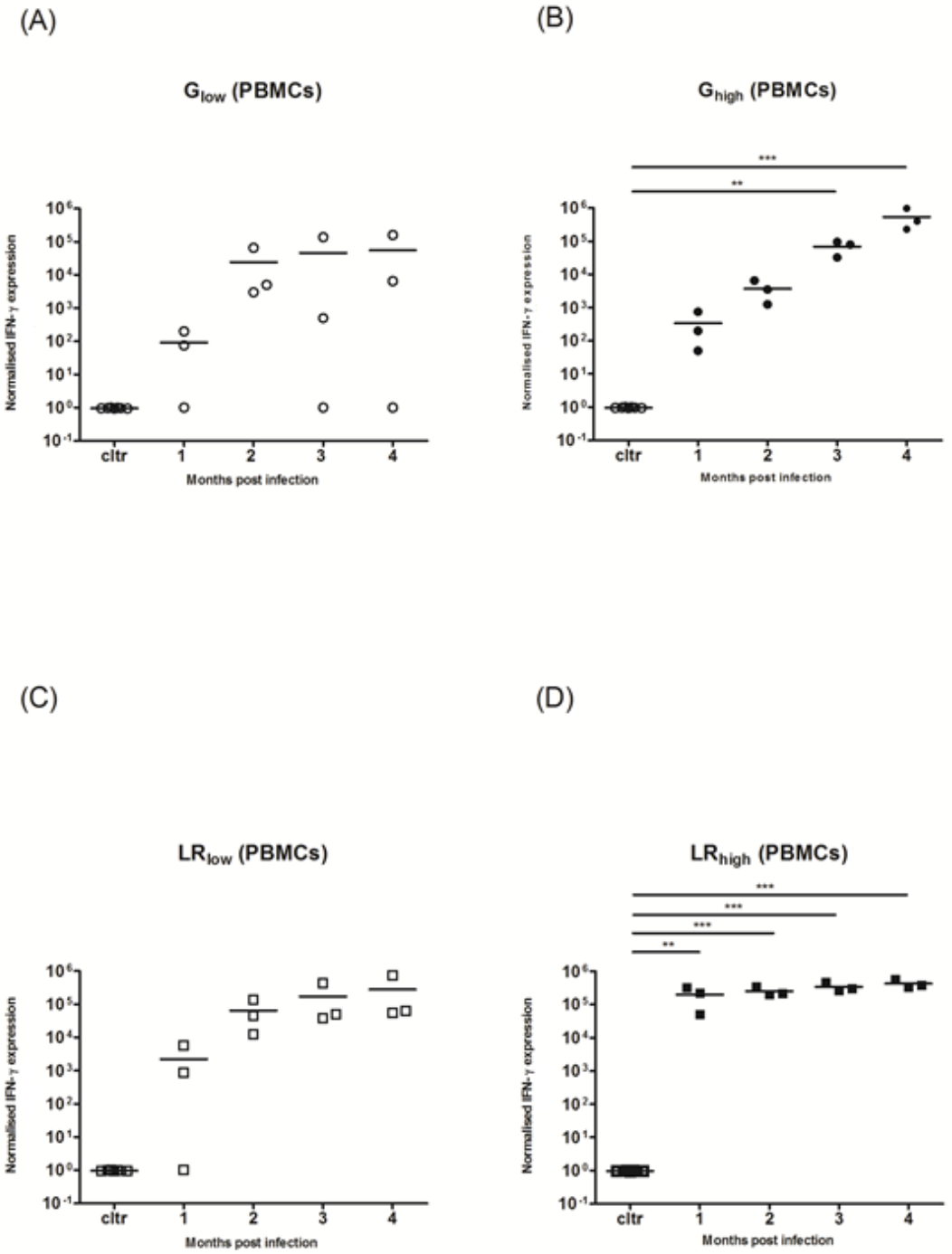


Figure 6.4 : Relative normalized IFN-γ expression in PBMCs after a single IPB-G or IPB-LR inoculation. PBMCs were isolated monthly from pigs orally infected with (A) a low or (B) a high dose of the IPB-G strain (G_{low} and G_{high}) or (C) a low or (D) a high dose of the IPB-LR strain (LR_{low} and LR_{high}). Cells were restimulated *in vitro* with TLA and IFN-γ mRNA was quantified with RT-PCR. The thick lines indicate the group mean. The significance level: * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$.

Parasite load in tissues

At 120 dpi, the parasite load was determined in heart and striated skeletal muscles by qPCR and a bioassay, while in brain by qPCR only. In animals infected with the IPB-LR strain, the highest parasite load was found in brain followed by heart (Table 6.3). Interestingly, the inoculation dose did not prominently affect the distribution or load in the tissues as it was the case between both IPB-G groups. Besides brain and heart, also intercostal muscles and the longissimus dorsi were consistently positive in qPCR, whereas heart of all 6 animals was also positive in the bioassay.

A different pattern was seen in pigs inoculated with the IPB-G strain (Table 6.3), where a clear effect of the inoculation dose on the parasite distribution and load in the tissues was found. When inoculated with the low dose, the parasite was present in more tissues and in higher amounts than when inoculated with the high dose. However, even when inoculated with the low dose, the longissimus dorsi and psoas major remained negative in all three animals in this group. Summarizing, animals inoculated with the high dose of IPB-G showed the lowest amount of *T. gondii* DNA in their tissues. Brain, gastrocnemius and the longissimus dorsi were negative, whereas for diaphragm and psoas major only one sample was positive in the bioassay and qPCR, respectively. These results strongly suggest a dose-dependent decreased burden of the IPB-G strain in the examined tissues following inoculation, pointing towards an immune-mediated reduction of the parasite load.

Table 6.3: Parasite load (number of bradyzoites per 1E+08 cells) by qPCR after inoculation with two *T. gondii* strains, in comparison with bioassay (number of positive/total tested). Groups: a single low or high dose of the IPB-G strain (G_{low} and $G_{high)}$ 120 dpi; a single low or high dose of the IPB-LR strain (LR_{low} and LR_{high}); a high dose of the IPB-G strain, followed 60 dpi by a high dose of the IPB-LR strain (G_{high}/LR_{high}); a high dose of the IPB-G strain ($G_{high1/2t}$) 60 dpi; a high dose of the LR strain, followed 60 dpi by a high dose of the IPB-G strain (LR_{high}/G_{high}). Tissues: Br (brain), Ha (heart), Di (diaphragm), Ic (intercostal m.), Mg (gastrocnemius m.), Ld (long. dorsi m.), Pm (psoas major m.). (nt = not tested).

	qPCR							Bioassay						
Group	**Br**	**Ha**	**Di**	**Ic**	**Mg**	**Ld**	**Mp**	**Br**	**Ha**	**Di**	**Ic**	**Mg**	**Ld**	**Mp**
G_{low}	2/3	2/3	2/3	3/3	2/3	0/3	0/3	nt	2/3	1/3	1/3	0/3	0/3	0/3
average	1.1E+05	8.5	20.6	44.6	3.0	0.0	0.0							
SD	1.1E+05	8.4	29.9	13.4	2.7	0.0	0.0							
G_{high}	0/3	1/3	0/3	2/3	0/3	0/3	1/3	nt	2/3	1/3	0/3	0/3	0/3	0/3
average	0.0	1.0	0.0	21.2	0.0	0.0	2.3							
SD	0.0	1.0	0.0	19.1	0.0	0.0	4.0							
LR_{low}	3/3	3/3	3/3	3/3	2/3	3/3	3/3	nt	3/3	3/3	1/3	0/3	0/3	0/3
average	378.8	169.6	8.5	23.4	52.4	28.7	189.1							
SD	94.9	228.7	12.2	17.5	45.4	29.9	244.9							
LR_{high}	3/3	3/3	2/3	3/3	3/3	3/3	2/3	nt	3/3	1/3	1/3	0/3	1/3	1/3
average	1340.0	592.2	7.8	29.4	35.0	72.6	67.8							
SD	322.8	281.1	11.6	30.5	8.6	28.5	68.0							
G_{high}/LR_{high}	3/3	3/3	3/3	2/3	1/3	0/3	2/3	nt	3/3	2/3	1/3	0/3	0/3	1/3
average	489.1	70.7	19.2	16.7	8.1	0.0	66.3							
SD	614.0	51.0	17.9	22.3	14.1	0.0	102.2							
$G_{high\ 1/2t}$	2/3	3/3	1/3	0/3	1/3	1/3	0/3	nt	2/3	1/3	0/3	0/3	0/3	0/3
average	1742.7	16.2	5.8	0.0	2.2	6.7	0.0							
SD	1570.9	26.1	10.1	0.0	3.9	11.6	0.0							
LR_{high}/G_{high}	3/3	3/3	1/3	0/3	1/3	0/3	1/3	nt	2/3	1/3	0/3	1/3	0/3	1/3
average	5950.5	105.3	1.67	0.0	0.8	0.0	1.6							
SD	8708.4	128.5	2.90	0.0	1.5	0.0	2.7							

6.4.2 Parasite tissue load and immune response in a subsequent infection model with two *T. gondii* strains

In order to assess if the low parasite load observed in some tissues after infection with the IPB-G strain was related to an immune response, in a second experiment animals were first infected with the high dose of one strain, followed 60 days later with the high dose of the other strain (Table 6.1). Since we hypothesized an effect of the inoculation with the IPB-G strain, an additional control group was included in the study inoculated with the high dose at 60 dpi and 60 days before euthanasia.

GRA-7 and TLA-specific antibody response

As in the first experiment, inoculation with the IPB-LR strain induced higher GRA7- and TLA-specific IgM production than with the IPB-G strain, independently from the order of inoculation (Figure 6.5A and 6.5B). This was most pronounced for the TLA-specific IgM response (Figure 6.5B). The presence of a clear TLA-specific IgM response after the second inoculation with the IPB-LR strain was remarkable and suggests differences in antigen expression between both strains (higher immunogenicity, different antigens or other reasons), leading to the induction of a primary immune response against various TLA antigens. Interestingly, the increase in TLA-specific IgM levels in the IPB-G infected animals upon initial inoculation at day 60 ($G_{high1/2t}$) was higher in comparison with G_{high} or G_{low} groups (Figure 6.3A), suggesting maturation of the immune system. Similar to our findings of the first experiment (Figure 6.2C, 6.2D; Figure 6.3C, 6.3D), the GRA7- and TLA-specific IgG antibodies appeared within two weeks following the primary inoculation with the IPB-G or IPB-LR strain (Figure 6.5C,D). A pronounced booster response against GRA7 occurred upon the heterologous challenge at 60 dpi in both re-infected groups, as evidenced by a much faster increase in IgG levels in contrast to animals from the $G_{high1/2t}$ group (Figure 6.5C). The TLA IgG was not boosted following the heterologous infections in both challenged groups (Figure 6.5D). However, these

distinct IgG responses were more pronounced by the challenge with the IPB-LR strain than with the IPB-G strain.

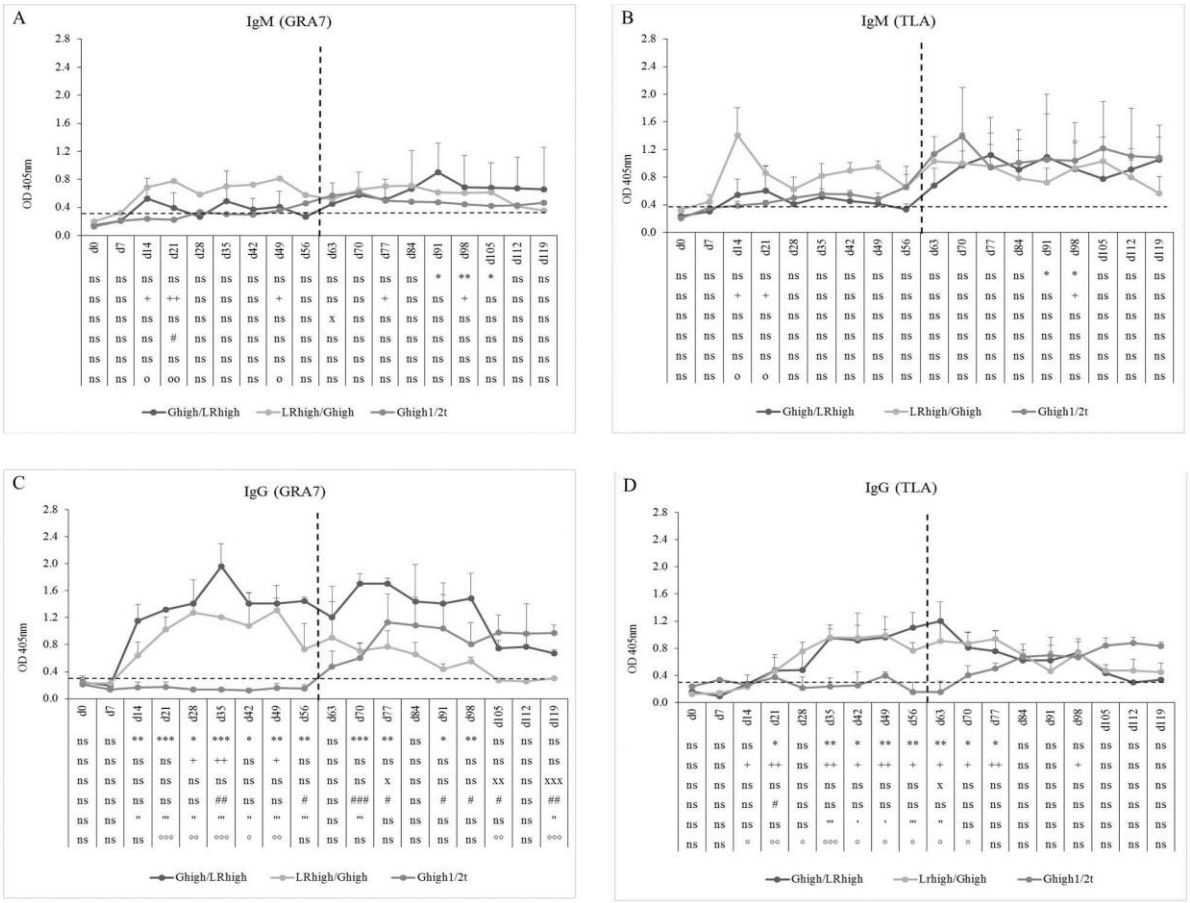


Figure 6.5 : GRA7- and TLA-specific IgM and IgG responses after inoculation with IPB-G and IPB-LR *T. gondii* strains. IgM (A+B) and IgG (C+D) antibody response towards *T. gondii* GRA7 (A+C) and TLA (B+D) in animals after a consecutive infection with a high dose of IPB-G and IPB-LR strains. Groups: G_{high}/LR_{high}, LR_{high}/G_{high}. Piglets infected after 60 days with a high dose IPB-G served as a control ($G_{high1/2t.}$)The horizontal dashed line indicates the cut-off value based on the mean of the negative animals, and the vertical one the time point of reinfection. The results represent a mean of the infected group ± SD; * G_{high}/LR_{high} versus controls, + LR_{high}/G_{high} versus controls, x $G_{high1/2t}$ versus controls, # G_{high}/LR_{high} versus $LR_{high}/G_{high,}$ ‘ G_{high}/LR_{high} versus $G_{high1/2t}$, ° LR_{high}/G_{high} versus $G_{high1/2t}$: P < 0.05, ** or ++ or xx or ## or ‘‘ or °°: P < 0.01; *** or +++ or xxx or ### or ‘‘‘ or °°°: P < 0.001, ns: not significant.

TLA-specific IFN-γ mRNA responses in PMBCs and spleen

IFN-γ responses after the initial inoculation with both *T. gondii* strains (Figure 6.6) were comparable with those in the first experiment (Figure 6.4). In two of the three animals receiving the IPB-G strain as a first inoculation (G_{high}/LR_{high}), IFN-γ mRNA expression could not be detected 1 mpi in the PBMCs recall assay with TLA. However, from 2 mpi all three animals showed a significantly ($p < 0.05$) to highly significant ($p < 0.001$) increased IFN-γ mRNA level (Figure 6.6A), similarly to the expression seen in animals receiving the IPB-LR strain as a first inoculum (LR_{high}/G_{high}) (Figure 6.6B), even though the latter showed a more homogenous response from 1 mpi onwards ($p < 0.01$). In both groups the IFN-γ mRNA expressions remained significantly elevated ($p < 0.05$ to $p < 0.001$) during the experiment and no additional increase was seen after inoculation with the heterologous strains. Slightly lower yet significant ($p < 0.05$) IFN-γ production was observed in group $G_{high1/2t}$, inoculated for the first time at 60 dpi (Figure 6.6C).
While no detectable cytokine production was found at 120 dpi in the spleen of animals from both infection groups in the first experiment (data not shown), in the heterologous infection model significant IFN-γ transcript levels were detected 60 days after the second infection for the G_{high}/LR_{high} ($p < 0.05$), $G_{high1/2t}$ ($p < 0.01$) and LR_{high}/G_{high} ($p < 0.001$) (Figure 6.7A and 6.7B).

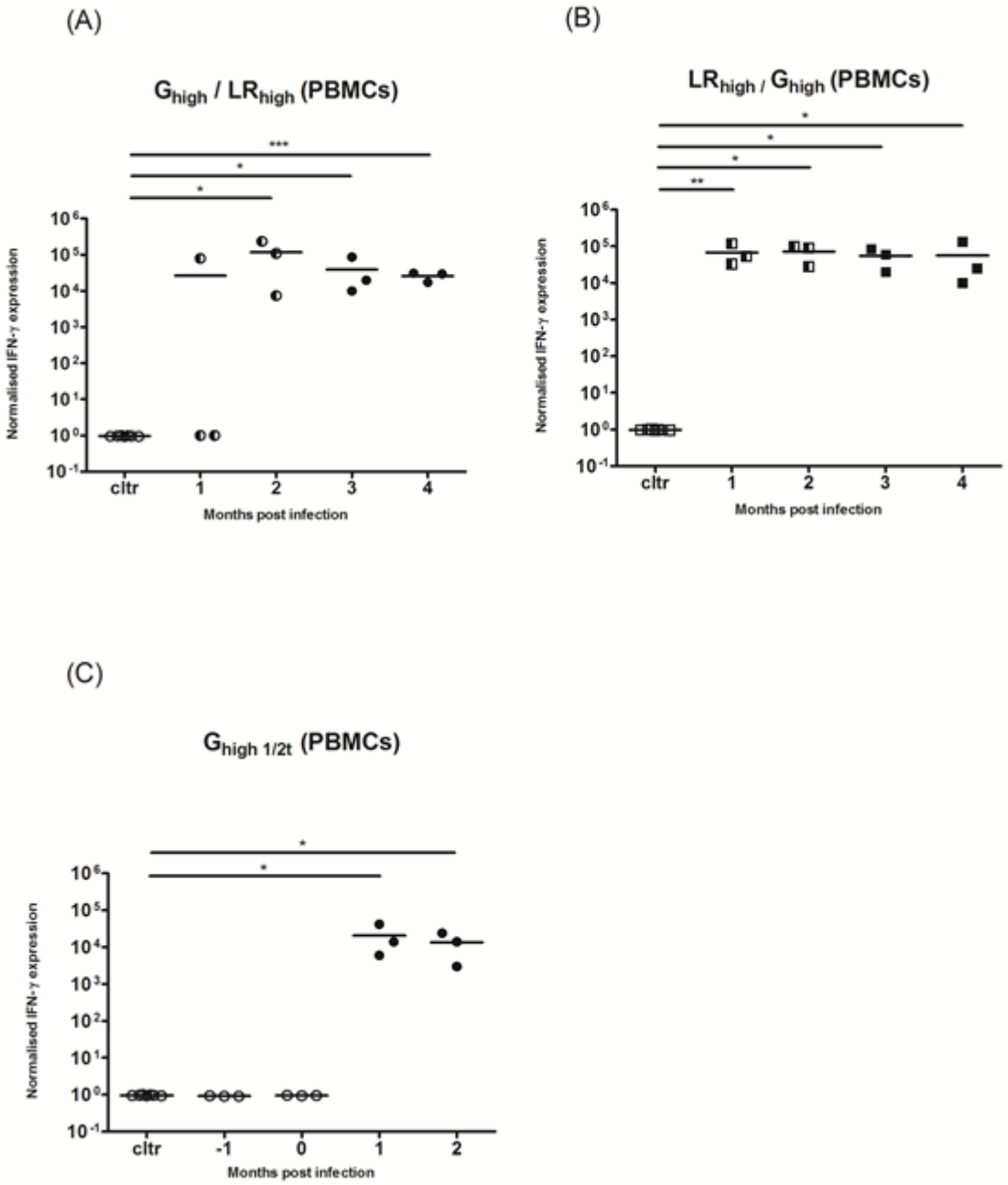


Figure 6.6 : Relative normalized IFN-γ expression in PBMCs after a consecutive IPB-G and IPB-LR inoculation. PBMCs were isolated monthly from pigs orally infected with (A) a high dose of the IPB-G strain followed 60 days later by a high dose of the IPB-LR strain (G_{high}/LR_{high}) or (B) reversed infection model (LR_{high}/G_{high}). Piglets infected with a high dose of the IPB-G strain at day 60 served as a control (C) ($G_{high1/2t}$). Cells were restimulated *in vitro* with TLA and IFN-γ mRNA was quantified with RT-PCR. The thick lines indicate the group mean. The significance level: * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$.

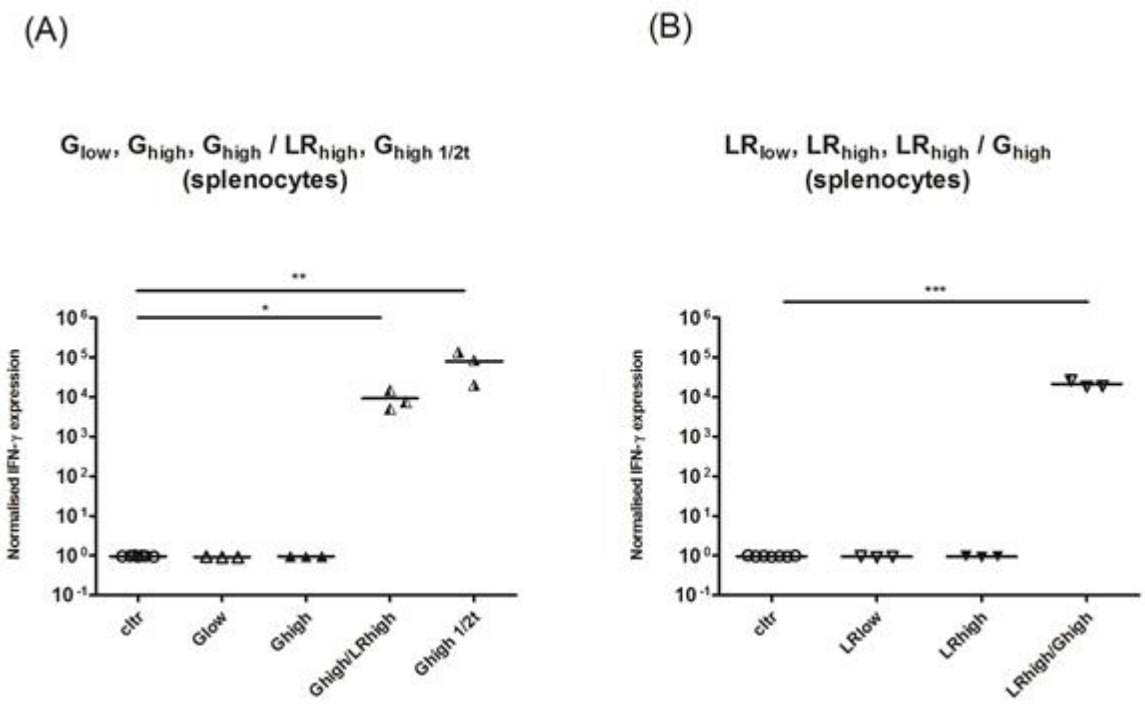


Figure 6.7 : Relative normalized IFN-γ expression in splenocytes after a single or consecutive IPB-G and IPB-LR inoculation. Splenocytes were isolated from pigs orally infected with (A) a low dose of the IPB-G strain (G_{low}), a high dose of the IPB-G strain (G_{high}), a high dose of the IPB-G strain followed 60 days later by a high dose of the IPB-LR strain (G_{high}/LR_{high}), a high dose of IPB-G at day 60 ($G_{high1/2t}$), or (B) a low dose of the IPB-LR strain (LR_{low}), a high dose of the IPB-LR strain (LR_{high}) or a high dose of the IPB-LR strain followed 60 days later by a high dose of the IPB-G strain reversed infection model (LR_{high}/G_{high}). Cells were isolated at 2 mpi (group: $G_{high1/2t}$) or 4 mpi (groups: G_{low}, LR_{low}, G_{high}, LR_{high}, G_{high}/LR_{high}, LR_{high}/G_{high}) and restimulated *in vitro* with TLA. IFN-γ mRNA was quantified with RT-PCR. The thick lines indicate the group mean. The significance level: * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$.

Parasite load in tissues

To assess the parasite load in tissues animals were euthanized 60 days after a first ($G_{high1/2t}$ group) or second infection (G_{high}/LR_{high} and LR_{high}/G_{high} groups). Interestingly, animals receiving only the high dose of the IPB-G strain ($G_{high1/2t}$) showed a parasite distribution and load (14 tissue samples negative in a bioassay) in between those

observed at 120 dpi with the IPB-G low dose (10 tissue samples negative in a bioassay) and the high dose (17 samples negative) in the first experiment (Table 6.3). This could suggest that the reduction in parasite load induced by the IPB-G strain was already appearing at 60 dpi upon inoculation with the high dose. The tissue distribution at 120 dpi in animals, which first received the IPB-LR high dose and 60 days later the IPB-G high dose, could also be explained by this phenomenon. At 120 dpi the animals showed a different parasite load and tissue distribution (12 samples negative) than in the first experiment (1 and 2 samples negative after infection with the low and the high dose, respectively). Animals receiving first the IPB-G high dose and 60 days later the IPB-LR high dose, showed a wider parasite tissue distribution and a higher parasite tissue load (7 samples negative), more comparable to animals receiving only the IPB-LR strain.

6.4.2 The involvement of $CD4^+$ and $CD8^+$ T cells in the strain-dependent IFN-γ production

Results of the above experiment supported our hypothesis that the IPB-G strain reduced the parasite burden. Since many studies suggested an important role for IFN-γ responses in controlling *T. gondii* infections, in a next experiment we compared the kinetics of IFN-γ producing T cell subsets in blood, following infection with the high dose of both strains in an *in vitro* TLA recall assay. Depending on the *T. gondii* strain, differences in the kinetics of circulating IFN-γ producing T cell subpopulations were observed (Figure 6.8). The $CD3^+CD4^+CD8\alpha^-$ and $CD3^+CD4^+CD8\alpha^{dim}$ represent porcine T-helper cells, while $CD3^+CD4^-CD8\alpha^{bright}$ cells are cytotoxic T cells (Gerner et al., 2015). Animals inoculated with the IPB-LR strain showed at 21 dpi a significant increase in the $CD3^+IFN\text{-}\gamma^+$ T cell subsets ($CD4^+CD8\alpha^-$, $CD4^+CD8\alpha^{dim}$ and $CD4^-CD8\alpha^{bright}$ T cells), with the $CD4^+CD8\alpha^-$ T-helper cells (up to 22.2 ± 8.3 % of $CD3^+$IFN-γ producing cells) being most prevalent (Figure 6.8A). This latter population remained stable, whereas the $CD4^-CD8\alpha^{bright}$ population gradually increased from 9.3 ± 0.87 % of the IFN-γ^+ producing

cells at 28 dpi to > 40 % (41.1 ± 17.4 %) at 98 dpi (Figure 6.8C). In contrast, the percentage $CD4^+CD8\alpha^{dim}IFN\text{-}\gamma^+$ cells gradually decreased from 16.4 ± 1.4 % to < 2.1 ± 0.8 % at the end of the experiment (Figure 6.8B). In animals inoculated with the IPB-G strain, a similar increase in the percentage of $CD4^+CD8\alpha^{dim}IFN\text{-}\gamma^+$ cells at 21 dpi to 10.4 ± 0.4 %, and a subsequent gradual decrease to 2.2 ± 0.5 % was seen (Figure 6.8B). The $IFN\text{-}\gamma^+$ within $CD4^+CD8\alpha^-$ and $CD4^-CD8\alpha^{bright}$ T cells gradually increased, reaching significantly higher levels (22.6 ± 11.9 % and 18.7 ± 9.5 %, respectively) at 84 dpi as compared to 0 dpi (Figure 6.8A, 6.8C). Intriguingly, the increase of the latter T cell population was clearly less pronounced in the G_{high} ($p < 0.05$) than in the LR_{high} group ($p < 0.01$).

The difference in circulating $CD4^-CD8\alpha^{bright}$ T cell populations between both high dose groups seems to be reflected on the long term in the significantly higher ($p < 0.05$) percentage of $CD4^-CD8\alpha^{bright}$ $IFN\text{-}\gamma^+$ T cells in the popliteal lymph nodes (LN) in the LR_{high} group than in the G_{high} group at 98 dpi (Figure 6.9C). Additionally, a similar difference in the percentage of the same population between both groups can be detected in the mesenteric LN. However, in mediastinal LN, which drain heart and diaphragm, a higher percentage of $CD4^-CD8\alpha^{bright}$ $IFN\text{-}\gamma^+$ T cells was found in the G_{high} group, although not significantly different from the LR_{high} group. The distribution of the other T cell subpopulations in different lymphoid tissues shows a comparable pattern: a higher percentage of the $CD4^+CD8\alpha^-IFN\text{-}\gamma^+$ T was found in the mesenteric ($p < 0.05$) and popliteal ($p > 0.05$) LN of the LR_{high} group than in the G_{high} group (Figure 6.9A). Likewise for the $CD4^-CD8\alpha^{bright}$ T cells, a reverse situation was noticed in the mediastinal LN. Regarding the $CD4^+CD8\alpha^{dim}IFN\text{-}\gamma^+$ cells, relatively low percentages were detected in both infected groups. The highest counts were found in the mesenteric and popliteal LN in the LR_{high} group, followed by the mesenteric LN in the G_{high} group, whereas they were nearly absent in the other sampled LN (Figure 6.9B).

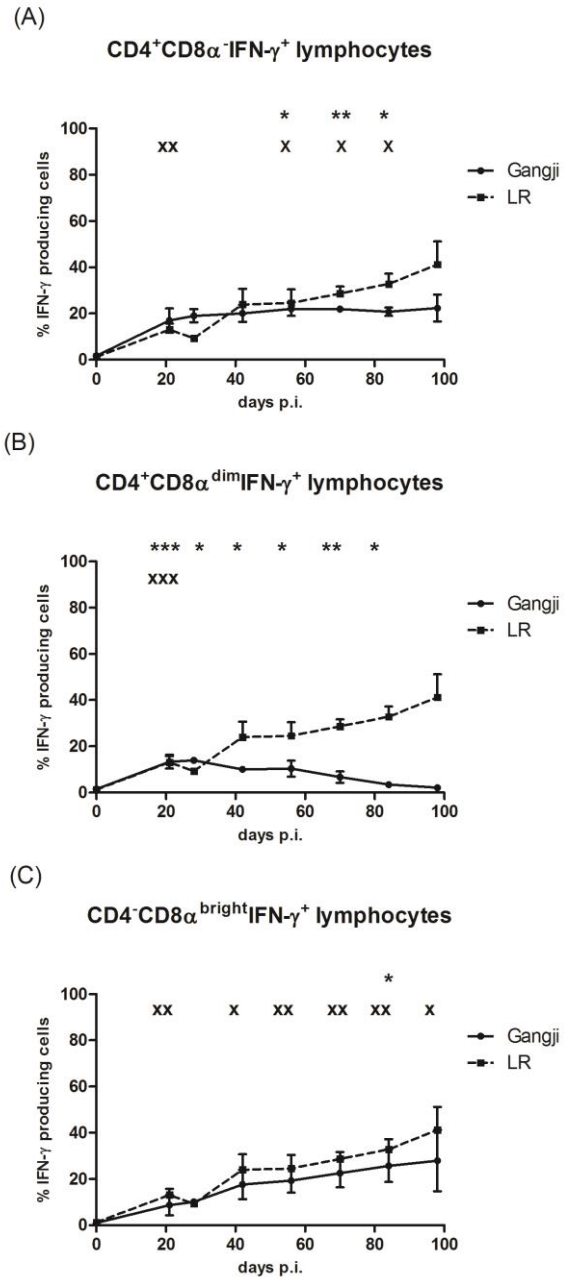
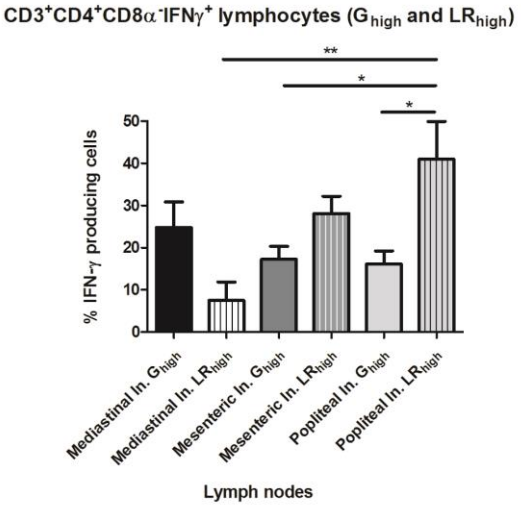
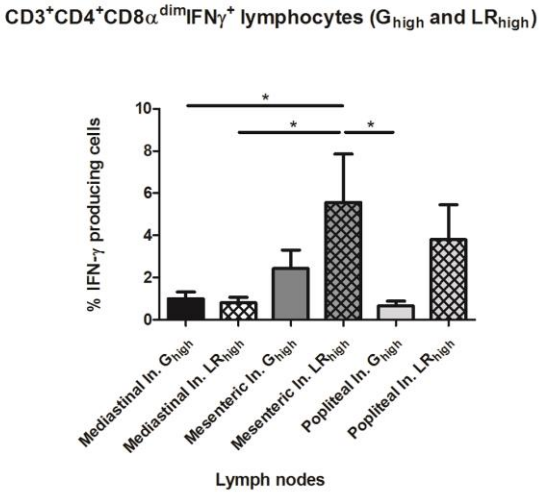


Figure 6.8 : The percentage of IFN-γ$^+$ T lymphocyte subsets in PBMCs after a single IPB-G or IPB-LR inoculation. IFN-γ$^+$ T lymphocyte subsets in PBMC's of pigs after oral infection with a high dose of the IPB-G (G_{high}) or the IPB-LR strain (LR_{high}). Cells were restimulated *in vitro* with TLA and demonstrated by flow cytometry following triple staining for IFN-γ, CD3, CD4 and CD8 (A). IFN-γ$^+$ cell populations were identified as (A) $CD3^+CD4^+CD8\alpha^-$IFN-γ$^+$, (B) $CD3^+CD4+CD8\alpha^{dim}$ IFN-γ$^+$, (C) $CD3^+CD4^-CD8^{bright}$IFN-γ$^+$ lymphocytes. The results represent mean percentages ± SD for each group; * (IPB-G) or x (IPB-LR) : P < 0.05, ** or xx: P < 0.01; *** or xxx: P < 0.001 in comparison with day 0.

(A)


CD3+CD4+CD8α-IFNγ+ lymphocytes (Ghigh and LRhigh)
**
*
*
% IFN-γ producing cells
50
40
30
20
10
0
Mediastinal ln. Ghigh
Mediastinal ln. LRhigh
Mesenteric ln. Ghigh
Mesenteric ln. LRhigh
Popliteal ln. Ghigh
Popliteal ln. LRhigh
Lymph nodes

(B)


CD3+CD4+CD8αdimIFNγ+ lymphocytes (Ghigh and LRhigh)
*
*
*
% IFN-γ producing cells
10
8
6
4
2
0
Mediastinal ln. Ghigh
Mediastinal ln. LRhigh
Mesenteric ln. Ghigh
Mesenteric ln. LRhigh
Popliteal ln. Ghigh
Popliteal ln. LRhigh
Lymph nodes

(C)

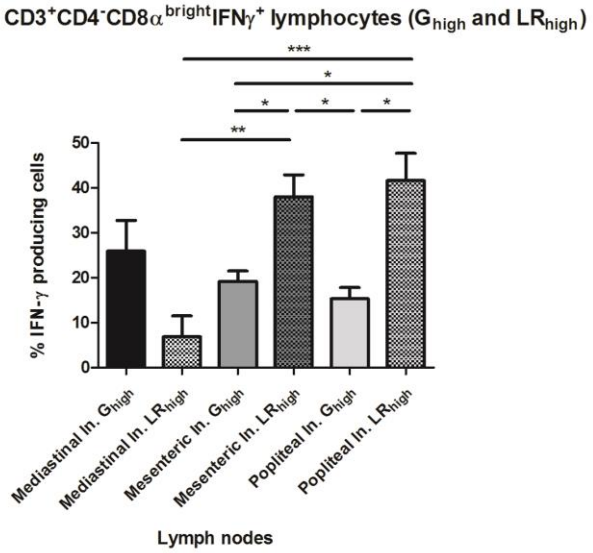

CD3+CD4-CD8αbrightIFNγ+ lymphocytes (Ghigh and LRhigh)
***
*
*
*
*
**
% IFN-γ producing cells
50
40
30
20
10
0
Mediastinal ln. Ghigh
Mediastinal ln. LRhigh
Mesenteric ln. Ghigh
Mesenteric ln. LRhigh
Popliteal ln. Ghigh
Popliteal ln. LRhigh
Lymph nodes

Figure 6.9 : The percentage of IFN-γ^+ T lymphocyte subsets from lymph nodes after a single IPB-G or IPB-LR inoculation. IFN-γ^+ T lymphocyte subsets in leucocytes from peripheral lymph nodes of pigs 98 dpi with a high dose of the IPB-G (G_{high}) or the IPB-LR strain (LR_{high}). Cells were restimulated *in vitro* with TLA and demonstrated by flow cytometry following triple staining for IFN-γ, CD3, CD4 and CD8 (A). IFN-γ^+ cell populations were identified as (A) $CD3^+CD4^+CD8\alpha^-$IFN-γ^+, (B) $CD3^+CD4+CD8\alpha^{dim}$ IFN-γ^+ and (C) $CD3^+CD4^-CD8^{bright}$IFN-γ^+ lymphocytes. The results represent mean percentages ± SD for each group; * (IPB-G) or x (IPB-LR) : $P < 0.05$, ** or xx: $P < 0.01$; *** or xxx: $P < 0.001$ in comparison with day 0.

6.5 Discussion

In the performed experiments, we compared the single or subsequent infection in pigs inoculated with either a high or a low dose of the IPB-G and the IPB-LR strains for the tissue specific parasite load and the accompanying immune response. The IPB-G strain has a mixed type I/II genotype, while the IPB-LR strain has a classic type II genotype (Jongert et al., 2008; Dubey et al., 2012b). The antibody response against GRA7 and TLA, which are frequently used in serological assays in different species, was monitored until 120 dpi to confirm the successful inoculation and persistence of the infection (Figure 6.2 and 6.3). Overall, for both IgM and IgG, independent from the infection dose, the GRA7-specific antibodies were detected very soon after the initial infection, starting from 10 dpi (Figure 6.2). Similar to previous results (Verhelst et al., 2015), we detected a late TLA-specific IgG response from 28 to 35 dpi onwards (Figure 6.3). However, results of the present study demonstrated that strain and dose are important factors to consider, since primary GRA7- and TLA-specific antibody responses could be detected earlier during infection and were higher upon inoculation with a high dose of the IPB-LR strain as compared to the IPB-G strain. A low dose on the other hand resulted in a later and less prominent seroconversion. Interestingly, whereas primary antibody responses were comparable in the heterologous challenge model, a clear IgM response was seen after the challenge with the heterologous strains, indicating exposure to other antigens, presumably due to the genetic diversity of both strains

(Figure 6.5). Burrells et al. (2015) described a significant TLA-specific IgG increase after challenge of pigs with the heterologous strain M4 upon inoculation with the S48 strain. Strikingly, the challenge was performed with oocysts, after an initial inoculation with tachyzoites, stressing the expression of related or identical variability antigens in correlation to the parasite stage and the strain.

Together with a robust humoral response following *T. gondii* infection, a strong innate and cellular immune reaction is well described in mouse and human models, involving several populations of immune cells as well as different activation pathways (Miller et al., 2009; Andrade et al., 2013; Gazzinelli et al., 2014; Sturge and Yarovinsky, 2014). To date, it is well known that innate immune cells (macrophages, dendritic cells (DC's) and neutrophils) are involved in the acute stage of the infection by triggering the myeloid-differentiation primary response protein 88 (MyD88) signalling pathway after uptake and intracellular recognition of the parasite by CC-receptor 5 (CCR5) or Toll-like receptor (TLR) 11 and 12 in mice; TLR7, 8 and 9 in human and TLR7 and 9 in other mammals like pigs (Miller et al., 2009; Andrade et al., 2013; Koblansky et al., 2013; Gazzinelli et al., 2014). In particular interferon regulatory factor 8 (IRF8)-dendritic cells, activated by the uptake of the parasite's protein profilin, are crucial for the induction of IL-12 secretion in mice. Human and porcine DCs and monocytes are activated by the recognition of the parasite's ssRNA and DNA via TLR7 and TLR9, respectively, prior to their pro-inflammatory cytokine response (Uneishi et al., 2012; Andrade et al., 2013).

Consequently and irrespective of the activated TLRs, the DC-driven IL-12 production leads to the activation of T-helper 1 cells and Natural Killer (NK) cells (Sturge and Yarovinsky, 2014). The latter massively produce IFN-γ, which not only continuously activates macrophages via a positive feedback mechanism, but also elicits the expression of the GTPases. The GTPases family includes four subfamilies: the very large inducible GTPases (VLIG), the Mx proteins, the immunity-related GTPases (IRGs) such as p47 or p65, and the guanylate-binding proteins (GBPs). The p47 IRG offers a robust protection against intracellular pathogens, being recruited to the parasite attachment

site at the host cell (MacMicking, 2004; Taylor et al., 2004; Liesenfeld et al., 2011). Subsequently, a lethal damage to the parasitophorus vacuole (PV) is induced, leading to the rupture of the infected cell and release of the parasite into the cytosol (Gazzinelli et al., 2014). The infected cell undergoes necrosis, simultaneously with an enhanced local immune response.

Thus, the continuous production of IFN-γ seems to be necessary in maintaining a delicate balance between the host immune system and the parasite's evasion strategies. Additionally, this cytokine plays a pivotal role in controlling both the acute and chronic phase of infection, as it facilitates stage conversion from the tachyzoite to the bradyzoite in acute toxoplasmosis and suppresses the opposite conversion during chronic infection (Denkers, 1999). Likewise, we detected a significant increased IFN-γ production by PBMC's after inoculation with two different *T. gondii* strains (Figure 6.4), which corroborates our previous results when inoculating pigs with the IPB-G strain (Verhelst et al., 2015). Here, we demonstrated a time- and dose-dependent increase in IFN-γ mRNA expression upon infection with the IPB-G strain. Several studies focused on experimental infection in pigs reported a time-dependent increase of IFN-γ levels in serum, supernatant from cultured PBMCs and IFN-γ mRNA expression in PBMCs and intestinal lymphoid tissues (Solano Aguilar et al., 2001; Dawson et al., 2004; Dawson et al., 2005, Verhelst et al., 2015).

On the contrary, the inoculation with the low dose of the IPB-LR strain was almost as potent in inducing a relatively fast and strong IFN-γ production by PBMCs as the high dose of the same strain, which resulted in high IFN-γ mRNA levels at already 2 mpi, that were maintained until 120 dpi. Interestingly, the IFN-γ mRNA production in the LR_{high} dose group did not show any increase over time, implying reaching the maximum capacity from 1 mpi onwards. In line with our findings, IL-12 (IL-12p35 and IL-12p40) mRNA expression was not detected in PBMCs in the acute phase of the infection (7 and 14 dpi) in an earlier study in pigs (Dawson et al., 2005).

Importantly, we conducted the studies in a homogenous pig population in order to exclude host diversity; however, as the strains are maintained by serial passage in mice, their virulence might be altered compared to the original isolate. Therefore, it is tempting to speculate that the high IFN-γ production together with the lower parasite counts in the porcine tissues originate from a coevolution towards host tolerance and reduced virulence, as suggested earlier by others (Gazzinelli et al., 2014). Furthermore, looking at the total IFN-γ expression following reinoculation with the heterologous *T. gondii* strain in the second experiment, no obvious difference between the groups could be observed (Figure 6.6). In the G_{high}/LR_{high} group we detected an initial increase, which was followed by a steady decrease after the challenge. The IFN-γ production profile in the reversed infection model (LR_{high}/G_{high}) supports our previous findings, showing a constant IFN-γ detection over time. Interestingly, in some animals basal or low level of cytokine mRNA were detected at 1mpi, followed by a substantial increase at the later time points, similar to the single infection experiment (Figure 6.4A and 6.6A). However, the final IFN-γ concentration at the end of the experiment upon a heterologous challenge was ten times lower than after a single high dose inoculation. We could speculate that the primary infection with a mixed genotype I/II strain, characterized by a high acute virulence and long-term STAT3 and STAT6 activation, partially modulates the immune response upon the challenge with genotype II strain. As the result, the initial impairment of the Th1 response after the challenge leads to a lower than in a single infection model IFN-γ production, and elimination of a certain fraction of the parasites. Consequently, a reduced amount of the tachyzoites disseminate to convert into bradyzoites. The latter has been shown by a lower parasite load 60 dpi challenge than in IPB-LR experiment (Table 6.3 and Figure 6.6).

In regard to the involvement of immune cells in controlling the parasite's dissemination to the tissues and the chronic phase of *T. gondii* infection, different populations seem to play a role. As described earlier and analogous to the acute infection stage, the production of IFN-γ is gradually taken over from the innate immune cells by T

lymphocytes (Guan et al., 2007). Experimental infections in mice (Jongert et al., 2010; Suzuki et al., 2012) demonstrated the importance of $CD4^+$ and $CD8^+$ IFN-γ producing T cells in maintaining a chronic *T. gondii* infection, but the exact contribution of each subset remains unknown. Miller et al. (2006) describes higher production of IFN-γ by murine $CD4^+$ cells upon *in vitro* stimulation by infected macrophages or by TLA, but admits that the higher protective potential against dissemination of the parasite by $CD8^+$ or $CD4^+$ lymphocytes is not simply expressed by the amount of this cytokine. Indeed, it seems that IL-4 and IL-10 cytokines, produced by $CD4^+$ lymphocytes in addition to IFN-γ, might down regulate this protective capacity against the parasite. In line with that, due to their IFN-γ-independent cytolytic activity, the role of primed $CD8^+$ T cells in the host's immunity during chronic toxoplasmosis has been widely acknowledged (Wang et al., 2005; Suzuki et al., 2012; Sa et al., 2013). In pigs, only a few experiments identified $CD8^+$ and $CD4^+CD8^+$ cells in the acute phase of the infection as the major source of the IFN-γ production (Solano Aguilar et al., 2001; Dawson et al., 2005). The additive or synergistic effect of $CD4^+$ T cells on the activity of the $CD8^+$ T cell population should not, however, remain neglected. In our study, regardless of the strain, the $CD4^-CD8\alpha^{bright}$ T cell subset contained the most IFN-γ positive cells, followed by the $CD4^+CD8\alpha^-$ subset, whereas the $CD4^+CD8\alpha^{dim}$ T cell subset showed very few IFN-γ positive cells (Figure 6.8). Additionally, the $CD4^-CD8\alpha^{bright}$ population showed a temporal increase in IFN-γ production in animals infected with IPB-LR, while the percentage of this subset was rather declining from 4 wpi onwards, when infected with the IPB-G. The IFN-γ production resulting from the induced toxoplasmosis in pigs and the involvement of the different lymphocyte populations are in line with other studies, where the *in vitro* cytokine profile was investigated until 14 (Dawson et al., 2005), 40 (Solano Aguilar et al., 2001) or 56 dpi (Verhelst et al., 2011). However, opposite to the pig model, in murine experiments only two T lymphocyte subsets were differentiated ($CD4^+$ and $CD8^+$). Furthermore, the extent of the cellular response was positively

correlated with the infection dose and the time-interval from the inoculation, and was higher when induced by the strain with a greater tissue persistence.

When considering the parasite load in the tissues and the viability of the cysts in the bioassay, a clear correlation was found between the amount of detected DNA and the dose of the used strain (Table 6.3). In general, we observed a decline in the concentration of the parasite's DNA in animals when inoculated with a high dose of the IPB-G, but not the IPB-LR strain. This dose- and time-dependent decline is prominently present in different tissues in the G_{high} group in comparison with G_{low} and $G_{high1/2t}$ groups, indicating that the effect of the high dose is particularly visible after a longer infection time. These results are in line with the findings of Verhelst et al. (2011), where neither parasite DNA nor viable parasites were detected in certain muscle tissues six months after initial infection with the IPB-G strain. In the same study, brain and heart of all animals remained infectious as determined by a bioassay and qPCR. However, these findings are opposite to results obtained in rats and cats, showing that inoculation with increasing amounts of tachyzoites or bradyzoites resulted in a decreased survival rate or in a higher number of tissue cysts, respectively (De Champs et al., 1998; Cornelissen et al., 2014). In addition and similar to our results, others reported a reduction in parasite burden in strain-vaccinated and challenged pigs (Kringel et al., 2004; Garcia et al., 2005; Jongert et al., 2008; Burrells et al., 2015). In these experiments vaccination with oligonucleotides, antigens or infection with attenuated strains can enhance Th1 responses to elicit sufficient protection during the acute phase of the infection, resulting in a lower parasite burden in comparison with the infected control animals. Referring to that, the strains used in our study differ greatly in terms of genetic background and associated virulence, both in mice and in pigs. Therefore, we have grounded scientific reasons to believe that the observed differences in the parasite load upon infection with both strains, especially in the heterologous co-infection model, are not coincidental.

For most tissue samples this dose- and strain-dependent reduction in the number of the tissue cysts in qPCR is consistent with the bioassay in mice. It is noteworthy that a few qPCR-positive samples were negative in the bioassay, indicating that parasite DNA, but not viable parasites were present. Consequently, these results show a substantially higher sensitivity of the qPCR method used here above the bioassay, since it has been optimized and successfully applied for the detection of the parasite in various human or animal tissues, with a detection limit of 2-4 tissue cysts in 100 g of sample (De Craeye et al., 2011). Conversely, both techniques gave positive results on all samples derived from animals upon single inoculation with the IPB-LR strain. Therefore, we can assume that the reduced parasite load occurred due to the earlier infection with the IPB-G strain in the group G_{high}/LR_{high}. This phenomenon does not seem to be limited to type II strains (Velmurugan et al., 2009; Suzuki et al., 2012), but is also common in type I strains (Burrells et al., 2015).

Summarizing, the groups infected with the IPB-LR strain can serve as a classical model of *T. gondii* persistence in its intermediate host. The prominent production of parasite-specific antibodies, consistent amounts of IFN-γ and activation of cytotoxic T lymphocytes on the one hand and well-distributed DNA concentration together with isolation of the viable parasite on the other hand, clearly prove an established balance between the host immunity and the pathogen's activity. The parasite's persistence appears to be beneficial for the two, under conditions that the immunocompetent host can resist the immunomodulation by *T. gondii*. On the contrary, as the partial or total removal of the tissue cysts was observed in the IPB-G infected animals together with the increasing IFN-γ production profile on both the mRNA and protein level, we propose that the IPB-G strain induces a robust immune reaction in the host in the early phase of the infection. This IFN-γ-mediated response in pigs can lead to the resistance of the host to parasite invasion by elimination of the tissue cysts during the chronic infection. Further experiments to unravel the nature of this resistance are warranted as

it could serve an important role in vaccination strategies and in the risk assessment for food safety and human health.

6.6 Acknowledgements

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Chapter 7 Immune responses against antigen fractions in acute and chronic *Toxoplasma gondii* infection in pigs

Adapted from:

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7.1 Abstract

Toxoplasma gondii is an ubiquitous intracellular protozoan parasite, which infects one third of the global population. The consumption of raw or undercooked pork is considered as one of the main sources of disease in humans.

The objective of this study was to investigate the *in vitro* activation of T-lymphocytes by fractionated *T. gondii* antigens, expressed as Interferon-γ (IFN-γ) production. The infection experiments were performed in seronegative pigs, which were orally inoculated with 6000 tissue cysts of IPB-Gangji (IPB-G) strain or IPB-LR strain. The PBMC's were isolated from the infected and control animals on multiple time points during the acute and chronic phases of infection. Subsequently, PBMC's were *in vitro* restimulated with native *T. gondii* antigens, fractionated prior to use by the continuous elution-electrophoresis and subdivided into 6 pools. The produced IFN-γ was quantified by ELISA in the cell culture supernatant of animals infected with IPB-G or IPB-LR, and showed reasonable differences between both infection groups. In general, there was a detectable IFN-γ concentration in the supernatant of the cultured PBMC's and isolated lymphocytes from different tissues, produced by the animals from both groups upon stimulation with the TLA-pools. However, the animals infected with the IPB-LR strain produced in general much higher quantities of the cytokine than pigs inoculated with the IPB-G, except for the duodenal lymph nodes, and that after stimulation with each TLA-pool. The muscles, heart and brain tissues collected from the animals at euthanasia were tested with a qPCR to investigate the parasite load with regard to the strains, the tissues and the time points in the infection course.

This study demonstrates that the amount of the produced IFN-γ upon *in vitro* stimulation by TLA pools varies considerably between the groups infected with different strains of *T. gondii* and between the origin of the cells used in the antigen recall assay. This divergence is possibly correlated with the activation state of the PBMC's *in vivo*, and, thus, the distribution and the persistence of the parasite within the host during acute and chronic toxoplasmosis.

7.2 Introduction

Toxoplasma gondii (*T. gondii*) is an obligatory intracellular parasite with a worldwide distribution among mammals and birds. According to global estimations, 30 to 50% of the world human population is infected by *T. gondii* and the main source of infection is foodborne (Tenter et al., 2000; Robert-Gangneux and Dardé, 2012, Flegr et al., 2014). The infection in humans is associated with severe to fatal clinical symptoms in newborns or immunocompromised patients, and the global infection burden in infected individuals is estimated to one million disability-adjusted life years (DALY's) per year (Torgerson and Mastroiacovo, 2013). While certain animal species, such as sheep and goats, may develop severe clinical signs upon naturally infection, asymptomatic carriers such as pigs also contribute to the transmission from livestock towards humans.

Nevertheless, even though a variety of diagnostic techniques exist as explained in the literature review (Chapter 2), these tests still cannot guarantee a reliable and fully efficient parasite detection (Opsteegh et al., 2016a; Algaba et al., 2017). The latter represents a considerable challenge for the food safety. Therefore, efforts should rather be directed towards prevention and elimination of the infection in meat-producing animals. Among the different preventive options, vaccination appears to be one of the most appealing methods.

The majority of vaccination studies have been performed in mouse models, using a wide range of formulations and different combinations of recombinant or native parasitic antigens or DNA-vaccines (Vercammen et al., 2000; Letscher-Bru et al., 2003; Jongert et al., 2007; Dubey et al., 2012a; Wu et al., 2012; Cao et al., 2015; Wagner et al., 2015; Yin et al., 2015). It appeared that the multivalent vaccines (Vercammen et al., 2000; Li, et al., 2011; Wu et al., 2012; Cao et al., 2015; Yin et al., 2015) seemed to provide better protection against the challenge than monovalent (Letscher-Bru et al., 2003; Jongert et al., 2007). Consequently, focus moved to the identification of the highly immunogenic protein clusters within total lysate of antigens (TLA) or a crude extract

from the whole parasite. Recent findings indicated that a prolonged survival of *T. gondii* infected mice can be achieved upon a systemic priming and a subsequent challenge with TLA, together with a reduction of the parasite tissue cysts in murine brain (Wagner et al., 2015). These promising results made the assessment of the efficacy of TLA based vaccines in other species such as pigs very desirable.

Considering the outcome of experimental vaccination studies in pigs, namely a reduction of *T. gondii's* viability and/or decrease of the parasite DNA burden upon a vaccination and a challenge with a viable strain, the virulence and the strains' genotype played a critical role (Dubey et al., 1991; Dubey et al., 1994; Kringel et al., 2004; Garcia et al., 2005; Jongert et al., 2008; Burrells et al., 2015). Irrespective from the study and the experimental conditions, a polarized Th1-immune response was induced in vaccinated animals, expressed as a predominant IFN-γ production, associated with a reduced cysts formation in the porcine tissues.

Several infection studies in pigs revealed more insights into the early and chronic events following the infection, where a correlation was found between the intensity of the immune response during the *in vitro* exposure to the parasitic antigens and the persistence of the parasite within the host (Verhelst et al., 2011; Verhelst et al., 2015; Jennes et al., 2017).

In the present study native *T. gondii* antigens were evaluated based on *in vitro* induction efficiency of IFN-γ production by pigs experimentally infected with two genotypically different strains. The TLA crude extract was fractionated prior to use by continuous elution-electrophoresis and subdivided into pools with increasing molecular weight. Each pool containing distinct proteins was applied in an antigen recall assay on mononuclear cells, isolated from blood or lymphoid tissues of infected pigs. The IFN-γ generated by the pools was assessed in the supernatant of the cultured cells by cytokine ELISA.

Being the primary cytokine regulating and enhancing the pathogen's survival, the amount of produced IFN-γ was related to the strain-dependent distribution and the

persistence of the parasite within the host's tissues. The variability in the extent of the reaction towards the broad range of the parasitic antigens in several lymphoid tissues, as well as the use of two strains with a different genetic background, could possibly reveal the time-, tissue- and strain-dependent response of the host to the pathogen.

7.3 Materials and methods

7.3.1 Animals and experimental design

Indoor-raised Belgian Landrace piglets were blood sampled at 2 weeks of age to test for the absence of anti-*T. gondii* serum antibodies (IgM and IgG) by indirect immunofluorescence assay (IFA), (Verhelst et al., 2015, Jennes et al., 2017). At weaning age (3w), 46 seronegative piglets were randomly assigned to the acute phase (n=36) and the chronic phase (n=10) experiments. During the experiments, all animals were housed in isolation units at the Faculty of Veterinary Medicine (UGent, Belgium) as approved by the Ethical Committee of the faculties Veterinary Medicine and Bioscience Engineering at Ghent University (EC 2015/102; Biosafety permit nr: AMV/11062013/SBB219.2013/0145).

Acute phase experiment

Thirty-six animals were divided into 10 groups of 3 animals each and one uninfected control group (n=6) (Table 7.1). Animals from five groups were orally inoculated at d 0 with 6000 tissue cysts of *T. gondii* IPB-strain, while five other groups were inoculated with an equal dose of the IPB-LR strain. The six control animals received an inoculum prepared from the homogenized brains from naive mice. The animals were sampled at d 0 and subsequently euthanized at the following time points: 2, 4, 8, 14 and 28 days post infection (dpi) in order to evaluate *in vitro* IFN-γ production in a *T. gondii* antigen recall assay (Table 7.1). The control group was euthanized at 35 dpi to exclude the risk of cross-contamination during sample processing. The following samples were

collected and analyzed for both the cellular immune response (see 7.3.4 and 7.3.5) and the detection of the parasite DNA (see 7.3.6): PBMC's, bone marrow, mediastinal and duodenal, jejunal and ileal mesenteric lymph nodes.

Chronic phase experiment

Eight animals were randomly assigned to one of 2 infection groups, of which one was orally inoculated with 6000 tissue cysts of the IPB-G strain (n=4), and to the other with the same dose of the IPB-LR strain (n=4); two animals served as uninfected controls. The humoral and cellular immune responses were monitored until 168 dpi by two-weekly blood sampling in infected and control animals. The presence of *T. gondii* specific antibodies was detected by IFA, GRA7 ELISA and TLA ELISA. The *in vitro* cytokine production by the isolated and antigen stimulated PBMC's was determined in supernatant of the cells by an IFN-γ ELISA. The *T. gondii* cyst persistence in the porcine tissues (M. psoas major, M. gastrocnemius, diaphragm, Mm. intercostales, M. longissimus dorsi, heart, brain) was evaluated at 168 dpi by qPCR.

Table 7.1 : Experimental design of the acute phase and chronic phase T. *gondii* infection experiments.

Study	Strain	Dose tissue cysts	Group	Animals (n)	Duration (dpi)
acute phase	G	6000	G_2	3	2
			G_4	3	4
			G_8	3	8
			G_{14}	3	14
			G_{28}	3	28
	LR	6000	LR_2	3	2
			LR_4	3	4
			LR_8	3	8
			LR_{14}	3	14
			LR_{28}	3	28
	/	/	control	6	35
chronic phase	G	6000	G_{168}	4	168
	LR	6000	LR_{168}	4	168
	/	/	control	2	168

G: IPB-G strain; LR: IPB-LR strain.
Groups acute phase: G_2, G_4, G_8, G_{14}, G_{28} and LR_2, LR_4, LR_8, LR_{14}, LR_{28}: pigs inoculated at d0 with 6000 tissue cysts of the IPB-G or IPB-LR strain, respectively, and euthanized at 2, 4, 8, 14 and 28 dpi.
Groups chronic phase: G_{150} and LR_{150}: pigs inoculated at d0 with 6000 tissue cysts of the IPB-G or IPB-LR strain, respectively, and euthanized at 150 dpi.
Control groups: pigs inoculated at d0 with homogenized brain tissue of naive mice and euthanized at 35dpi (acute phase) and 150 dpi (chronic phase).

7.3.2. *T. gondii* strains

In the experiments the IPB-Gangji (IPB-G) and the IPB-LR *T. gondii* strains were used for the pigs inoculation, and the RH-strain for the preparation of the parasitic antigens (Jennes et al., 2017). The strains were maintained at the National Reference Laboratory for Toxoplasmosis (Scientific Institute for Public Health, Brussels, Belgium) by a serial

passage in Swiss mice, under approval from the Ethical Committee (nr 20140704-01) and the European regulation on the use of the laboratory animals (2010/63/EU). Tissue cysts were collected from the brain of IPB-G and IPB-LR *T. gondii* inoculated mice, in order to prepare the inoculum for the experimental study of pigs, as described further. The tachyzoites from the ascites of the RH-infected mice were harvested for the preparation of the parasitic antigens.

T. *gondii* bradyzoites

The strains of *T. gondii* were maintained in Swiss mice by peritoneal inoculation with pepsin-treated infectious material (such as placenta). The mice inoculated 6 weeks earlier were euthanized by cervical dislocation and the brains were collected from individual animals. The tissues were homogenized in PBS supplemented with penicillin/streptomycin (1%).

The homogenized brain tissue was inspected using phase-contrast microscopy for the quantification of the tissue cysts. Subsequently, approximately 6000 cysts of each strain were suspended in 10 ml of sterile phosphate buffered saline (PBS) and used for the inoculation of the piglets within 8 hours after cysts isolation. The inoculum for the negative pigs was prepared from the brains of the naive mice. The infectious material was stored at 4°C until inoculation of the pigs.

T. *gondii* tachyzoites lysate antigen (TLA)

TLA or the total lysate antigen is a crude antigen mixture extracted from *T. gondii* tachyzoites of the RH strain (type I). The tachyzoites of the RH strain were maintained by serial passages of approximately 10^7 to 10^8 tachyzoites/ ml into the peritoneal cavity of female Swiss mice. Subsequently, the intra-peritoneal fluid containing the tachyzoites was collected and diluted (1:1) in sterile PBS containing penicillin-streptomycin (1%). The suspension was first purified by differential centrifugation at

800g (Centrifuge Sigma®3-16K Fisher Bioblock Scientific, Newtown, United Kingdom), then filtrated through a 5µm syringe filter (MilleX®SV, Merck Millipore, Overijse, Belgium), and further sonicated (Ultrasonic disintegrator (MSE, Leicester, United Kingdom) on ice. Finally, the parasite extract was centrifuged to remove cell debris and the pellet was resuspended in PBS. After the determination of the protein content by bicinchoninic acid assay (BCA-assay), the lysate was used for antigen identification in SDS-PAGE and Western blot (chapter 7.3.3).

7.3.3. Fractionation of *T. gondii* antigens

SDS-PAGE electrophoresis and Western blot

To distinguish the band pattern within the TLA-extracts, the lysate samples collected from RH-infected mice were diluted (1:1) in loading buffer, containing 5% v/v ß-mercaptoethanol for the disulphide bonds reduction, denatured by heat treatment (5 min, 95°C), and then run (Power PAC 300, Bio-Rad) on a 12% SDS-PAGE with a 4% stacking gel (10 min, 90 V, followed by 60 min, 130 V). For the visualization of the electrophoresis the gel was stained with Coomassie during 30 min at RT, while for the subsequent Western blotting the proteins were transferred on a polyvinylidene fluoride (PVDF) membrane (Millipore) at 130V for 1 h. The membrane after blotting and blocking overnight with 5% (w/v) BSA, 0.3% (v/v) Tween 80 in PBS, was incubated during 1 h at RT with a pooled porcine (IPB-G or IPB-LR infected) or murine (RH-infected) serum sample diluted 1/100 in 0.5% (w/v) BSA in PBS, and developed with the horse radish peroxidase (HRP)-conjugated goat anti-pig IgG or goat anti-mouse IgG (Bethyl Laboratories Inc., Montgomery, USA), respectively at the dilution ratio 1/1000. Between each step, blots were washed three times for 5 min in 0.3% (v/v) Tween® 20 while shaking. Finally, luminol enhancer solution (Pierce®ECL Western Blotting Substrate, Thermo Fisher Scientific, Gent, Belgium) was applied to visualize the bands by ChemiDOC TM MP Imaging System machine (Bio-Rad) for image processing (Image

Lab. 4.0.1, Bio-Rad). The protein bands on Coomassie-stained gels (Figure 7.1A) and the developed blots (Figure 7.1B) were identified, and their molecular weight (MW) was determined by the comparison with marker proteins of the known molecular size (All Blue Marker Precision Plus Protein^{TM} All Blue Standard (Bio – Rad) and MagicMark XP Western Protein standard (Thermo Fischer Scientific), respectively).

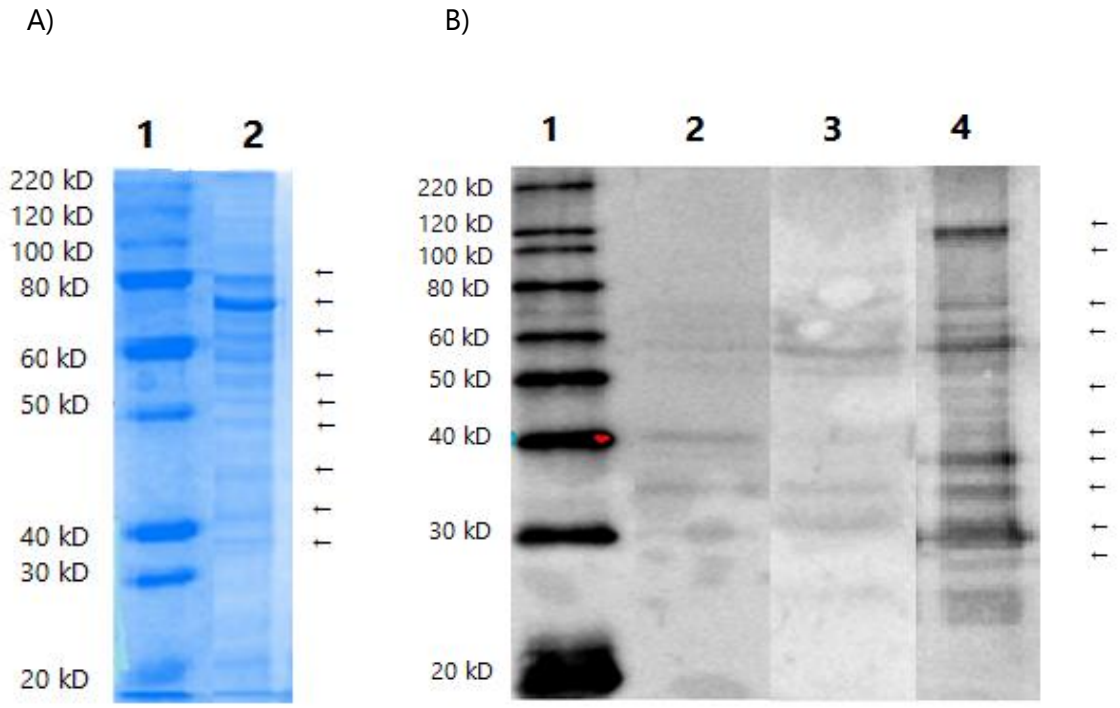

Figure 7.1 : TLA detection by **A)** SDS-PAGE (lane 1: marker; lane 2: TLA), and **B)** Western blot upon detection with porcine (IPB-G or IPB-LR infected) or murine (RH-infected) serum (lane 1: marker; lane 2: serum from IPB-LR infected pigs at 28 dpi; lane 3: serum from IPB-G infected pigs at 28 dpi; lane 4: serum from RH-infected mice at 3 dpi). The arrows indicate the bands assigned to *T. gondii* antigens.

From the analysis of the pattern observed in the samples, the bands correspond presumably to the following proteins (according to the decreasing MW): myosin B of 114 kD (Gómez de León et al., 2014), heat shock protein 82 (HSP90) of 82 kD (Hye-Jin et al., 2003), heat shock protein 70 (HSP70) of 70 kD (Miller et al., 2000), rhoptry 18

(ROP18) of 62.3 kD (Qu et al., 2013), coronin of 54 kD (Guang-Yuan et al., 2009), articulin of 47 kD (Gómez de León et al., 2014), surface antigen 3 (SAG3) of 43 kD (Amerizadeh et al., 2013), excreted-secreted antigen (ESA) of 41 kD (Nockeman et al., 1998), SAG-related sequence 3 (SRS3) of 36.2 kD (Amerizadeh et al., 2013), rhoptry 9 (TgROP9-p36) of 36 kD (Reichmann et al., 2002), surface antigen 5 (SAG5) of 35 kD (Amerizadeh et al., 2013), dense granules 7 (GRA7) of 29 kD (Neudeck et al., 2002), surface antigen 1 (SAG1) of 28 kD (Marques et al., 2012), surface antigen 4 (SAG4) of 23 kD (Amerizadeh et al., 2013) and surface antigen 2 (SAG2) of 22 kD (Lau and Fong, 2008). However, the majority has been detected with the RH-murine serum, while only several with the porcine serum, namely: HSP90 (82 kD), HSP70 (70 kD), ROP18 (62.3 kD), coronin (54 kD), SAG3 or ESA (41-43 kD), SRS3 or ROP9 OR SAG5 (35-36 kD), GRA7 or SAG1 (28-29 kD). Since serum from the IPB-G infected animals revealed more bands than serum collected from the IPB-LR inoculated pigs, the pooled serum from the first group was used in the further steps.

Continuous-elution electrophoresis

Following the characterization of TLA lysate in SDS-PAGE and Western blot, continuous-elution electrophoresis (Model 491, Prep Cell, Bio-Rad) was performed to further identify antigens in the lysate. First, an optimal gel concentration for continuous-elution electrophoresis was selected by comparing in SDS-PAGE the separation of TLA lysate proteins using SDS gel concentrations from 5 to 12%. Next, the recognition pattern by Abs from sera from animals formerly infected with different *T. gondii* strains (IPB-LR and IPB-Gangji) was identified and compared in Western blotting (data not shown). Finally, we calculated the Rf (relative mobility) with a preferable value between 0.55 and 0.6, in order to determine the optimal separation conditions for TLA proteins, according to the formula:

$$Rf = \frac{migration\ distance\ of\ the\ protein\ of\ interest}{migration\ distance\ of\ the\ tracking\ dye}$$

Upon optimization, the continuous electrophoresis-elution was performed (300V, PAC 300, Bio-Rad) on a TLA lysate (0.5 mg/ml) after dilution in loading buffer (1:1) containing 5% v/v ß-mercaptoethanol and a heat treatment (5 min, 95°C). During the electrophoresis a ring-shaped sample migrated down the gel through the frits and dialysis membrane (6 kD, Bio-Rad), to the elution tube in the centre of the cooling core. The subsequent samples were collected in separate liquid fractions (3 ml), and stored cooled at +4°C until further analysis with Western blot.

Based upon the band pattern of TLA fractions collected by the continuous-elution electrophoresis and recognized in Western blotting by the *T. gondii* antibody positive porcine sera 6 distinct antigen pools were formed with the following MW ranges: 20-40 kD (P1), 40-55 kD (P2), 55-65 kD (P3), 65-80 kD (P4), 80-100 kD (P5), 100-120 kD (P6) (Figure 7.2). Since pools 2 and 3 contained many fractions and multiple bands, they were subdivided into smaller fractions: P2A and P2B, and P3A, P3B and P3C, respectively, as in Figure 7.3.

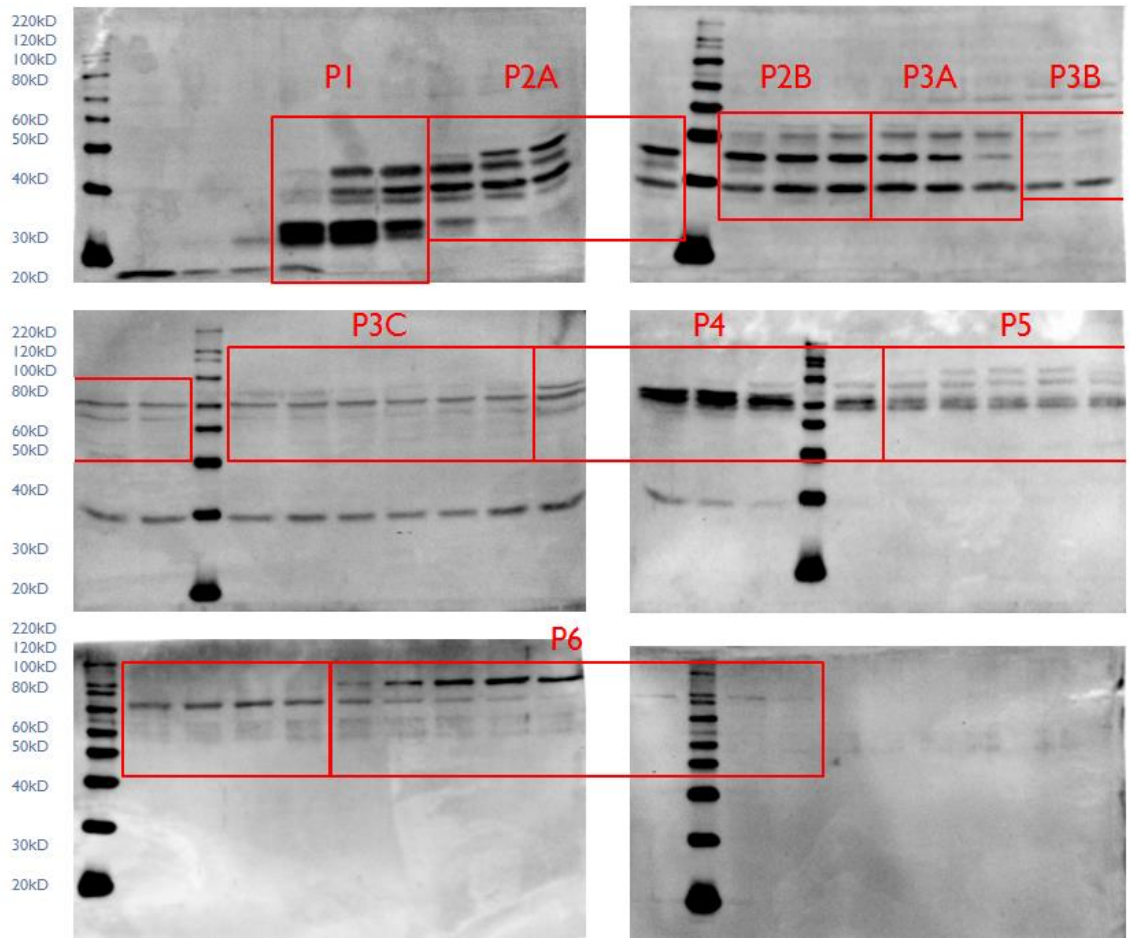

Figure 7.2 : TLA fractionated in separate samples and assigned to pools (P1 - P6) after continuous- elution electrophoresis with Prep Cell, demonstrated by Western blot (detection with serum from IPB-Gangji infected animals at 28 dpi). Pools: P1 (20-40 kD), P2 (40-55 kD), P3 (55-65 kD), P4 (65-80 kD), P5 (80-100 kD), P6 (100-120 kD).

Upon the identification of the proteins and assigning to the pools, the isolated fractions were dialyzed in ultra-pure water (UP) and concentrated, using Amicon Ultra filtration columns (10 kD cut-off; Merck-Millipore, Overijse, Belgium) during subsequent centrifugation steps. An aliquot of each fraction was collected for the SDS-PAGE analysis, which demonstrated a clear pattern of an increasing MW and multiple bands per pools, indicating various parasitic proteins per fraction (Figure 7.3).

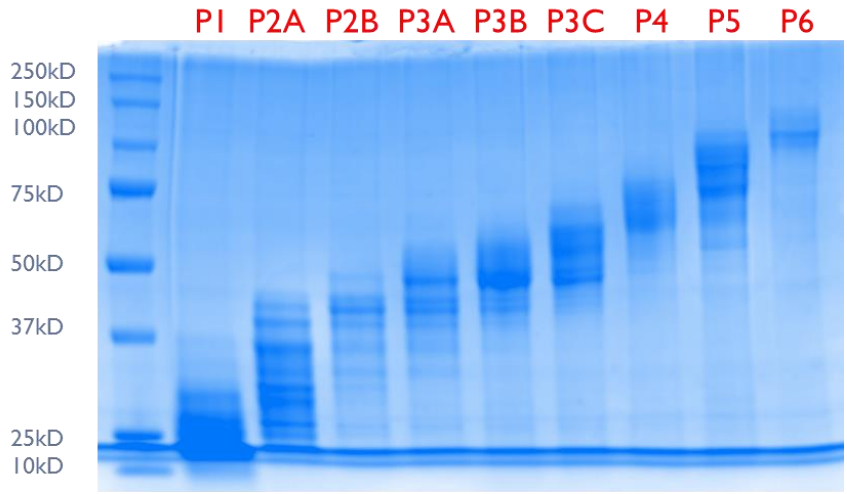

Figure 7.3 : TLA fractionated in pools (P1 - P6) after continuous- elution electrophoresis with Prep Cell, demonstrated by SDS-PAGE.

Finally, the pools were resuspended in culture medium, filtrated and used in the *in vitro* assay with the porcine PBMC's and lymphocytes for the evaluation of their stimulating potential, assessed by the cytokine production.

7.3.4. Detection of IFN-γ response in infected animals

Isolation and stimulation of PBMC's and lymphocytes

PBMC's (lymphocytes and monocytes) were separated from blood based on density gradient centrifugation (800 × *g* at 18°C, 25 min) with Lymphoprep™ (Axis Shield, Olso, Norway; density: 1.077 ± 0.001 g/ml; osmolality: 290 ± 15 mOsm) and isolated as described previously (Jennes et al. 2017). Lymphocytes from the peripheral lymph nodes (mediastinal, duodenal, jejunal and ileal mesenteric lymph nodes) were isolated from the lymphoid tissue according to a previously optimized protocol (Verhelst et al., 2011). Finally, all cell populations were resuspended at a concentration of 10^7 cells/ml in complete medium, supplemented with fetal calf serum (5%), non-essential amino acids (1%), Na-pyruvate (100 µg/ml), L-glutamine (292 µg/ml), penicillin (100 IU/ml),

streptomycin (100 µg/ml) and kanamycin (100 µg/ml)). Cells were cultured in a 96-wells culture plate (10^6 cells/well) for 72 h upon stimulation with the six isolated TLA pools (10 µg/ml) or the mitogen concanavalin A (ConA, Sigma-Aldrich, USA; 5 µg/ml) as a positive control. Complete medium without antigens or mitogen was the negative control.

IFN-γ ELISA

The supernatant collected after 72h incubation was transferred from the cultured cells to another empty 96-well culture plate for the detection and quantification of the produced IFN-γ in a sandwich-ELISA (Fisher Scientific, Erembodegem, Belgium). In brief, the plates were coated with mouse anti-pig IFN-γ monoclonal antibodies (mAbs), blocked with the assay buffer for 1 hour at RT, whereafter the prepared standards (2500 pg starting concentration) or supernatant dilution (1/5) in assay buffer were pipetted in duplicate into the wells. Detection of the porcine IFN-γ was performed with biotin-conjugated working Abs, followed by the subsequent addition of streptavidin-HRP solution, TMB substrate and stop solution to each well. The cytokine concentration (pg/ml) was calculated from the obtained OD-values measured at the absorbance 450nm, using a 4-parameter curve fit with Delta Soft software.

7.3.5 Humoral immune response

The parasite-specific IgM and IgG production was evaluated by GRA7- and TLA-ELISA and by IFA, as described previously (Jennes et al., 2017). Blood was bi-weekly sampled from the jugular vene. For GRA7- and TLA-specific antibodies, serum was tested at a dilution of 1/50 on plates coated with 10 µg/ml of antigen. Antigen-specific IgM and IgG were detected with the goat anti-pig IgM- and IgG-HRP conjugate (Bethyl Laboratories Inc., Montgomery, Texas, USA), respectively, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as substrate-chromogen solution

(Jennes et al., 2017; Verhelst et al., 2015). Previously determined sera from one positive and three negative control animals were included in the assay on each plate. The absorbance was measured at 405 nm (TECAN Spectra Fluor, Tecan Group Ltd., Männedorf, Switzerland) and the cut-off value was calculated as the mean OD_{405} of the negative controls + 3 x its standard deviation (SD).

For IFA, the IgM and IgG antibodies against *T. gondii* were identified on slides coated with formalin-fixed tachyzoites (RH-strain) (Toxo-Spot IF, Biomérieux, Marcy-l'Etoile, France), incubated with a 1/50 dilution of serum samples and developed with a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-swine IgM(μ) or IgG (H+L) (KPL, Maryland, USA) as previously described (Jennes et al., 2017). Fluorescence of the stained tachyzoites was evaluated by fluorescence microscopy (Carl Zeiss, Germany), in comparison with positive and negative reference sera.

7.3.6 Detection of parasite DNA in the tissues

The tissues of the infected and control animals in the chronic phase experiment were tested for the detection of the parasite DNA by a highly sensitive MC-qPCR technique, recently optimized in the WIV-IPB institute on the samples from the former experiments of our research group (Algaba et al., in press). The model developed by Opsteegh et al. (2010) is further improved due to an increased sensitivity and efficiency, by the reduction of the incubation times, and, above all, it is suitable for the use in the diagnostic laboratories by the ISO validation (ISO 17025). Briefly, the recent method combines a specific DNA extraction and concentration step, by the hybridization of specific, biotin labelled probes to a *T. gondii* target sequence with a capture with streptavidin coated paramagnetic beads, in addition to co-capture of cellular r18S as a non-competitive PCR inhibition control. The release of the captured target DNA is processed upon UVb light (290-315nm, peak at 305nm) exposure during 5 min by designing the capture probes with a UVb cleavable spacer between the biotin and the

oligonucleotides (5' PC Biotin). The following tissues were collected and tested with this MC-qPCR: brain, heart, diaphragm, Mm. intercostales, M. gastrocnemius, M. longissimus dorsi and M. psoas major.

The DNA samples from both experiments were tested in duplex (*T. gondii* and r18S) and in simplex (*T. gondii*) on Bio-Rad CFX 96 Thermocycler Detection System (Bio-Rad, Hercules, CA, USA), according to the following protocol: denaturation and activation of the Taq polymerase at 95°C for 3min, followed by 41 cycles at 95°C for 15 sec and 60°C for 20 sec. The obtained values of the crossing point (Cp) for each sample were analyzed with the CFX Manager Software, supplied with the device (Bio-Rad).

7.3.7 Bioassay in mice

In addition to the parasite DNA detection by MC-qPCR (see chapter 7.3.6), the viability of *T. gondii* in the tissue samples collected at the end of the chronic phase experiment was evaluated by the bioassay in mice, as performed earlier (Verhelst et al. 2011, Jennes et al., 2017). Hereto, homogenized samples (50 g for brain and 100 g for other tissues) underwent a trypsin (0.25%) digestion during 1h and 2h respectively, followed by filtration, washing with sterile physiological solution supplemented with gentamycin (0.4mg/ml) in order to remove the trypsin and resuspension in 10 ml of PBS supplemented with gentamycin. Finally, 1 ml of the suspension was injected intraperitoneally into 5 Swiss white female mice (Ethical Committee permit: 20140704-02). Six weeks after the inoculation the blood from mice was tested by IFA for the presence of *T. gondii* IgG antibodies, or by qPCR in lungs and ascites in case of acute toxoplasmosis, when the bioassay was pre terminated due to reaching the human end points. The parasite DNA was then detected by qPCR as for the porcine samples (see chapter 7.3.6).

7.3.8 Statistics

The IFN-γ concentration per pool at different time points, and the Cp value per tissue in the infected groups are presented as means ± SD. A one-way Analysis of Variance (ANOVA) and post hoc Bonferroni's Multiple Comparison Tests were performed to discriminate between infected and control groups for cytokine response and the parasite load (GraphPad Prism 5). A *p*-value <0.05 was considered statistically significant.

7.4 Results

7.4.1. Kinetics of IFN-γ secretion by PBMC's and peripheral lymphocytes of *T gondii* infected pigs upon *in vitro* restimulation with TLA pools and TLA.

Acute phase experiment

High IFN-γ concentrations could be detected already 8 and/or 14 dpi following *in vitro* restimulation of lymphocytes. This IFN-γ production dramatically decreased by 28 dpi. Regardless of the strain pigs were inoculated with, the highest peaks in the IFN-γ concentration were seen for PBMC's (Figure 7.7) with values reaching 28800 pg/ml, followed by mediastinal (Figure 7.8), duodenal (Figure 7.4) jejunal (Figure 7.5) and ileal mesenteric (Figure 7.6) lymph node cells within the range of individual values between 2400 and 6000 pg/ml, while the lowest (<1000 pg/ml) were notified for bone marrow (Figure 7.9). For most time points this IFN-γ concentration was even beyond the detection limit of the ELISA standard dilution.

The pools inducing the strongest response in the PBMC's were the fractions 1, 4, 6 and 3. The response was more pronounced for the animals from the IPB-LR group (23650 ± 8920 pg/ml, 20300 ± 14740 pg/ml, 19890 ± 15430 pg/ml, 11620 ± 14930 pg/ml, respectively) than for the same pools in the IPB-G group (20200 ± 14890 pg/ml, 20260

± 14800 pg/ml, 15740 ± 14010 pg/ml, 19400 ± 16290 pg/ml, respectively). However, due to the excessive inter-animal variation within the same group, these differences were not significant.

The IFN-γ induction profile for mediastinal lymphocytes by the TLA pools showed lower concentrations than for PBMC's and a rather equal distribution of IFN-γ production. Although the maximum individual values reached above 6000 pg/ml for several TLA-fractions at 8 dpi in both infected groups, the group averages were not higher than 2500 pg/ml (2410 ± 3110 pg/ml, 2500 ± 3040 pg/ml, 2060 ± 3410 pg/ml, 2275 ± 3225 pg/ml, 236 ± 320 pg/ml, 2040 ± 3430 pg/ml for the pools 1-6, respectively) in the IPB-LR group, and only occasionally higher than 4000 pg/ml (2590 ± 2990 pg/ml, 4030 ± 3405 pg/ml, 850 ± 720 pg/ml, 3890 ± 3260 pg/ml, 2200 ± 3290 pg/ml, 2270 ± 3250 pg/ml for the pools 1-6, respectively) in the IPB-G group (Figure 7.8).

Other alterations in reference to PBMCS's were that TLA stimulation resulted in higher IFN-γ concentrations than the fractionated pools, and that IPB-LR infection induced similarly elevated IFN-γ concentrations at 8 and 14 dpi, with the exception of pools 5 and 6 which peaked only at 14 dpi (2815 ± 3000 pg/ml and 4010 ± 3450 pg/ml, respectively). In the IPB-G group, as mentioned above, IFN-γ reach the highest value also at 8 dpi, whereafter the cytokine concentration dropped considerably. By the last sampling time point (28 dpi) the production was at a low level in all the infected animals, irrespective of the strain.

The cytokine production levels in the antigen recall assay in the mesenteric lymph nodes showed another pattern in the distinct segments of the intestine (Figures 7.4 – 7.6). In general, except for the duodenal lymph nodes, the responses were slightly or predominantly higher for the IPB-LR strain infected animals than for the IPB-G inoculated group. Referring to that, immune cells isolated and cultured from the jejunal and ileal mesenteric lymph nodes of IPB-LR group released the cytokine in up to 80 times higher amounts than the IPB-G group (Figure 7.5 and Figure 7.6). The strongest responses were induced by the pools 1 (2040 ± 3430 pg/ml versus 28 ± 32 pg/ml) 2

(2050 ± 3420 pg/ml versus 35 ± 30 pg/ml), 4 (2065 ± 3410 pg/ml versus 40 ± 43 pg/ml), and 5 (2040 ± 3430 pg/ml versus 25 ± 27 pg/ml) at 14 dpi for the jejunal lymph nodes, and pools 1 (1240 ± 990 pg/ml versus 346 ± 503 pg/ml) and 2 (1460 ± 1030 pg/ml versus 255 ± 218 pg/ml) at 8 dpi for the ileal lymph nodes, both from IPB-LR animals in comparison with IPB-G animals, respectively.

However, the lymphocytes activity observed in the duodenal mesenteric lymph nodes showed clearly an opposite trend, with a higher cytokine expression in the IPB-G infected animals (Figure 7.4). While all the pools seemed to induce a modest IFN-γ production (with a maximum of 2100 pg/ml) in the IPB-LR group at 14 dpi, in the IPB-G infected animals several TLA fractions stimulated the cells to a more excessive cytokine levels until above 4000 pg/ml (pool 1: 3880 ± 3260 pg/ml; pool 2: 4005 ± 3455 pg/ml; pool 3: 4060 ± 3360 pg/ml and pool 5: 4040 ± 3400 pg/ml).

In addition, several other interesting observations were noticed, when considering the TLA pools and their stimulation capacity towards the distinct cell populations in the acute phase experiment. Hence, not only pool 3 but also other pools, such as pools 1, 2 or 6 were involved in this early response. The total lysate (TLA) had a very clear IFN-γ inducing capacity for the cells from mediastinal lymph nodes in animals from both infected groups, and to a lesser extent for the lymphocytes from jejunal and ileal mesenteric lymph nodes in IPB-G inoculated pigs. Surprisingly, most individual pools showed consequently a stronger activation than TLA, in particular in PBMC's and duodenal lymph nodes. As mentioned earlier, cells isolated from the bone marrow did not show any noticeable pattern in terms of cytokine production.

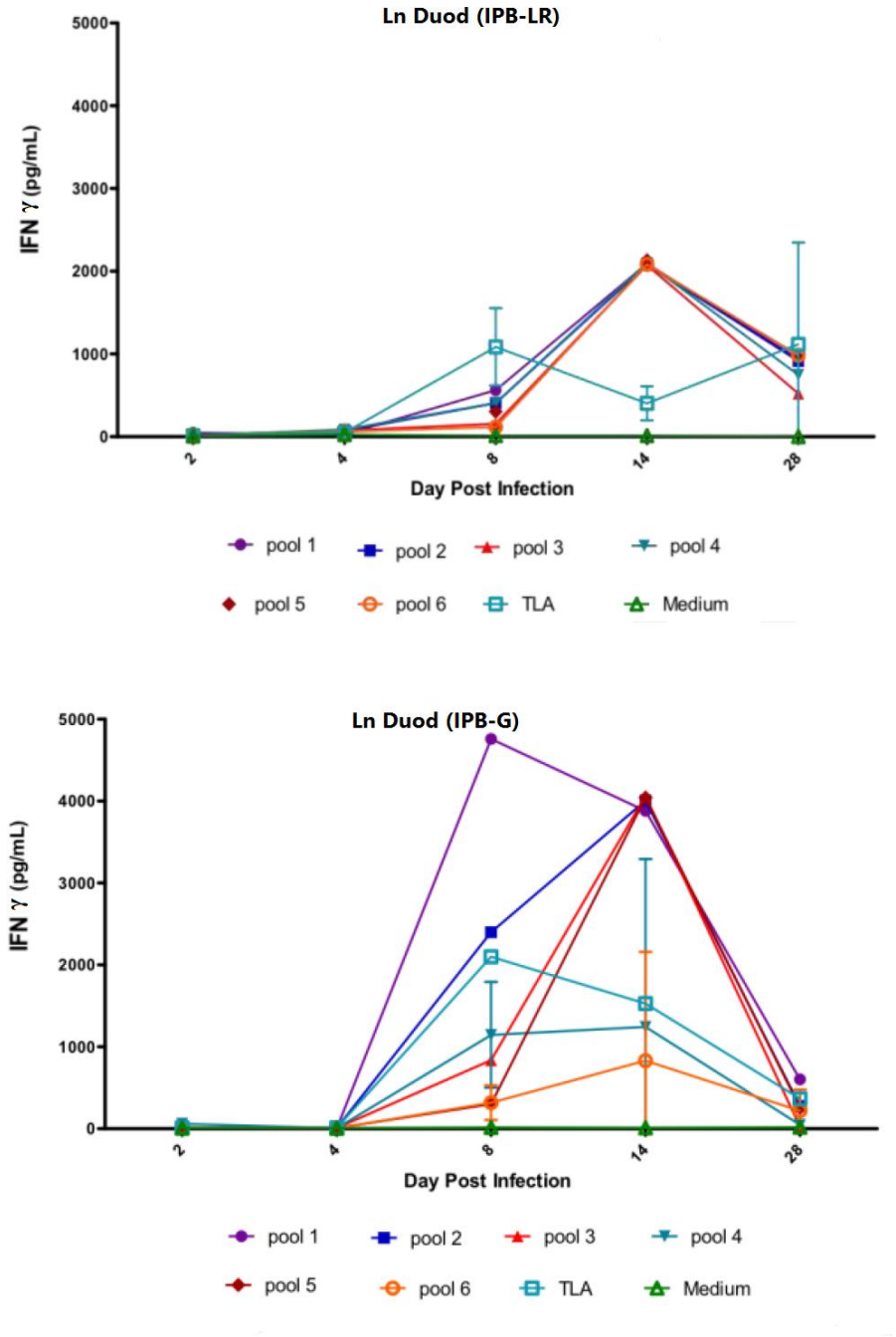

Figure 7.4 : Kinetics of the IFN-γ concentration (pg/ml) in the supernatant of lymphocytes isolated from duodenal lymph nodes (Ln Duod) of *T. gondii* IPB-LR (upper panel) and IPB-G (lower panel) strain infected pigs after *in vitro* stimulation with TLA pools.

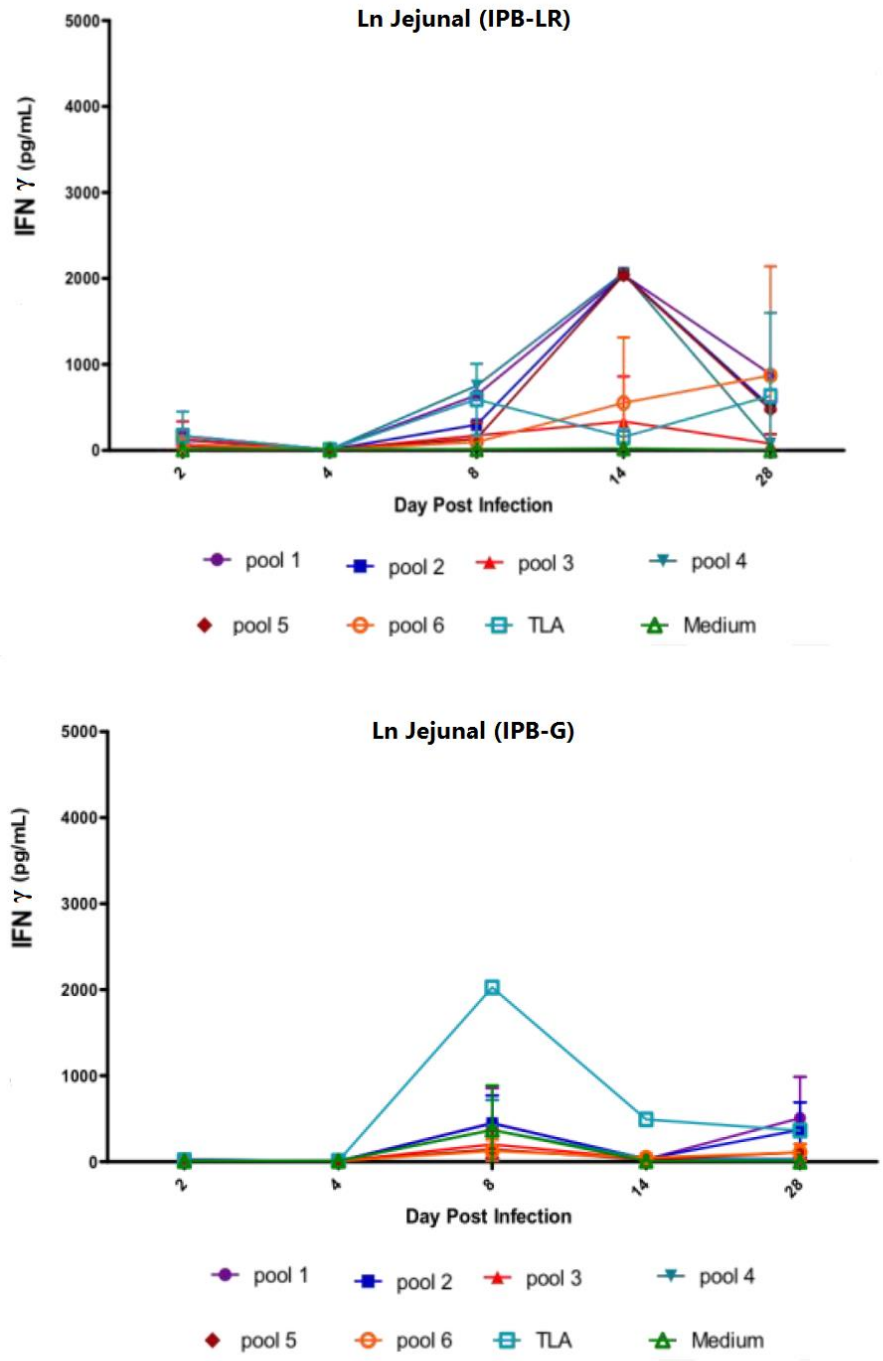

Figure 7.5 : Kinetics of the IFN-γ concentration (pg/ml) in the supernatant of lymphocytes isolated from jejunal lymph nodes (Ln Jejunal) of *T. gondii* IPB-LR (upper panel) and IPB-G (lower panel) strain infected pigs after *in vitro* stimulation with TLA pools.

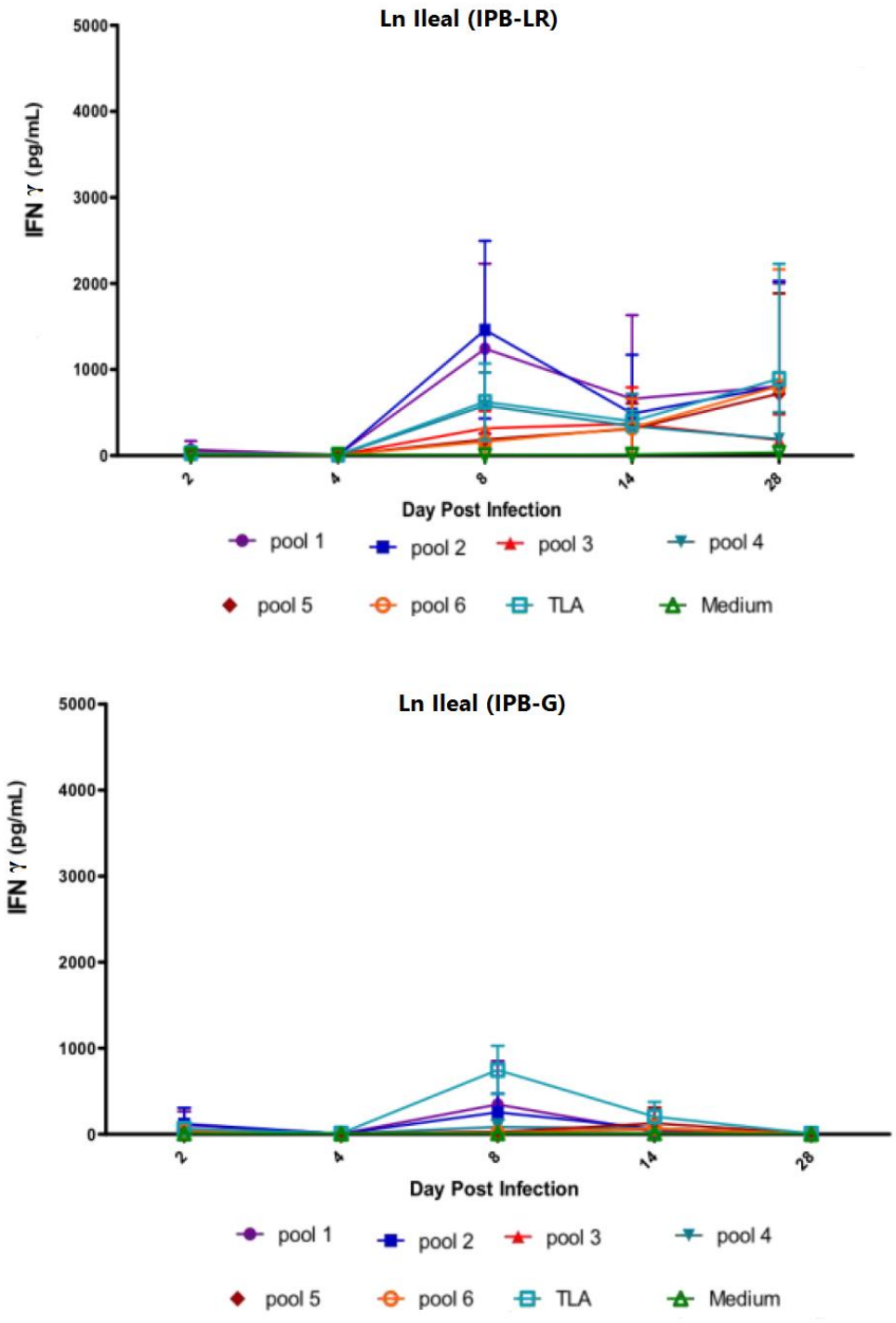

Figure 7.6 : Kinetics of the IFN-γ concentration (pg/ml) in the supernatant of lymphocytes isolated from ileal lymph nodes (Ln Ileal) of *T. gondii* IPB-LR (upper panel) and IPB-G (lower panel) strain infected pigs after *in vitro* stimulation with TLA pools.

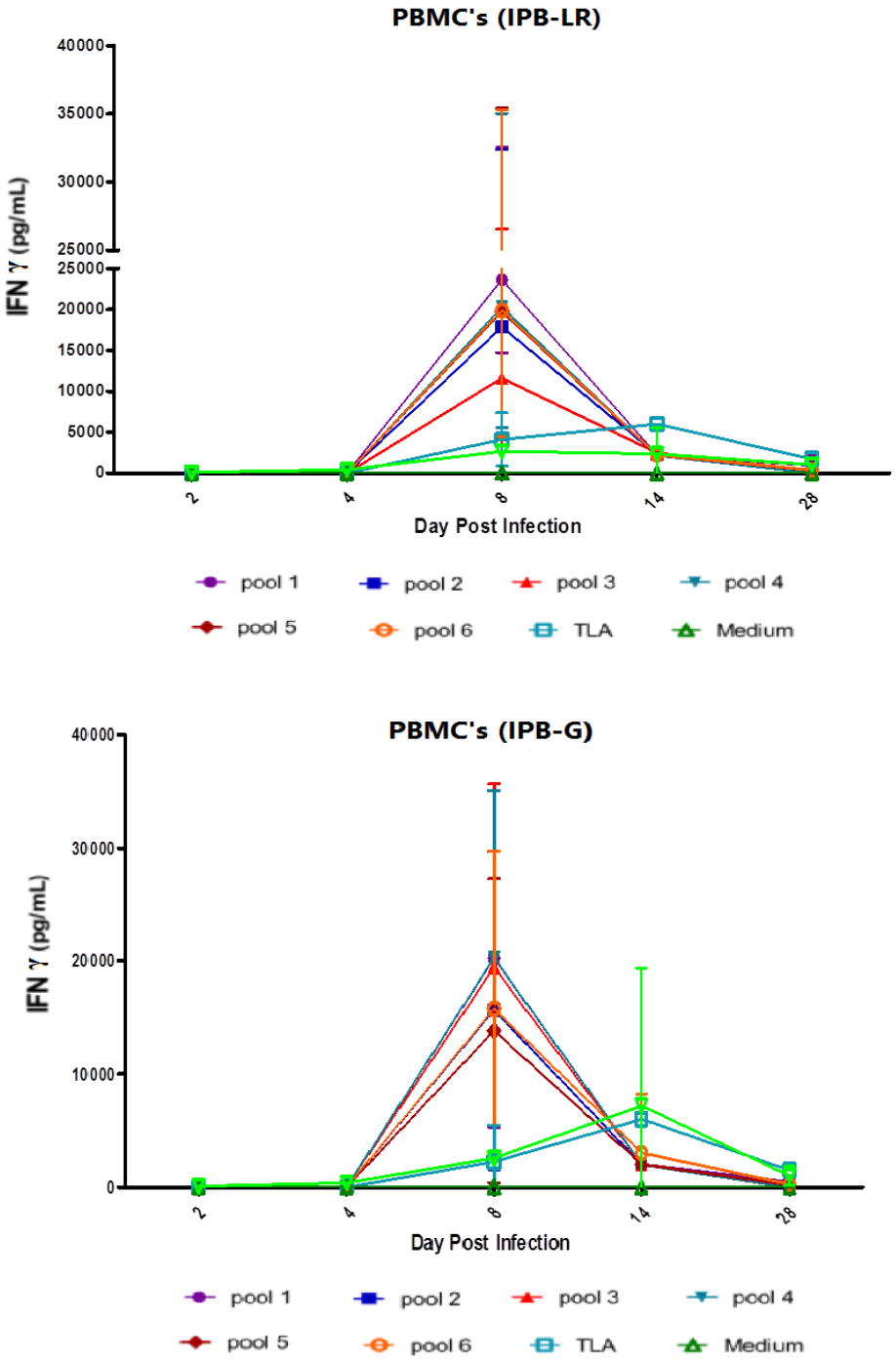

Figure 7.7 : Kinetics of the IFN-γ concentration (pg/ml) in the supernatant of PBMC's isolated from with *T. gondii* IPB-LR (upper panel) and IPB-G (lower panel) strain infected pigs after *in vitro* stimulation with TLA pools (acute phase experiment).

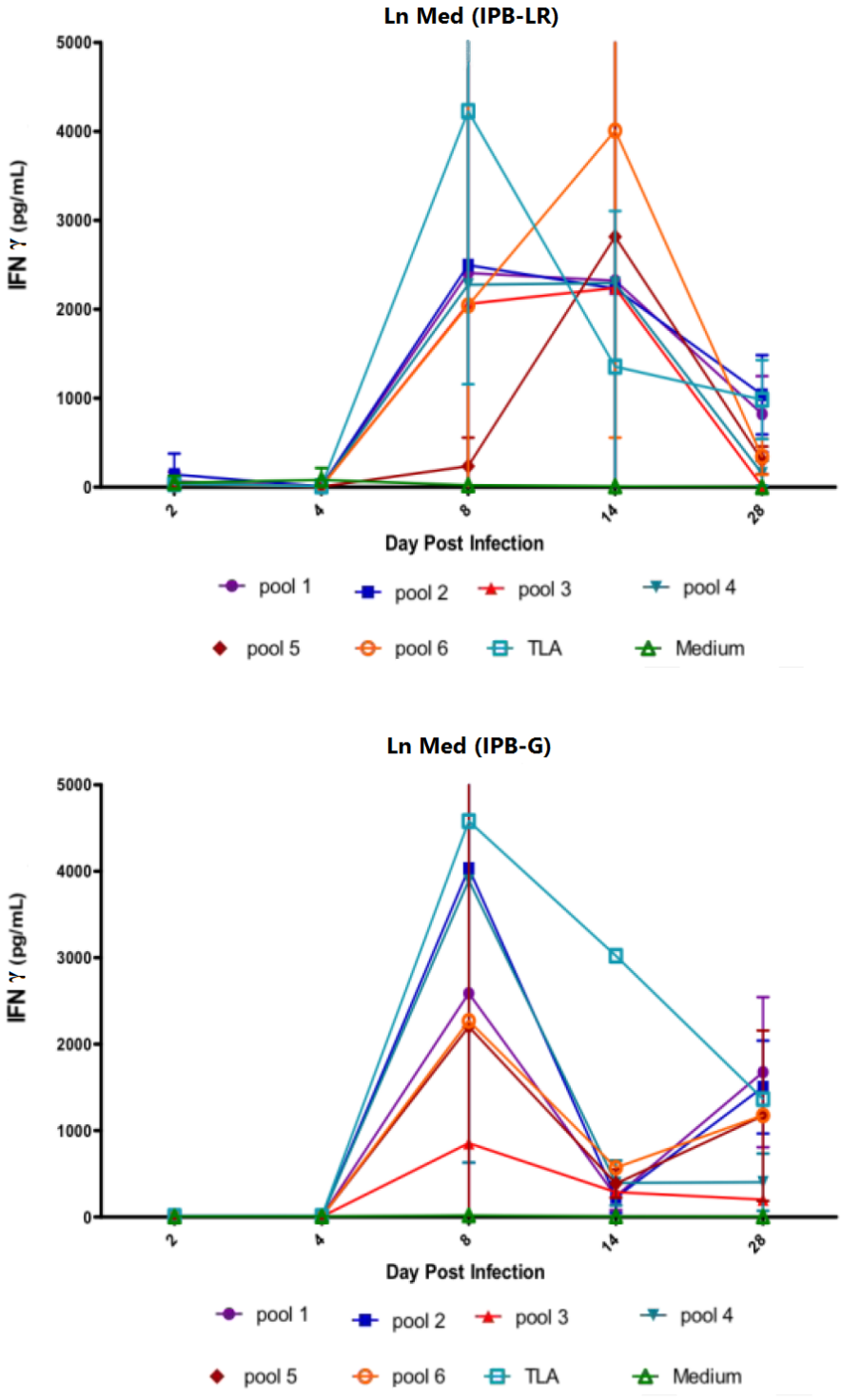

Figure 7.8 : Kinetics of the IFN-γ concentration (pg/ml) in the supernatant of lymphocytes isolated from mediastinal lymph nodes (Ln Med) of *T. gondii* IPB-LR (upper panel) and IPB-G (lower panel) strain infected pigs after *in vitro* stimulation with TLA pools.

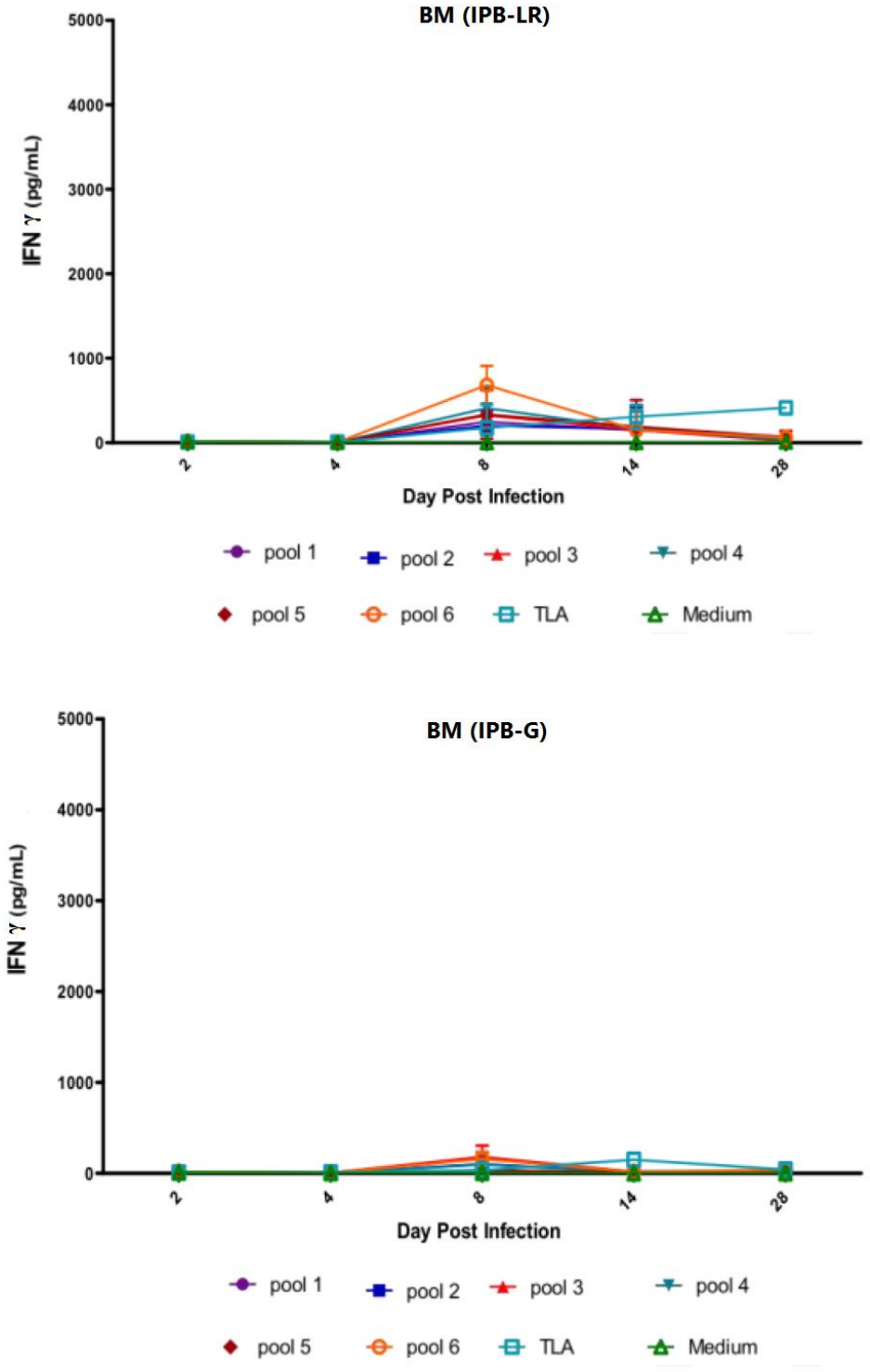

Figure 7.9 : Kinetics of the IFN-γ concentration (pg/ml) in the supernatant of lymphocytes isolated from bone marrow (BM) of *T. gondii* IPB-LR (upper panel) and IPB-G (lower panel) strain infected pigs after *in vitro* stimulation with TLA pools.

Chronic phase experiment

In this study pigs were followed until 168 dpi. As mentioned in material and methods, blood was two-weekly sampled and PBMC's were restimulated *in vitro* with TLA pools to determine their IFN-gamma inducing effect. In the IPB-LR group, the cytokine concentration clearly increased between 42 and 70 dpi upon stimulation with pool 3 until approximately 5000 pg/mL on 56 dpi, followed by a decrease to almost baseline values between 84 and 126 dpi (Figure 7.10 upper panel). For the other pools this first IFN-γ peak occurred later, was lower and of shorter duration. A second elevation in the detected cytokine concentration occurred at 140 dpi and was more pronounced for pools 1 and 5, whereas stimulation with pool 3 induced an intermediate increase.

In the IPB-G group, the IFN-γ concentration started to increase 70 days post infection (dpi), which was clearly later than in the IPB-LR group and irregularly rose until 140 or 154 dpi, depending on the pool used for stimulation (Figure 7.10 lower panel). In contrast to IPB-LR infected pigs, there was no dip in the IFN-gamma secretion between 84 and 126 dpi.

Although the re-stimulation with pool 3 induced a clear response and showed the highest concentration 168 dpi, higher values were seen for one or several of the other pools between 98 and 154 dpi.

Since TLA pool 3 induced a high IFN-γ response, we subdivided pools 2 and 3 in several smaller fractions (P2A and P2B; P3A, P3B and P3C, respectively) to investigate whether the IFN-γ response could be further restricted to a certain molecular weight fraction. Stimulation assays were performed on selected animals from both infection groups between 168 and 182 dpi. Nevertheless, no significant differences in the antigen recall assay and the subsequent cytokine production was observed for these fractions at the later time points (data not shown).

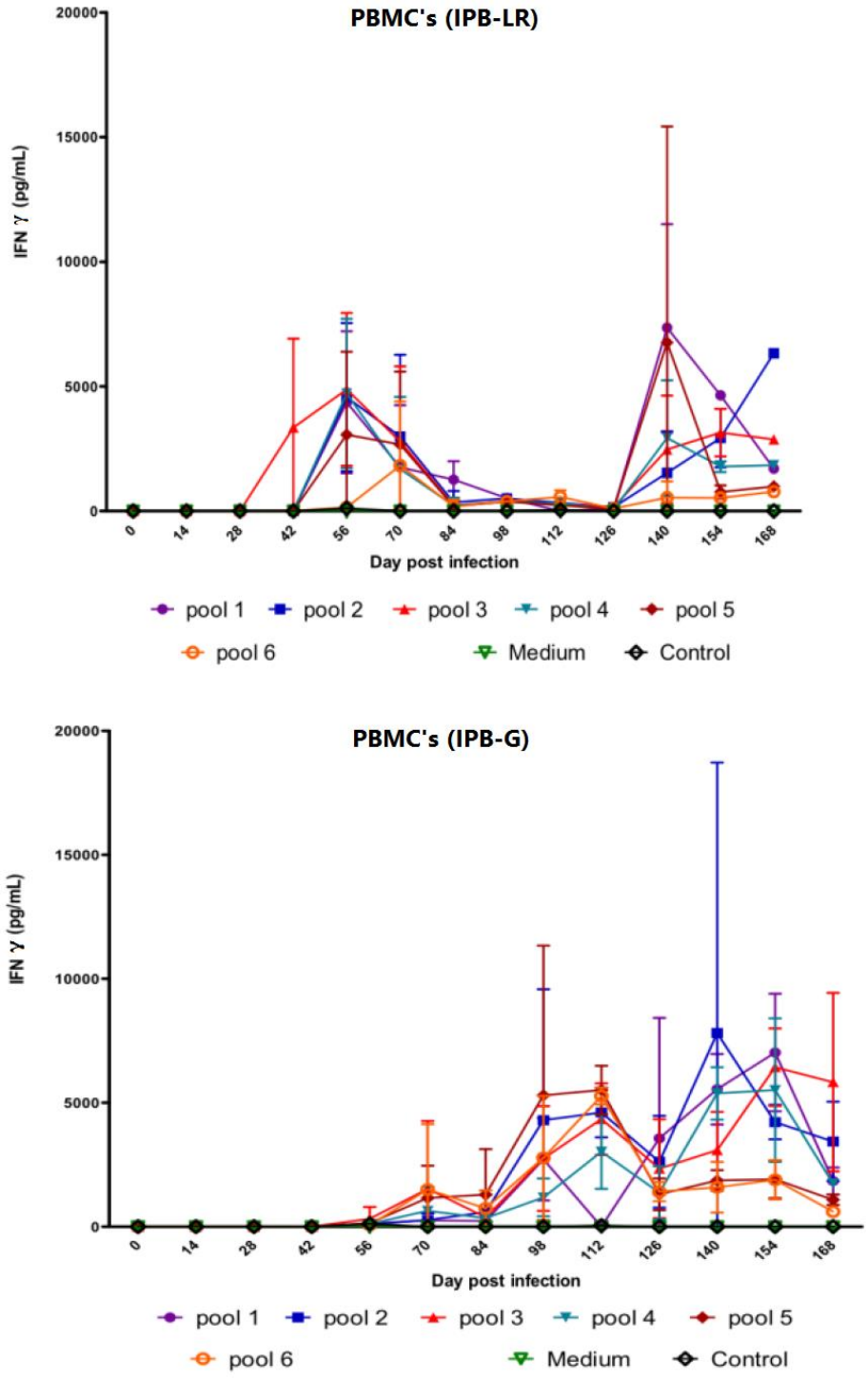

Figure 7.10 : Kinetics of the IFN-γ concentration (pg/ml) in the supernatant of PBMC's isolated from with *T. gondii* IPB-LR (upper panel) and IPB-G (lower panel) strain infected pigs after *in vitro* stimulation with TLA pools (chronic phase experiment).

7.4.2. Parasite load and viability in the porcine tissues

At the euthanasia, the white blood cells isolated from blood, bone marrow and the lymphoid tissues (mediastinal lymph nodes, duodenal, jejunal and ileal mesenteric lymph nodes) were tested with the qPCR for the detection of the *T. gondii* DNA. The parasite load was calculated using Cp or the point at which the amplification curve crosses the vertical threshold line. The amplification data were expressed as ΔCp (delta crossing point) or the difference in Cp between the total number of the cycles (n=41) in the qPCR and the average number of cycles per sample in duplex and in simplex assessment.

Chronic phase experiment

From the results per group, the animals inoculated with the IPB-G strain of *T. gondii* showed on average a lower, however, not significant, parasite load in different tissues compared to the IPB-LR infected groups (Figure 7.11). The largest differences between both groups were notified for the M. gastrocnemius (ΔCp: 3.95 ± 3.81 for IPB-LR and ΔCp: 0.29 ± 0.57 for IPB-G) and the diaphragm (ΔCp: 5.9 ± 5.23 for IPB-LR and ΔCp: 3.66 ± 3.3 for IPB-G).

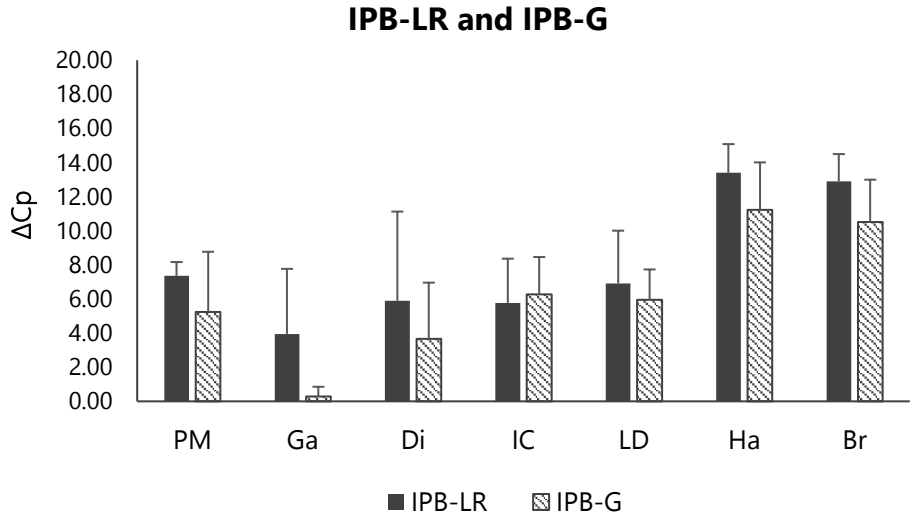

Figure 7.11 : Average parasite load determined in the tissues of the pigs inoculated with IPB-LR and IPB-G in the chronic phase experiment. ΔCp (crossing point): 41 (total number of cycles) – average Cp per sample. Samples: PM: M. psoas major; Ga: M. gastrocnemius; Di: diaphragm; IC: Mm. intercostales; LD: longissimus dorsi; Ha: heart; Br: brain.

Equally in both groups, the highest parasite load was detected in brains and hearts of the inoculated animals (Figure 7.11). The samples from the control pigs included in the study tested negative (data not shown).

The results of the bioassay revealed the same trend, namely in the majority of the samples (83.4%; 15/18) collected from the IPB-LR inoculated pigs, the presence of the viable parasite was detected upon IFA or qPCR in murine samples (Table 7.2). Among the tissues derived from the IPB-G infected animals, 75% or 18/24 contained viable *T. gondii* bradyzoites, as evidenced by bioassay in mice. In the following samples in both inoculation groups no or a diminished number of viable parasites was detected: M. gastrocnemius, diaphragm, Mm. intercostales and M. longissimus dorsi.

Table 7.2 : Bioassay and MC-qPCR results in the tissues of the pigs inoculated with IPB-LR (LR 1- LR 3) and IPB-G (G 1 – G 4) in the chronic phase experiment.

IPB-LR

	Bioassay							MC-qPCR						
	PM	Ga	Di	Ic	LD	Ha	Br	PM	Ga	Di	Ic	LD	Ha	Br
LR 1	5/5	5/5	2/5	5/5	5/5	5/5	np	++	++	-	++	++	+++	+++
LR 2	5/5	5/5	2/5	1/5	1/5	5/5	np	++	+	++	+	++	+++	+++
LR 3	5/5	0	0	0	1/5	5/5	np	++	-	++	++	+	+++	+++

IPB-G

	Bioassay							MC-qPCR						
	PM	Ga	Di	Ic	LD	Ha	Br	PM	Ga	Di	Ic	LD	Ha	Br
G 1	5/5	0	0	1/5	1/5	5/5	np	++	-	-	++	+	+++	++
G 2	5/5	5/5	4/5	4/5	5/5	5/5	np	++	-	+	++	++	+++	+++
G 3	5/5	0	3/5	5/5	5/5	5/5	np	++	+	+	++	++	+++	+++
G 4	1/5	0	4/5	0	0	1/5	np	-	-	++	+	+	++	++

PM: M. psoas major; Ga: M. gastrocnemius; Di: diaphragm; IC: Mm. intercostales; LD: longissimus dorsi; Ha: heart; Br: brain. Bioassay: + : positive; - : negative; MC-qPCR: + : $\Delta Cp < 5$; ++ : $5 < \Delta Cp < 10$; +++ : $\Delta Cp > 10$; - : $Cp = 0$; np: not performed.
(one animal from IPB-LR group was euthanized before the end of the experiment due to animal welfare reasons and the bioassay and MC-qPCR data were therefore withdrawn from the IPB-LR group results).

However, the data from the individual animals showed even a more pronounced divergence between the two strains. Apart from the three samples in the animal LR3, where no viable parasites were detected in any of the assayed mice, there were also other samples (1 in LR1 and 3 in LR2) with a decreased number of mice showing *T. gondii* specific antibodies (2/5 and 1-2/5, respectively). When considering the bioassay from the IPB-G pigs, the number of samples showing a reduced amount of the viable parasites is considerably expanded: 25% (3/24) showed no viable bradyzoites, another

8 samples (or 33.3%) had a reduced number of mice positive in IFA or in qPCR. The most noticeable bio-assay results in this group were observed for the animal G4, where for none of the tissues all 5 injected mice showed seroconversion (Table 7.2).

In contrast with the group's averages per tissue (Figure 7.11), the data of MC-qPCR per single animal confirmed the main findings of the bioassay in mice (Table 7.2). Not only more samples collected from the IPB-G group (5/24, 21% against 2/24, 8.3% in IPB-LR) showed no amplification of the 529 bp DNA fragment of *T. gondii*, while the house-keeping gene performed well, excluding by this the failure of the assay; similarly, the ΔCp values were also within the lowest range (ΔCp < 5) in 6 (25%) tissues of the IPB-G group, while in 3 (16.7%) samples in the IPB-LR group. Surprisingly, even the heart and brain tissues did not show the highest ΔCp in some IPB-G infected animals, while it was evidently the case in the opposite group.

7.5 Discussion

The diagnosis of *T. gondii* infection in an intermediate host can be made by a variety of indirect and direct techniques, with their specific characteristics to investigate the immune responses of the host towards the parasite. The most convenient as well as the most frequently used techniques are the serological assays for the detection of the *T. gondii*-specific antibodies (Chapter 2). Despite the numerous advantages of antibody detection, this humoral response plays just a partial role in the resistance against the parasite and, as such, does not reflect the complexity of the immune mechanisms upon a natural or experimental infection. Hence, multiple parameters associated with toxoplasmosis should be evaluated to obtain a full insight of the immune reactions. As highlighted in Chapter 2, the first line of the host's defense relies on the cells of the innate immune system such as DC's, NK's and macrophages. In the further stage of the infection also the cells from the acquired immune system are involved, namely different subpopulations of the T-lymphocytes and the B-cells (Filisetti and Candolfi, 2004;

Murphy et al., 2008; Miller et al., 2009; Denkers, 2010; Cohen and Denkers, 2014; Sturge and Yarovinsky, 2014).

Hereto, in the present study we detected and monitored IFN-γ production by the lymphocytes in the acute and chronic *T. gondii* infection model in experimentally infected pigs, in response to the *in vitro* native antigen stimulation. In accordance with the earlier studies in pigs (Verhelst et al., 2011, Verhelst et al., 2015; Jennes et al., 2017), we focused on the TLA from the tachyzoites of the RH-strain as the source of the parasite antigens in the recall assay.

It is well known that the complex antigenic composition of *T. gondii* varies strongly depending on the strain and the developmental stage of the parasite (Kotresha and Noordin, 2010; Khammari et al., 2014). Certain antigens have clearly a stage-specific expression pattern, while others are common during each developmental stage. The most frequently used *T. gondii* proteins for diagnostic or experimental purposes are either tachyzoite-derived, as in our study, or recombinantly produced surface antigens (SAGs), electrodense secretory organelles such as matrix antigen (MAG), microneme proteins (MICs), rhoptry proteins (ROPs), and dense granule (GRAs) (Kotresha and Noordin, 2010). Among others, SAG1 is a membrane-associated antigen, while MAG1 is present in both stages: tachyzoite and bradyzoite. The GRA proteins (GRA2, GRA6, GRA7 and GRA8) are not related to the stage but rather to the time-interval, namely they are expressed in the acute phase of the infection, except for GRA1, which is reported to be a marker of the chronic stage (Kotresha and Noordin, 2010).

Further, we quantified the IFN-γ production as the major cytokine associated with toxoplasmosis in an intermediate host, upon identification of the antigens by one-dimensional SDS-PAGE and Western blot. Other studies have shown the utility of these techniques for testing of human and animal serum samples and identification of the most immunodominant parasitic antigens (Hafid et al., 1991; Marcolino et al., 2000; Fatoohi et al., 2004; Kotresha and Noordin, 2010; Khammari et al., 2014).

However, it has been proven that the induction of the cytokine production *in vitro* is in a relation to the strains used, due to the antigenic variation among the strains (Rodgers et al., 2005). In our study we used TLA prepared from tachyzoites of the RH-strain in a heterologous antigen recall assay. However, it is challenging to predict the magnitude of the divergence between the inoculation (IPB-G and IPB-LR) and restimulation strain (RH) without performing a genetic analysis, since we expect different expression of the same antigens and/or variability of the present epitopes. Nonetheless, as detected cytokine is the product of collateral recognition of multiple epitopes, the previous application of the lysate in a comparable experimental setup resulted in a strong immune response, as determined via IFN-γ mRNA expression by qPCR and via the intracellular IFN-γ detection by flow cytometry, irrespective from the strain (Verhelst et al., 2011, Verhelst et al., 2015; Jennes et al., 2017).

To better understand which molecular fraction of the highly heterogeneous crude extract has the highest potential to elicit a robust immune response, we applied continuous-elution electrophoresis to separate TLA proteins in an SDS-PAGE on MW and as such to collect different MW ranges. The subsequent application of these TLA pools in *in vitro* antigen stimulation of the PBMC's and isolated mononuclear cells (lymphocytes and antigen-presenting cells) allowed to test the full repertoire of the parasite proteins, comparable to a mutual use of the membrane and the soluble antigens, but without any prior selection of the targets, when using recombinant antigens (Kotresha and Noordin, 2010; Khammari et al., 2014). One of the restrictions of the continuous-elution electrophoresis technique, apart from the denaturing conditions prior to the SDS-PAGE and the manipulation of the sample, is that a partial mixing of one extract into the other is nearly inevitable; in that way the cells are exposed to a broader spectrum of proteins than strictly the MW range of a pool (Hafid et al., 1991; Marcolino et al., 2000; Fatoohi et al., 2004; Kotresha and Noordin, 2010; Khammari et al., 2014).

Based on the IFN-γ responses during the acute phase of the infection, we can hypothesize that the highly activated PBMC's, producing the foremost largest amounts of the cytokine, origin presumably from the systemic lymphocytes activation in the acute phase of the infection. The ingested bradyzoites initiate a local innate immune response in the intestines, but upon their conversion to tachyzoites, they disseminate in blood and are taken up by, among others, macrophages and dendritic cells. These populations will migrate to the lymphoid organs and present antigens to lymphocytes, so that a more systemic immune reaction can be expected. Consequently, the highest activation of PBMC's was demonstrated at 8 dpi, while immediately after this stimulation is passed on to the lymphocytes from the mediastinal, jejunal and ileal mesenteric lymph nodes, but to a much lesser extent. As the tachyzoites eventually reach the peripheral tissues and convert back to the bradyzoites, cyst formation obviously attracts primed immune cells to target the parasitic antigens (Filisetti and Candolfi, 2004; Murphy et al., 2008; Miller et al., 2009; Denkers, 2010; Cohen and Denkers, 2014; Sturge en Yarovinsky, 2014).

The results from the intestinal lymphoid tissue gave an indication that the duodenum might be the most preferable site of the *T. gondii* IPB-G strain for the initial host tissue invasion. Consequently, the concentration of the detected IFN-γ for the IPB-G was clearly higher in the duodenum draining lymph nodes than for the IPB-LR strain (Figure 7.4). Macroscopically, duodenum in IPB-G infected group was surrounded by massively inflamed lymph nodes at 8 and 14 dpi, as compared to jejunum and ileum (data not shown). On the contrary, in the IPB-LR infected group the lymph nodes in the duodenum area were less enlarged than in the jejunum and ileum as from 8 dpi. Apart from these findings, the cytokine production in other mesenteric lymph nodes was very comparable between those draining distinct segments of the intestine, and repeatedly higher for the IPB-LR strain infected animals than for the IPB-G inoculated group (Figures 7.5 – 7.6).

The acute phase of the infection seemed to involve the majority of fractionated proteins, namely the pools 1, 2, 4, 5 and 6. The highest cellular responses were initiated, although with some inter-strain variations by the pools 1, 4 and 6 in PBMC's, 2, 4 and 6 in mediastinal lymph nodes, 1, 2, 4, and 6 in duodenal lymph nodes, 1 and 4 in jejunal lymph nodes, and 1 and 2 in ileal lymph nodes. On the contrary, in the chronic phase experiment throughout the whole study pool 3 was the most immunodominant fraction in inducing IFN-γ stimulation in PBMC's. These preliminary data do not allow to identifying separated proteins, included within the molecular size ranges for the pools (20-40, 55-65 and 80-100 kDa respectively). Based on the published data and the band pattern obtained during the SDS-PAGE and Western blot, we can, however attribute these ranges to some parasitic antigens such as: heat shock protein 82 (HSP90) of 82 kDa (Hye-Jin et al., 2003), rhoptry 18 (ROP18) of 62.3 kDa (Qu et al., 2013), coronin protein of 54 kDa (Guang-Yuan et al., 2009), excreted-secreted antigen (ESA) of 41 kDa (Nockeman et al., 1998), SAG- related sequence 3 (SRS3) of 36.2 kDa (Amerizadeh et al., 2013); rhoptry 9 (TgROP9-p36) of 36 kDa (Reichmann et al., 2002), surface antigen 5 (SAG5) of 35 kDa (Amerizadeh et al., 2013), dense granules 7 (GRA7) of 29 kDa (Hiszczyńska-Sawicka et al., 2010), surface antigen 1 (SAG1) of 28 kDa (Marques et al., 2012), surface antigen 4 (SAG4) of 23 kDa (Amerizadeh et al., 2013) and surface antigen 2 (SAG2) of 22 kDa (Lau and Fong, 2008).

The majority of the listed antigens was applied in the experimental vaccination studies in different species (Jongert et al., 2007; Jongert et al., 2008; Li et al., 2011; Wu et al., 2012; Yin et al., 2015, Hu et al., 2017; Zhu et al., 2017). However, to date, no vaccine based on the use of a single protein or DNA target has been proven to prevent the intermediate host from *T. gondii* infection. Nevertheless, some successful attempts have been undertaken to partially or fully clear the parasite from the tissues of the infected animals upon immunization and challenge with a heterologous strain (Katzer et al., 2014; Burrells et al., 2015). Having in mind that the native antigens more efficiently mimic the immune response in a naturally infected host, immunization of

naïve piglets with isolated fractions from the viable parasite, and subsequently followed by a challenge with one of the previously applied strains, will be performed as a future prospect of this study.

Additionally, the pigs infected with IPB-LR showed an earlier elevated IFN-γ response than pigs infected with IPB-G. Due to intermittent cytotoxic activity of $CD8^+$ and macrophages towards the tissue cysts during a chronic infection, as postulated by others (Kim, 2015), a continuous antigen exposure activates the memory cells within the PBMC's, what results in a gradual increase in the concentration of the detected IFN-γ in both infection groups, as determined by the cytokine ELISA (Figures 7.10).

Summarizing, it is without any doubt that IFN-γ plays a crucial role in the survival of the parasite in the intermediate host; therefore, the observed differences in the potency of the immune response in time and between the strains could perhaps be correlated with the tissue distribution and parasite load, as earlier described (Jennes et al., 2017).

In the course of a common oral inoculation the parasite undergoes a stage conversion and intense multiplication in the intestinal tissues, followed by dissemination and encysting in the systemic tissues. In the earlier studies of a chronic *T. gondii* infection in pigs we investigated the parasite's distribution in brain, heart, diaphragm, intercostal and several skeletal muscles (Verhelst et al., 2011, Verhelst et al., 2015; Jennes et al., 2017). In a parallel study of an acute infection in sheep the parasites seemed to diminish in the intestinal tissues and appear in the systemic (lymphoid) tissues, while the heart tested positive in all inoculated animals and was, thus, the most reliable sample for demonstrating the presence of the parasite (Verhelst et al., 2015).

In our chronic infection study, the presence of parasitic DNA was detected in the majority of the tissues in the IPB-LR and IPB-G infected animals. However, we clearly noticed an inter-strain difference, when considering the parasite load (ΔCp values) in the distinct tissues. Although the group averages were rather similar due to the substantial SD values, the individual results showed more prominent divergences in the

amount of the detected parasite DNA. The trend of parasite reduction in the tissues of the IPB-G inoculated pigs, previously investigated and described by our research group (Verhelst et al., 2011; Jennes et al., 2017), was here confirmed, but this time by a more sensitive detection method.

In the earlier studies *T. gondii* detection was performed with the qPCR assay, characterized by the detection limit of 2-4 tissue cysts in 100 g of the tissue sample (De Craeye et al., 2011); opposite to that, the here applied MC-qPCR was optimized to detect 1 single cyst in 100 g of a tissue (Algaba et al., 2017). Even though several hundreds of bradyzoites are normally present per tissue cyst, the optimized technique reaches as few as 65 tachyzoites per tissue sample, highlighting the diagnostic value of this assay.

When comparing the most recent data of the MC-qPCR with the results of the bio-assay in mice, a similar conclusion can be drawn about the presence of the viable parasites upon inoculation of pigs with both strains: nearly 60% of the sampled tissues showed a reduced viability of the parasites of the IPB-G strain, as compared to 44% for the IPB-LR inoculated animals. Additionally, these findings were in agreement with the results of the MC-qPCR for the particular tissues, where none or few parasites were detected, resulting in the low ΔCp value. Overall, the results of both techniques showed a 100% specificity, but the sensitivity of MC-qPCR was slightly higher: 94.12% (80.32 - 99.28 95% CI), as compared to the bioassay: 86.49% (71.23 - 95.46 95% CI) (Algaba et al., in press). This could explain the higher detection rate of the parasite DNA in the tissues samples, which were considered negative or weak positive in bioassay (0-4/5 mice). However, it is noteworthy that among these samples, numerous (8 for the IPB-G and 3 for the IPB-LR) showed a low ΔCp value in addition to a reduced number of seropositive mice.

We would like to remark that despite these interesting results, it remains a subject of discussion, whether the isolated cells, especially these collected in the early stage of the infection, were still under the influence of the *in vivo* activation by the parasite, or

rather became reactivated by the exposure to the fractionated TLA. Indeed, at some of the early time-points (≤ 8 days) no memory cells will yet be developed. Furthermore, the exposure of the cells to TLA of a heterologous strain than the originally inoculated might decrease the efficiency of the stimulation (Rodgers et al., 2005).

As a future recommendation, in order to compare the activation status of the cells stimulated *in vitro* and *in vivo,* the cells from the same tissues and time points after infection but under non-stimulated conditions, should be analyzed by in a gene expression study such as RNAseq to fully understand tissue- and strain-specific immune mechanisms within the host.

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Chapter 8 General discussion and future prospects

8.1 Introduction

The objective of this doctoral thesis was to estimate the prevalence of *T. gondii* in pigs in Belgian herds and to gain novel insights into the molecular and immunological interactions between the porcine host and the pathogen in an experimental infection model.

To address and investigate these aspects, the setup of this thesis was divided into four main aims: (1) estimation of the true prevalence of *T. gondii* infection in Belgian swine; 2) evaluation of the strain and dose effects on the host's immune response and the parasite burden and viability; 3) investigation of the effect of reinfection with a heterologous strain on the immune responses and the parasite burden; 4) initial assessment of a potential vaccine candidates out of Total Lysate Antigen fractions by *in vitro* IFN-γ induction.

8.2 True prevalence and risk factors associated with porcine *T. gondii* infection

As mentioned in the literature review, nearly half (30-63%) of human foodborne toxoplasmosis originates from consumption of meat from infected livestock or game (Kijlstra and Jongert, 2008; Batz et al., 2012; Robert-Gangneux and Dardé, 2012; Opsteegh et al., 2016b). The incidence could be estimated as odds ratio or an approximate risk in a retrospective case-control study and the information obtained via a questionnaire, by identifying the potential risk factors among the subjects of the study. Hence, it should theoretically be possible to quantify the incidence rate of the foodborne toxoplasmosis by well-defined inclusion criteria and exclusion of the cases of CT, organ or blood transplants (Torgerson and Mastroiacovo, 2013).

Among domestic animal species worldwide, pork is indicated as one of the major meat sources associated with human foodborne *T. gondii* infection (Havelaar et al., 2015; Scallan et al., 2015). Therefore, the assessment of the infection rate in pigs is important for the development of prevention strategies of this zoonosis via pork.

Despite the numerous serological and molecular studies addressing *T. gondii* prevalence in multiple species, and pigs in particular (Dubey, 2009b; Guo et al., 2015b), along with the recommendations of the food safety institutions on the foodborne transmission mode of the infection (EFSA report 2007, Hill et al., 2008; Opsteegh et al., 2016b), no standardized approach has been developed so far for the implementation on national or international level. One of the difficulties is the impressive range of the available diagnostic techniques and of the targeted parasitic antigens, each with their own advantages and drawbacks in terms of sensitivity, specificity, and the practical implications. Nonetheless, the detection of *T. gondii*-specific antibodies against single, chimeric or multiple, native or recombinant parasitic antigens in serological assays could be a first, relatively fast, affordable and feasible indication for a recent or former exposure of the host to the parasite (Mancianti et al., 2010; Bhattacharyya et al., 2013). Despite these obvious benefits, the currently available serological assays still could be improved, as any of the systemic (e.g. sampling strategy), technical (e.g., assay procedure, antigen used) or biological factors (genetic strain, dose, viability of the pathogen, host's diversity) may have a substantial impact on the test outcome (Greiner and Gardner, 2000; EFSA, 2011).

In **Chapter 4** we provided a first estimation of the true and apparent prevalence of *T. gondii* infection in the porcine population in Belgium. To our knowledge, no earlier report on the number of *T. gondii* infected pigs had been made for Belgian herds. In our study we described the presence of the parasite-specific IgG in the Flemish and Walloon regions, using a recombinant and a native antigen.

When considering the choice of the antigens applied in this study, the combined use of multiple parasitic antigens (TLA), next to a single recombinant GRA7 protein, should increase the chance of T. *gondii* antibody detection, and therefore, provide a more reliable result (Bokken et al., 2015). Indeed, whereas the TLA-ELISA detected more frequently *T. gondii*-specific antibodies than the GRA7-ELISA, also some of the GRA7-seropositive animals were TLA-seronegative. Although GRA7-ELISA achieves

satisfactory sensitivity and specificity on animal sera, it also may yield false positive results from the cross-reaction with other species of the *Sarcocystidae*. accidentally ingested by pigs (Jacobs et al., 1999; Terkawi et al., 2013; Gondim et al., 2017). However, this reported cross-reaction might equally contribute to a higher detection rate in TLA-ELISA.

Yet, none of the two applied tests could serve as a gold standard, thus, false positive or false negative results cannot be excluded, when determining the apparent prevalence in the selected populations. Indeed, despite the obvious practical benefits, the currently available serological assays still could be improved. Although all the samples were collected, stored and processed in the same way, still a certain grade of variation was possible, affecting the final outcome of the assays. As mentioned earlier, any of the systemic (e.g. sampling strategy), technical (e.g., assay procedure, antigen used, operator) or biological factors (genetic strain, dose, viability of the pathogen, host's diversity) may have a substantial impact on the test outcome (Greiner and Gardner, 2000; EFSA, 2011). Having this in mind, to refrain from the imperfectness of the serological tests in general, and from the limitations of the use of a recombinant or a native antigen in this particular case, we performed a Bayesian statistical analysis to estimate the true prevalence.

This model requires prior information, derived from expert knowledge or previously published diagnostic data, to estimate the probability of two (or more) assays for a given disease, namely, the sensitivity and specificity of the applied tests, and correlation between the outcome of the assays in infected and non-infected animals (Branscum et al., 2004; Berkvens et al., 2006; Basso et al., 2013; Bokken et al., 2015). The statistical model developed by Branscum (2004) was adapted to fit into our study by not taking into consideration the conditional probability but to apply covariances between the two tests for infected and non-infected animals; as both assays used in our study reflect an equivalent information on the humoral immune response towards *T. gondii*, and both rely on the recognition of the proteins expressed by the same infectious stage

(tachyzoites), the results show possibly a certain degree of correlation. Additionally, the prior information about the tests' sensitivity and specificity, included in the adapted model, is derived from naturally infected individuals, therefore we could assume that the estimated true prevalence may be considered as accurate when applied on a population which also undergoes a natural infection, as in our study.

Our results demonstrated a significantly higher total, between-herd and within-herd apparent prevalence of anti-*T. gondii* IgG in Wallonia than in Flanders ($P < 0.001$) in both tests. Likewise, the estimated true prevalence confirmed our apparent findings across all data. Although the number of the sampled herds and animals within farms was limited, especially for the Walloon region, we have reasons to believe that the obtained results are a realistic estimation of the seroprevalence of *T. gondii* in the Belgian pig population. The type of farm management and its impact on the prevalence is strictly correlated with the extent of the outdoor access and the presence of the cats on farm, as clearly demonstrated by the studies in The Netherlands (Kijlstra et al., 2004; Meerburg et al., 2006; van der Giessen et al., 2007; Kijlstra et al., 2008). Here, only 0.0-0.4% of conventionally raised pigs tested positive, as opposed to 1.2-10.9% of the organic and 4.7-5.6% of free-range animals. Since the management type for the collected samples in our study is not confirmed, we can only speculate on the possible reason for the substantial discrepancies in the seroprevalence between both regions.

Summarizing, we described here the first results on the prevalence of *T. gondii* in Belgian pigs. Furthermore, the apparent prevalence and the specificity of both serological assays used in our study differed significantly between two sampled regions. These findings imply that serological tests are a valuable tool for indications on the infection status of animals, especially in large-scale populations, but due to their limitations, the statistical models such as Bayesian analysis could be a better alternative (Basso et al. 2013; Bokken et al., 2015). The non-equal distribution of the samples on Flemish farms, a substantial difference in the number of the included farms between Flanders and Wallonia, and no exact background data on the farms' management type

should be considered as drawbacks of this study. Moreover, the serological assays in general, despite their advantages in terms of feasibility, do not demonstrate the presence and the distribution of the parasite within the tissues. Referring to that, as the parasite was successfully isolated from seronegative animals (Opsteegh et al., 2016a and 2016b), we can hypothesize that antibodies can be beyond the detection level, do not persist life-long in domestic animals as in humans, or not all the infections in certain species (such as cows and horses) lead to developing antibodies against the pathogen (Opsteegh et al., 2011, Opsteegh et al., 2016a and 2016b). On the other hand, antibodies could be produced as a result of the exposure to the parasite but without establishment of an infection, or, as discussed further, the humoral response persist, while the clearance of the cysts in the host's tissues might occur. This implies that the number of the infected animals could be easily under- or overestimated, which might increase the risk on the transmission of the zoonosis to the consumer or exclude seropositive animals from the food chain, respectively. Consequently, the detection of *T. gondii* DNA or the viable parasites in the tissues of farm animals is more valuable for the prediction of the human infection's risk than serological methods. We elaborated further on this in **Chapter 5, Chapter 6** and **Chapter 7**.

8.3 Parasite burden and tissue distribution in the acute and chronic infection model

As postulated in **Chapter 4** and in 8.2, serology on its own has a high diagnostic value for the detection of the ongoing infection within the herd, irrespective of the use in the apparent or true prevalence calculation. However, the presence (or absence) of *T. gondii*-specific antibodies does not provide sufficient information on the strain virulence, the distribution within the host and the parasite load per tissue. Moreover, a poor correlation between the serological status of pigs determined by ELISA and the direct parasite's detection via bioassay or molecular techniques is not unusual, as demonstrated by Belgian research group (Verhelst et al., 2011) and recently confirmed

in an EFSA report (Opsteegh et al., 2016b). Since the incidence of naturally acquired porcine toxoplasmosis is difficult to predict and distinguish, due to the lack of clear clinical symptoms, we performed a series of inoculation experiments in weaned *Toxoplasma*naïve piglets to perform a multi-parameter analysis on both the parasite's distribution and viability, as well as on the induced immune responses of the host (see 8.4). The findings of these studies, addressing the second and third aim of this doctoral thesis, were described in **Chapter 5**, **Chapter 6** and **Chapter 7**.

The first investigation of *T. gondii* dissemination upon experimental infection with the IPB-G strain was focused on the comparison of the parasite load obtained by qPCR between several porcine tissues and the viability of the parasite at 6 wpi, as highlighted in **Chapter 5**. The results of the former studies revealed a significantly diminishing amount of the parasite DNA to undetectable levels in the skeletal muscles at 6 mpi, and the persistence of the cysts exclusively in brain and heart tissues (Verhelst et al., 2011; Verhelst et al., 2015). While the results of the bioassay were consistent with the qPCR in the chronic infection model, showing a substantial decrease in the infectious capacity over time, the outcome of the bioassay from samples collected at 6 wpi indicated a higher sensitivity of the bioassay above qPCR. Earlier studies have already mentioned the interference of porcine DNA with the qPCR's sensitivity, and a better performance of the bioassay (Garcia et al., 2006, Hill et al., 2006). Interestingly, heart and brain tested consistently positive in both techniques at the acute and chronic stages of the infection. Therefore, these tissues seem to be a reliable indicator to demonstrate the parasite's presence upon *T. gondii* infection in pigs. Although the heart consists of relatively limited amount of muscle tissue, it showed a reasonable concentration of the parasite in comparison with the bigger muscles. As observed in the subsequent studies, the amount of parasite DNA was clearly higher in the heart tissue of animals from the latter experiments (**Chapter 6** and **Chapter 7**) than from the former study (**Chapter 5**) due to the 2-fold difference in the inoculation dose (6000 versus 3000 tissue cysts, respectively). This is in line with the recently published EFSA

report, investigating the predilection sites in multiple species of livestock (Opsteegh et al., 2016b). Based on these novel findings, a hypothesis of the clearance of parasite infection from the edible tissues was formulated, which was further evaluated in the subsequent experiments. Additionally, we aimed to compare the surprising results obtained with the IPB-G strain with a genetically different IPB-LR strain to fully address the predefined second aim of the thesis. Hereto, we performed a study of each infection group in parallel, investigating both the inoculation dose and the strain effect on the final infectious capacity of the edible tissues in pigs at 4 mpi. **Chapter 6** describes the experimental setup and the major findings of the performed research. The obtained results emphasize the dose and strain-dependent parasite burden of *T. gondii* in the porcine tissues. Importantly, a clear correlation was found between the amount of detected DNA and a nearly 10-fold difference in the inoculation dose. However, this correlation was reversed when inoculated with a high dose of the IPB-G, since an absence or a strong decline in the parasite load was detected in the porcine tissues, which is of pivotal importance for the estimation of the parasite burden, and, hence, for the risk for human infection. On the contrary, upon inoculation with a low dose, the parasite was more prominent in terms of predilection sites and the DNA concentration. Yet, still some muscle tissues remained negative at 4 mpi, indicating the exceptional characteristics of the IPB-G strain even with the minimum inoculation dose. Reversely for the IPB-LR, the inoculated animals presented the highest parasite load in heart and brain, and no alteration of the distribution or load in the tissues in qPCR, as expected. Further, in the next experiment we investigated the effect of a heterologous challenge on the same parameters (G_{high}/LR_{high} and LR_{high}/G_{high} groups). Here we described the effect of the prior infection on the outcome of the subsequent challenge: as shown in the LR_{high}/G_{high} group, the animals, which were first inoculated with the IPB-LR strain, followed by the challenge with the IPB-G strain, showed a much lower parasite tissue distribution than observed after a single IPB-LR strain infection. These observations seem to be crucial for the understanding of the mechanisms of chronic *T.*

gondii infection in pigs, since they underscore the impact of the native antigens in the induction of the efficient immune response. The parasite distribution among the tissues and the amount of the detected DNA was much lower, yet not significantly different as with the IPB-LR strain alone. This striking observation has not been exclusively described for genotype II strains (Velmurugan et al., 2009; Suzuki et al., 2012), but also for type I strains (Burrells et al., 2015).

In parallel to the qPCR, the infectious capacity of the tissues from the single inoculation and heterologous challenge was evaluated by bioassay in mice. Interestingly, as described in **Chapter 6**, the detected reduction in parasite load in qPCR was in full agreement with the results of the bioassay. In contrast to the earlier study (Verhelst et al., 2011), the results proved a substantially higher sensitivity of the qPCR method than of the bioassay. The qPCR is a direct detection technique with many advantages, as it is very sensitive and specific, amplifying a parasite-specific DNA fragment, but also fast and relatively easy to perform and interpret (Homan et al., 2000). The here applied qPCR technique has been further optimized for the diagnosis of the parasite in human or animal tissues, with a detection limit of 2-4 tissue cysts per 100 g of sample (De Craeye et al., 2011). Nevertheless, we are aware that further reducing this threshold is desirable, as the dissemination of the tissue cysts per sample varies but is mainly very low. Additionally, the cysts are slowly replicating and encysted intracellularly, by which their detection might even be more difficult. As one tissue cyst contains on average hundreds to thousand(s) of single bradyzoites, the acute toxoplasmosis resulting from the cysts' reactivation and release, or the ingestion of the infected meat, has an exponentially increasing severity. Therefore, efforts have been made by the collaborating research group to further optimize the existing Magnetic Capture-qPCR technique for the isolation and enrichment of the tissue cysts from animal samples (Opsteegh et al., 2010). The recently optimized novel technique has a proven increased sensitivity in comparison with the bioassay and the conventional qPCR, reaching to 65 tachyzoites or a single tissue cyst per 100g of the sample (Algaba et al., 2017). The first

application of this assay on experimentally infected porcine tissues has been depicted in **Chapter 7**, describing the latest performed inoculation study in this dissertation. Unlike in the earlier studies, nearly all the tissues from the chronically IPB-G infected animals (168 dpi) tested positive for the presence of *T. gondii* DNA, similar to the IPB-LR infected group. The reason for that is the considerably increased sensitivity of the applied detection assay. However, the main findings concerning the parasite load and tissue distribution are in both groups in concordance with the previously observed trend, showing that the highest parasite load was detected in brains and hearts of inoculated animals from both groups. Strikingly, the individual results of the ΔCp values and the bioassay in mice, although with a certain variation among the animals and the tissues, confirmed our hypothesis that the IPB-G infected animals have a lower parasite load and a decreased infectious capacity of the tissues upon inoculation than the IPB-LR group (Verhelst et al., 2011, Verhelst et al., 2015, Algaba et al., 2017; Jennes et al., 2017).

As a confirmation of our results, others have described a reduction in parasite burden in pigs in vaccination and challenge experiments (Kringel et al., 2004; Garcia et al., 2005; Jongert et al., 2008; Burrells et al., 2015). In those studies, similar to our findings, an enhanced Th1 immune response seems to initiate host's protection mechanism, resulting in an elimination of the parasite. The immunological aspects associated with this crucial difference between the strains are further discussed (**Chapter 6, Chapter 7** and 8.4).

8.4 Strain dependent immune responses in the acute and chronic infection model

As illustrated by the qPCR results and the bioassay data on the parasite load and *T. gondii* viability in the examined tissues from the experimentally infected animals (**Chapter 5**, **Chapter 6** and **Chapter 7**), our results strongly suggest a dose-dependent decrease of the IPB-G strain burden in tissues following inoculation, pointing towards immune-mediated mechanisms. To address this assumption, we investigated different

immunological parameters along with the infectious capacity of the tissues. In each set of the experiments, humoral and cellular responses were evaluated via the detection of *T. gondii*-specific antibodies (IgM and IgG), and the quantification of the major cytokine (IFN-γ), expressed as mRNA or synthetized protein. For this, we focused on the systemic responses in serum and the *in vitro* activation of PBMC's or isolated lymphocytes upon stimulation with the parasitic antigens.

The involvement of the innate and acquired immune system was previously observed in several experimental infections in pigs, dominated by antibody production against the parasitic antigens, and by a Th1-type immune response (Solano Aguilar et al., 2001; Dawson et al., 2004; Kringel et al., 2004; Dawson et al., 2005, Garcia et al., 2005; Jongert et al., 2008; Verhelst et al., 2011; Verhelst et al., 2015). Our studies confirmed these findings, since irrespective from the strain and the infection dose, an early IgM and IgG GRA7-specific antibody production was initiated. In contrast with that, the TLA-response remained delayed in all the experiments, but this was compensated by the longer persistence of the elevated IgG titers against the lysate antigens. Novel information from our research included the dose and the strain effects on the primary GRA7- and TLA-specific antibody responses, which were detected earlier for the first and had a higher extent for the latter.

In addition to the enhanced humoral immunity, a polarized Th1-immune response is predominant during acute and chronic *T. gondii* infection in different hosts. Briefly, as extensively described in **Chapter 2**, the uptake of the parasite by antigen presenting cells (DC's or macrophages) leads via IL-12 and TNF-α secretion to the activation of other cell populations (Suss-Toby et al., 1996; Carruthers, 2002; Kasper et al., 2004; Gregg et al., 2013; Cohen and Denkers, 2014). Activation of this pro-inflammatory pathway requires interactions of PRR's, among which TLR's, on immune cells with parasite ligands (Miller et al., 2009; Andrade et al., 2013; Koblansky et al., 2013; Gazzinelli et al., 2014). From here, DC dependent IL-12 production activates Th1 and NK cells, which are responsible for the massive IFN-γ release (Sturge and Yarovinsky,

2014). The subsequent cytokine-dependent expression of GTPases leads to the recruitement of immunity-related GTPases (IRG's) and guanylate-binding proteins (GBP's) (Yarovinsky, 2014). These effector proteins target the parasite attachment site at the host cell and are directly involved in lethal damage to the parasitophorus vacuole (PV), causing the release and elimination of the parasite of the parasite (MacMicking, 2004; Taylor et al., 2004; Liesenfeld et al., 2011; Gazzinelli et al., 2014).

It is important to mention that IFN-γ-inducible IRG's are well studied in murine models, where 23 different genes have been identified to date. The data on the identification of porcine GTPases are scarce, but a high similarity to the human IRG's is mentioned (MacMicking, 2004). Only two porcine GBP's have been reported until now: GBP1 and GBP2, whereas in humans 7 different GBP's have been identified (MacMicking et al., 2004; Li et al., 2016). However, several IRG's have been found using Affymetrix GeneChip® Porcine Genome Array but a detailed study in pigs is lacking (Fossum et al., 2014).

Followed by the innate immune responses as the first defense line against the parasite's invasion, T-cells of the acquired immunity take over the IFN-γ production during the sub-acute and chronic infection stage. Experimental infections in mice (Jongert et al., 2010; Suzuki et al., 2012) demonstrated the importance of $CD4^+$ and $CD8^+$ IFN-γ producing T cells in maintaining a chronic *T. gondii* infection, but the exact contribution of each subset remains unknown. In line with that, due to their IFN-γ-independent cytolytic activity, the role of primed $CD8^+$ T cells in the host's immunity during chronic toxoplasmosis has been widely acknowledged (Wang et al., 2005; Suzuki et al., 2012; Sa et al., 2013). In pigs, only a few experiments identified $CD8^+$ and $CD4^+CD8^+$ cells in the acute phase of the infection as the major source of the IFN-γ production (Solano Aguilar et al., 2001; Dawson et al., 2005). Our data provide the first insights into the immune response during a chronic infection model in pigs and indicate the $CD8^+$ T cell subset with the highest percentage of IFN-γ positive cells, followed by the Th1 $CD4^+$ subset.

In perspective of future experiments in pigs, it would be desirable to focus on other immune cells, involved in the responses throughout the infection process such as, the immunosuppressive T regulatory (Treg) or Th17 cells. As recently shown in mice, the robust immune reaction expressed by the high IFN-γ levels in the acute phase of the infection severely reduces the activity of Tregs in an IL-2 dependent and IL-10 independent manner (Tenorio et al., 2011; Olguin et al., 2015). In a longitudinal clinical case study of human acquired cerebral toxoplasmosis a dual function of the Treg population was described, by simultaneous down regulation of $CD4^+$ and activation of pathogen-specific $CD8^+$ T lymphocytes (Rb-Silva et al., 2017). In human congenital infections not only the $CD4^+$ Treg cell population seems to be involved in the immune reaction triggered by *T. gondii*, but also a different subset of $CD4^+$ or $CD8^+$, namely Th17. The activity of this population is independent from IFN-γ, IL-4 and perforin activation, as their migration to the inflammation sites in initiated by certain chemokines. Interestingly, the results of the *in vitro* PBMC's stimulation with tachyzoites showed a higher percentage of $CD4^+$ IL17 producing cells above the $CD8^+$ (Silva et al., 2014). By investigating the fluctuations of the activity of Tregs and Th17 cells during the acute and chronic phase of the infection in a porcine model, along with the sensitive parasite detection in the tissues, we might determine whether these populations are also involved in the persistent immunity towards the parasite.

In the single-strain inoculation experiments of pigs performed by others (Solano Aguilar et al., 2001; Dawson et al., 2004; Dawson et al., 2005; Jongert et al., 2008; Verhelst et al., 2015), the significantly increased IFN-γ in serum and supernatant from cultured PBMCs, or expressed as IFN-γ mRNA in PBMCs and intestinal lymphoid tissues, was clearly correlated with the duration of the experiments. Likewise, we successfully demonstrated a time- and dose-dependent increase in IFN-γ mRNA expression upon infection with the IPB-G strain; the inoculation with the IPB-LR strain, led immediately to a strong and persistent IFN-γ production by PBMCs, regardless of the inoculation dose. When considering the cell or tissue specific IFN-γ responses shortly after the

inoculation, we can conclude that PBMCs were activated to a much higher extent than other investigated cell populations isolated from the lymphoid tissues (**Chapter 6** and **Chapter 7**). However, we postulate that these systemic responses arose from an initial T-cell activation within the peripheral lymphoid organs, upon parasitic antigen uptake and presentation by cells of the innate immune system. Consequently, the activated T-cells migrate through homing process via blood to multiple sites, and as such, were included in our PBMC's population. This hypothesis was confirmed by the results from the mediastinal lymph nodes, draining heart in the mediastinum and the posterior surface of diaphragm, where a higher IFN-γ concentration was detected, in comparison with the intestinal lymphoid tissues. As shown by the parasite detection via qPCR in our experiments (see 8.3) and by others (Opsteegh et al., 2016b), heart has been declared as one of the major predilection sites with substantially high parasite load, but it is still impacted by the inoculation dose, as clearly demonstrated in our studies. In addition, negligible cytokine levels were found in bone marrow in the first four weeks following the infection, implying that no or only few parasites migrate to the bone marrow.

The chronic infection model involved memory cells within the PBMC's, which are activated by re-exposure to antigens slowly released from the tissue cysts in the later phase of the infection. That could explain the gradual increase in the concentration of IFN-γ in both infection groups, as determined by ELISA and the qPCR (**Chapter 6** and **Chapter 7**).

Data obtained from the intestines revealed that the initial host tissue invasion presumably takes place in the proximal part of the gut (**Chapter 7**). These findings are in line with the parasite DNA detection in acute infections in sheep (Verhelst et al., 2014), where a gradual decrease in parasite load was observed from the proximal to distal segments of the intestines, in the early phase of the infection. Other experimental infections with a genotype III-strain (VEG) in pigs (Dawson et al., 2005) showed a significant expression of IFN-γ mRNA in the majority of the intestinal tissues at 7 dpi,

irrespective of the anatomical location, as compared to control animals. In line with our findings, one week later none of the intestinal tissues still contained increased cytokine mRNA, proving a partial resolution or redistribution of the infection from mucosal to systemic sites. Together with the clear cytokine profile in the acute infection model, also the high expression of the CD3 was observed, implying the involvement of the T-cells in this early response. Likewise, in our infection experiments (**Chapter 6**) we detected significantly increased $CD3^+$ IFN-γ^+, Th1 $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ lymphocytes in both infection groups in the early phase of the infection.

However, further research is desirable to reveal the populations and pathways involved in the early immune response against *T. gondii*. To start with, the innate regulation mechanisms and signalling pathways of the IFN-γ-expression, such as STAT1, MYD88, IRF1 or IRF8 should be elucidated in experimental models via RNAseq technology. These genes modulate the immune responses leading directly or indirectly to IFN-γ production by T-cells, and, by this, to resistance against the parasite (Denkers, 2003; Szabo et al., 2003, Hou et al., 2011; Yarovinsky, 2014). However, the mutual relations between these pathways are still not fully discovered.

Finally, the potency of several native fractions of antigens to trigger the immune response *in vitro* was investigated in the last experimental study (**Chapter 7**). By addressing the last aim of the thesis, first steps were taken towards the search for a potential vaccine candidate among the isolated protein fractions. Although in the acute phase of the infection the majority of the fractionated proteins induced *in vitro* cytokine production by PBMC's, in the chronic phase mainly pools 1, 3 and 5 were the foremost immunogenic fractions). Yet, performing future immunization and challenge studies on the selected fractions and the detailed analysis of the immunogenic epitopes would significantly contribute to the understanding of the mechanisms of the host-pathogen interactions on molecular level, and could serve as a diagnostic tool or a potential vaccine candidate for porcine toxoplasmosis.

Based on the mutual results of the detection of IFN-γ and the parasite DNA in tissues (**Chapter 6** and **Chapter 7**), we could speculate about maintaining a balance between the host defense mechanisms and the invasion of the parasite. Upon inoculation with the IPB-LR strain, the high IFN-γ production during the infection was associated with high counts of parasite DNA in the porcine tissues. On the contrary, in the IPB-G groups IFN-γ production was elevated in a later phase of the infection and resulted in a very low to undetectable parasite load in some tissues, implying that high IFN-γ levels can tip the balance in favour of the host. Intriguingly, based on our observations and unpublished data from the acute infection model with the same strains (Jennes et al., in preparation), we speculate that exposure to a high dose of the IPB-G strain is more effective in activating innate immunity at the primary sites of infection than the low dose of the same strain or the inoculation with the IPB-LR strain. The strain-dependent differences in the IFN-γ production profile may result from the genetic and thus, biological features of the used strains, indicating expression of variable virulence factors towards the intermediate host.

In our opinion and according to others (Hunter and Sibley, 2012), the IPB-LR as genotype II strain, activates another pathway than the IPB-G strain, which is an atypical genotype (mixed genotype I and II). The virulence factors initiating a pathway would be GRA15 (via NF-κB) for the former, and ROP18 (via STAT3/STAT6 pathway) for the latter. Consequently, the IPB-LR induces Th1 type of protective immunity and remains persistent in the chronic phase. In our view, the IPB-LR strain does not show an acute virulence but it is adapted to persist within the intermediate host and, as such, increase own survival. To support these speculations, we observed a much milder macroscopic manifestation upon inoculation with the IPB-LR strain (Jennes et al., in preparation).

We further hypothesize that resistance to the chronic infection in the IPB-G model results from the high acute virulence and the subsequent fast elimination of the tachyzoites before they can successfully multiply and disseminate. Consequently, fewer

parasites can survive the initial parasitaemia, which eventually will lead to reduced numbers of cysts in the tissues.

8.5 Conclusions and future prospects

This doctoral dissertation describes various aspects of the interactions between *T. gondii* and the pig as an intermediate host. In the quest of searching for answers to the scientific questions included in the aims of this study, we provided data on the apparent and true prevalence of porcine *T. gondii* infection in Belgium. Further, we elucidated and quantified the parasite dissemination in a series of experimental infections, and compared the detection of *T. gondii* DNA with the results on the viability of the parasite in the edible tissues via bioassay in mice. In the last experimental chapter we applied the recently developed detection technique, which is proven to be suitable for the diagnosis of human or animal toxoplasmosis. Moreover, we investigated various immune responses of the host towards the pathogen, in correlation with the infection dose, strain and time upon inoculation. We could identify the major cellular players, contributing to the massive IFN-γ production in the acute and chronic infection model. Finally, we identified the fractionated parasite-derived antigens, which will be evaluated for their immunogenic potential in the upcoming vaccination and challenge experiment. Although the results of the present work have added new insights into the host-pathogen interactions to extend our understanding of many aspects of porcine toxoplasmosis, further studies are needed to fill in the missing gaps in the knowledge gained.

First, the prevalence study underscored the potential impact of the farm characteristics on the prevalence of *T. gondii* among pigs, such as geographical location, distribution, amount and density of the animals, the size of the herd or the management type. In the ongoing study within our research group different parameters on herd level, including a questionnaire on biosafety regulations and seroprevalence status of the sampled animals are being investigated in relation to the risk for *T. gondii* infection in

the finisher pigs. Further investigation is, however, necessary to draw any conclusions on the risk factors and the transmission modes for *T. gondii* into the pig population in Belgium.

Secondly, as we demonstrated the involvement of phenotypically different T-cell populations in the cellular response following the infection, we would like to postulate to study these and other cell populations more in detail. The involvement of the cells of the innate immune system such as NK's cells, together with the T-cells classification in terms of MHC class or T-cell receptor (TCR), combined with the identification of the epitopes for the parasite recognition via epitope mapping technique, could help us better understand the acute events following the inoculation. On top of that, the expression of the genes, up- or downregulating the immune cells' activation at the predilection sites, should be analyzed by the gene expression study such as RNAseq.

Thirdly, we described the important differences between the strains used in this study. Based on our data we propose that the IPB-G strain has the capacity to induce a robust immune reaction in the host in the early phase of the infection. This IFN-γ-mediated response in pigs may potentially lead to elimination of the tissue cysts during the chronic infection. The obtained resistance against *T. gondii* infection by vaccination would greatly contribute to the decrease of infection risk for food safety and human health.

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Summary

Toxoplasma gondii is an ubiquitous parasite with a significant impact on human's health and livestock production. The infection in humans is mainly subclinical but it may lead to severe or fatal clinical symptoms in newborns and immunocompromised patients, while toxoplasmosis in domestic animals remains important due to a high rate of loss in animal production. The parasite has a complex life cycle, with three distinct developmental stages: tachyzoites, bradyzoites enclosed in the tissue cysts and the oocyst with sporozoites. Human toxoplasmosis is predominantly foodborne and originates from the consumption of raw or undercooked meat containing tissue cysts or from eating raw vegetables contaminated with oocysts.

Pork is an important source of toxoplasmosis due to the frequent consumption of fresh or poorly processed porcine meat products, a high susceptibility of pigs to the infection, and the numerous risk factors, contributing to the persistent prevalence of toxoplasmosis in the pig population. Therefore, an efficient control of *T. gondii* by prevention of the transmission via infected pork is strongly recommended. However, knowledge on the prevalence of porcine toxoplasmosis, the parasite burden in the edible tissues and immunological parameters indicating an acute or chronic infection, is incomplete.

Determining the *T. gondii* seroprevalence is a first step in estimating the risk related to consumption of pork. Following infection, the innate and adaptive immune responses do not prevent development of the tissue cysts, which are accepted to persist lifelong in a dormant state within muscles and brains of most intermediate hosts. Nevertheless experimental vaccination can considerably decrease the parasite burden in tissues. Therefore, the knowledge of the mechanisms regulating the interactions between the parasite and the porcine immune system in both the early and later stage of the infection, as well as the estimation of the parasite's persistence in porcine tissues on the long term, could substantially contribute to a better control and prevention of infection in pigs. Consequently, it could play a pivotal role for global food safety and human health by diminishing the risk of foodborne toxoplasmosis.

Part I of this thesis provides a comprehensive overview of the current literature on *T. gondii* as a successful and underdiagnosed parasite of humans and animals worldwide.

Chapter 1 describes the biology and the transmission routes of *T. gondii* towards the intermediate hosts. Attention was given to the zoonotic aspects of the infection, in particular the clinical symptoms and severe consequences in humans. Furthermore, the diagnostic methods were widely presented with their advantages and limitations, along with the therapeutic and preventive options.

Chapter 2 extensively highlights all the crucial players of the innate and adaptive immune responses, involved in the early and late immunity against the parasite. In order to gain insight in the host's defense mechanisms, the general pathogenesis was first depicted, followed by the subsequent immunological events in the acute, subacute and chronic phases of the infection.

In **Chapter 3** the role of the pig as the natural intermediate host for *T. gondii* and as the source of the zoonosis for humans is fully explained, together with the details on the infection's prevalence in various countries. Importantly, the risk factors contributing to the parasite's persistence in the pig population are listed, followed by recommended preventive measures to decrease the risk of the transmission towards humans.

Part II includes the aims of the experimental work that was performed during this PhD study. The focus of this thesis was to estimate the infection rate with *T. gondii* of the porcine population in Belgium, and to investigate the immune responses in pigs upon an experimental infection in relation to the parasite burden and its viability in edible tissues. Special attention was also given to the parasite antigens with the highest immunogenic potential in *in vitro* assays, as possible candidates for a porcine vaccine against *T. gondii*.

Part III describes in detail the experimental studies in order to meet the objectives of the study, and provides new insights in the host-parasite interaction in pigs in the acute and chronic infection model.

In **Chapter 4** we provide a first estimation of the apparent prevalence of porcine toxoplasmosis in Belgium, based on two serological assays (GRA7- and TLA-ELISA) and applied on 2263 serum samples from 251 conventional herds with intensive management system. Surprisingly, the results of both tests showed a significantly higher total, between-herd and within-herd apparent prevalence of anti-*T. gondii* IgG in Wallonia than in Flanders. Additionally, we employed a Bayesian model to assess the true within-herd and between-herd prevalence based on the results of both diagnostic tests to correct for the limitations of the serological assays in biological samples. The total true prevalence across all herds, the true between-herd prevalence reached and the true within-herd prevalence of infected farms were significantly higher in Wallonia than in Flanders. Further, based on the estimation of the assays' characteristics, the specificities of the serological tests were significantly higher in Flanders, and for the GRA7-ELISA in particular, while the sensitivities of both ELISA's were not significantly higher in Wallonia. This study is the first epidemiologic report on *T. gondii* infection seroprevalence in Belgium, with specific focus on a high burden in Wallonia.

In **Chapter 5** the major findings of the first experimental infection with *T. gondii* in pigs are presented. The cytokine expression in blood mononuclear cells and the antibody production were followed in seronegative piglets upon inoculation with the IPB-Gangji strain. Among the studied cytokines (IFN-γ, IL-4 and IL-10) only the expression of IFN-γ was elevated in comparison with the control animals or day 0. The serum antibody responses against native (*Toxoplasma gondii* total lysate antigen) or recombinant (rGRA1, rGRA7, rMIC3 and rEC2) parasitic proteins showed a clear rGRA7-reaction, while remaining beyond the detection level for other antigens. The parasite burden and viability was demonstrated via qPCR and bio-assay in all the sampled porcine tissues. However, in comparison with an earlier study, these findings are important in

relation to the parasite persistence in the tissues over time, and motivate the hypothesis of the parasite clearance in a chronic model of IPB-Gangji strain infection in pigs.

Chapter 6 follows the previous study but provides more information on the strain- and dose dependent outcome of the experimental infections in pigs. Here we performed homo- and heterologous infection experiments with two distinct *T. gondii* strains (IPB-LR and IPB-Gangji), and evaluated several parameters, such as the host's immune response on the one hand, and the parasite burden on the other hand. First, an extensive humoral response was observed against GRA7 and TLA antigens upon inoculation with both strains. For both IgM and IgG, the GRA7-specific antibodies were detected very soon after the initial infection, while the TLA-specific IgM and IgG appeared later but persisted longer and at high titers in the course of the infection. Second, the *in vitro* IFN-γ production by TLA-stimulated blood mononuclear cells was directly correlated with the infection dose of both strains. As investigated by flow cytometry upon intracellular staining of isolated blood mononuclear cells, $CD3^{+}CD4^{-}CD8\alpha^{bright}$ T-lymphocytes were predominantly involved in the production of IFN-γ. Finally, in homologous infection experiments we demonstrated a strain-dependent parasite persistence in the tissues that was inversely correlated with the infection dose. In the heterologous challenge experiment, consisting of a primary infection with the IPB-LR strain, followed by a challenge with the IPB-Gangji strain, a remarkable reduction of the parasite burden was observed. Therefore, our results strongly indicate a reduction in the amount of parasite DNA and viable cysts in porcine tissues over time due to the potential of the IPB-Gangji strain to elicit a strong immune response in the host.

In **Chapter 7** we proceeded with further investigating the host-parasite interactions upon acute and chronic experimental infections with the IPB-LR and IPB-Gangji strains, and with studying the direct effect on immunological parameters and parasite load. Based on the promising results of the former study and the substantial TLA-stimulation

of the blood mononuclear cells, we investigated the *in vitro* activation of T-lymphocytes by *T. gondii* antigens, fractionated prior to use by continuous elution-electrophoresis and subdivided into 6 pools. Induction of IFN-γ was determined by ELISA in supernatant of the stimulated blood mononuclear cells or lymphocytes isolated from the lymphoid tissues. In general, although not for all tissues, the animals infected with the IPB-LR strain produced higher quantities of IFN-γ than pigs inoculated with the IPB-Gangji, and that after stimulation with each TLA-fraction. Referring to that, the cells isolated from the mediastinal lymph nodes and duodenum in the IPB-Gangji infected group showed an opposite trend. This study demonstrated that the amount of the produced IFN-γ upon *in vitro* stimulation by TLA pools varies considerably between the groups infected with different strains of *T. gondii* and between cells isolated from different tissues.

Chapter 8 provides a general discussion, highlighting the main findings of each experimental study, as well as the conclusions and future perspectives for further research. This thesis describes the first estimation of the true and apparent prevalence of porcine toxoplasmosis in Belgian herds and the considerable differences between the two regions. However, as the information on the farm management is lacking, a follow-up study is recommended to take this constraint into account and elaborate further on the risk factors contributing to *T. gondii* prevalence in the porcine population.

The subsequent experimental studies in pigs provided novel information on the strain-, dose- and time-dependent outcome of infections in terms of the parasite distribution and viability, in relation to the parameters defining the host's immunity. The general findings from these studies confirmed our initial hypothesis, proving that the IPB-Gangji strain, by eliciting a potent immune response, can induce a substantial reduction of the parasite distribution in the tissues.

We therefore postulate that the IPB-Gangji strain could be used as a promising tool in lowering the risk for human toxoplasmosis. To achieve that, more insights should be

gained in regulation of the innate and adaptive immune responses of the host. It is beyond doubt, however, that the mechanisms up- and downregulating the porcine immune responses against the parasite are as complex as in mice or humans. Additionally, attention should be given to the parasitic antigens, generating the strongest humoral and cellular immune responses in the host, to identify the best candidate to be included into a potential vaccine in pigs. Therefore, further research is warranted to elucidate these aspects and to contribute to a better understanding of the underlying host-pathogen interactions.

Samenvatting

Toxoplasma gondii is een wereldwijd verspreide parasiet met een significante impact op de gezondheid van mens en dier. De infectie bij mensen verloopt meestal subklinisch maar kan geassocieerd worden met ernstige tot fatale klinische symptomen bij pasgeborenen of bij patiënten met een verzwakt immuunsysteem, terwijl toxoplasmose bij huisdieren belangrijk blijft omwille van de hoge verliezen in de dierlijke productie. De parasiet heeft een zeer complexe levenscylus met drie opeenvolgende ontwikkelingsstadia: tachyzoïten, bradyzoïten in de weefselcysten en de oöcysten met sporozoïten. Humane toxoplasmose wordt overwegend door voedsel overgedragen en zeer frequent door consumptie van rauw of onvoldoende doorbakken vlees.

Varkensvlees is hierbij een belangrijke bron van toxoplasmose door de uitgebreide consumptie van verse of onverwerkte varkensvleesproducten, een hoge gevoeligheid van varkens voor de infectie en de talrijke risicofactoren die bijdragen tot een blijvende prevalentie van toxoplasmose in de varkenspopulatie. Een efficiënte controle van de *T. gondii* infectie bij het varken kan dan ook de overdracht via besmet varkensvlees sterk reduceren. Hiertoe is goede kennis vereist van de prevalentie van toxoplasmose bij varkens, van de verspreiding van de parasiet in de varkensweefsels bestemd voor de consumptie en van de immunologische parameters die indicatief zijn voor een acute of chronische infectie essentieel.

Deel I van dit proefschrift geeft een uitgebreid overzicht van de huidige literatuur over *T. gondii* als een succesvolle en onderdiagnosticeerde parasiet van mens en dier.

Hoofdstuk 1 beschrijft de biologie en de transmissieroutes van *T. gondii* naar de intermediaire gastheren. Aandacht werd gegeven aan de zoönotische aspecten van de infectie, met name de klinische symptomen en ernstige gevolgen voor de mens. Bovendien werden de diagnostische methoden uitgebreid besproken met hun voordelen en beperkingen, samen met de therapeutische en preventieve interventies.

Hoofdstuk 2 beklemtoont alle belangrijke spelers van de aangeboren en adaptieve immuunresponsen, die betrokken zijn bij de vroege en late immuniteit tegen de parasiet. Om inzicht te krijgen in de afweer van de gastheer, werd eerst de algemene pathogenese besproken, gevolgd door immunologische responsen in de acute, subacute en chronische fase van de infectie.

In Hoofdstuk 3 worden de rol van het varken als intermediaire gastheer voor *T. gondii* en als bron van zoönose voor mensen volledig behandeld, samen met de prevalentie van de infectie bij varkens in verschillende landen. De risicofactoren die bijdragen aan de persistentie van de parasiet in de varkenspopulatie worden opgesomd, gevolgd door de aanbevolen preventieve maatregelen om het risico van de overdracht naar de mens te verminderen.

Deel II beschrijft de doelstellingen van het experimentele werk dat tijdens dit doctoraat werd uitgevoerd. De focus van dit proefschrift lag op het onderzoek van de *T. gondii* prevalentie in de varkenspopulatie in België en de studie van de immuunresponsen bij varkens na een experimentele infectie, rekening houdend met de spreiding van de parasiet binnen de gastheer en het daaruitvolgend infectieus karakter van de weefsels. Er werd ook bijzondere aandacht besteed aan de parasietantigenen met het hoogste immunogene potentieel in *in vitro* testen, omdat deze mogelijke kandidaten kunnen zijn voor ontwikkeling van een vaccin tegen *T. gondii* bij varkens.

Deel III beschrijft in detail de experimentele studies om de doelstellingen van dit doctoraat te bereiken, en biedt nieuwe inzichten in de gastheer-parasiet interactie bij varkens tijdens de acute en chronische fase van de infectie.

In **Hoofdstuk 4** geven we een schatting van de prevalentie van porciene toxoplasmose in België, gebaseerd op twee serologische testen (GRA7- en TLA-ELISA) en toegepast op 2263 serummonsters uit 251 conventionele bedrijven met een intensief managementsysteem. Verrassend genoeg toonden de resultaten van beide testen een

significant hogere tussen- en binnen-bedrijf prevalentie aan van anti-*T. gondii* IgG in Wallonië dan in Vlaanderen. Daarnaast hebben we een Bayesiaans model gebruikt om de echte binnen- en tussen-bedrijven prevalentie te beoordelen op basis van de resultaten van beide diagnostische testen, rekening houdend met de beperkingen van de serologische analyses in biologische monsters. De totale ware prevalentie over alle bedrijven, de ware prevalentie tussen de bedrijven en de ware prevalentie per indivudeel bedrijf waren eveneens significant hoger in Wallonië dan in Vlaanderen. Verder, op basis van een schatting van de karakteristieken van de testen, was de specificiteit van de serologische testen aanzienlijk hoger in Vlaanderen, en dit vooral voor de GRA7-ELISA, terwijl de gevoeligheid van beide ELISA's niet significant verschillend was in beide landsdelen. Deze studie is het eerste epidemiologische rapport van de seroprevalentie van *T. gondii*-infectie in België.

In **Hoofdstuk 5** worden de belangrijkste bevindingen van onze eerste experimentele infectie met *T. gondii* bij varkens gepresenteerd. Na inoculatie van seronegatieve biggen met de IPB-Gangji stam wordt de antilichaamproductie en de expressie van cytokine mRNA (IFN-y, IL-4 en IL-10) in bloed mononucleaire cellen gevolgd. Onder de bestudeerde cytokines, vertoonden alleen IFN-y mRNA een verhoogde expressie bij de geïnfecteerde in vergelijking met de controle dieren. De infectie induceerde ook een duidelijke serum antistoffenrespons tegen recombinant GRA7, terwijl geen respons werd waargenomen tegen natief *Toxoplasma gondii* totaal lysaat antigeen (TLA) of tegen rGRA1, rMIC3 en rEC2 parasitaire eiwitten. De verspreiding van de parasiet in verschillende weefsels, de hoeveelheid en de leefbaarheid van de parasiet in deze weefsels werden respectievelijk aangetoond met qPCR en bio-assay. Vergelijking met een eerdere studie deed vermoeden dat de IPB-Gangji *T. gondii* stam minder leefbaar werd naarmate biggen langer geïnfecteerd waren. Dit ondersteunde de hypothese van een “clearance” mechanisme in een chronisch model van de IPB-Gangji infectie bij varkens.

Hoofdstuk 6 geeft meer informatie over de stam- en dosisafhankelijke resultaten van experimentele infecties bij varkens. We hebben hier homo- en heterologe infectie-experimenten uitgevoerd met twee verschillende *T. gondii*-stammen (IPB-LR en IPB-Gangji) en vervolgens verschillende parameters geëvalueerd, zoals de gastheer-specifieke immuunrespons enerzijds, en de parasitaire hoeveelheiden in weefsels anderzijds. Ten eerste, na een inoculatie met beide stammen werd er een uitgebreide humorale reactie waargenomen tegen GRA7- en TLA-antigenen. Zowel GRA7-specifiek IgM als IgG werden zeer kort na de initiële infectie gedetecteerd, terwijl de TLA-specifieke IgM en IgG later verschenen maar langer bleven en aan hogere titers aanwezig waren in de loop van de infectie. Ten tweede, de *in vitro* IFN-γ-productie door TLA-gestimuleerde bloedmononucleaire cellen was direct gecorreleerd met de infectiedosis van beide stammen. Flowcytometrie na een intracellulaire kleuring van de geïsoleerde bloed mononucleaire cellen, toonde dat voornamelijk de $CD8^+$ T-lymfocyten verantwoordelijk waren voor de productie van IFN-γ. Ten slotte, hebben we de aanwezigheid van parasieten in de weefsels in homologe infectie-experimenten onderzocht. Er bleek een omgekeerde correlatie te zijn met infectiedosis voor de IPB-Gangji-stam, maar niet voor de IPB-LR stam. Een heterologe infectie met IPB-LR, gevolgd door de IPB-Gangji-stam, waarbij een opmerkelijke vermindering van het parasitaire DNA en de levensvatbare cysten in varkensweefsels werden waargenomen, toonden het potentieel van de IPB-Gangji stam om een sterke parasiet verwijderende immuunrespons in de gastheer op te wekken. Wij postuleren dat de IPB-Gangji stam gebruikt kan worden als een hulpmiddel om het aantal levensvatbare parasieten in eetbare weefsels te beperken en bijgevolg het risico op humane toxoplasmose te verlagen.

In het laatste experimentele **Hoofdstuk 7** werd verder gefocust op het onderzoek naar de interactie tussen gastheer en parasiet bij een acute en chronische experimentele infectie met de IPB-LR- en IPB-Gangji-stammen. Hierbij werd de relatie tussen IFN-γ responsen en het aantal parasieten in weefsels geanalyseerd. Gebaseerd op de

veelbelovende resultaten van de vorige studie waarbij infectie met IPB-Gangji en niet met IPB-LR verwijdering van de parasiet induceerde en de substantiële IFN-γ inductie in de mononucleaire cellen uit het bloed na herstimulatie met TLA, werd nagegaan of er meer immunogene TLA fracties konden geïdentificeerd worden. Hiervoor werden het totale TLA voorafgaand gefractioneerd met behulp van continue elutie-elektroforese, en onderverdeeld in 6 fracties verschillend in moleculair gewicht, die dan gebruikt werden in restimulatie van bloed mononucleaire cellen of van lymfocyten, geïsoleerd uit de lymfoïde weefsels van geïnfecteerde dieren. In het algemeen, produceerden lymfocyten van de dieren geïnfecteerd met de IPB-LR-stam hogere hoeveelheden IFN-γ dan deze geïnfecteerd met de IPB-Gangji en dat na stimulatie met elke TLA-fractie, met uitzondering van de cellen geïsoleerd uit de mediastinale lymfeklieren en duodenum.

Deze studie toont aan dat de hoeveelheid geproduceerde IFN-γ bij *in vitro* restimulatie door TLA fracties niet alleen aanzienlijk varieert tussen de groepen dieren die geïnfecteerd zijn met verschillende *T. gondii* stammen, maar ook tussen cellen geïsoleerd uit verschillende weefsels.

Hoofdstuk 8 omvat de algemene discussie, waarbij de belangrijkste bevindingen van de experimentele studies worden besproken, evenals de conclusies en toekomstperspectieven voor verder onderzoek. De hoge prevalentie van toxoplasmose op Waalse varkensbedrijven dient verder onderzocht te worden om de bepalende risicofactoren te onderkennen en preventieve maatregelen te formuleren.

De in deze thesis besproken verschillen tussen *T. gondii* stammen in immuunrespons en persistentie van de parasiet, bieden nieuwe inzichten in de infectie bij varkens. Daarnaast laat identificatie van de meest immunogene TLA fracties vervolgonderzoek toe naar het mechanisme dat de parasiet doet verdwijnen uit varkensweefsels.

Uit het onderzoek postuleren we dat de IPB-Gangji stam gebruikt kan worden om de immuniteit bij varkens te versterken en zo het risico op humane toxoplasmose te verlagen. Hiertoe, moet er meer inzicht verworven worden in de aangeboren en

adaptieve immuniteit van de gastheer tegen de parasiet. Het is echter ongetwijfeld zo dat de mechanismen die de immuniteit tegen de parasiet stimuleren of inhiberen bij het varken, even complex zijn als bij muizen of mensen. Daarnaast moet de aandacht worden gevestigd op de parasitaire antigenen die de sterkste humorale en cellulaire immuunresponsen in de gastheer genereren, om de beste kandidaat te identificeren als een mogelijk vaccin bij varkens. Daarom is verder onderzoek aanbevolen om deze aspecten te verduidelijken en op die manier bij te dragen tot een betere inzichten in de onderliggende gastheer-pathogeen interacties.

Curriculum vitae

Malgorzata Jennes was born on July 26th, 1982 in Lublin, Poland. In 2009, Malgorzata obtained the Master of Science degree in Veterinary Medicine from Ghent University, Belgium with distinction. In November 2009, Malgorzata started her doctoral study in the same Laboratory, where she actively participated in two projects funded by the Belgian Federal Public Service for Health, Food Chain Safety and Environment (grants RF 09/6213 and RF 13/6274), focused on multiple aspects of the natural and experimental infection with Toxoplasma gondii in pigs.

In addition to her doctoral study, Malgorzata held the position of the teaching assistant for the practical classes of immunology for the Bachelor students of Veterinary Medicine. She supervised several Master students of Veterinary Medicine, Bachelor students of the Laboratory Technology and Master Students of the Interuniversity Program Molecular Biology. In her daily work Malgorzata was involved in the detection of immune-mediated diseases in domestic animals, and in the leading of the Animal Welfare Unit, being responsible for the implementation of the Ethical Committee regulations on the use of the laboratory animals. As a representative of the PhD students' community from life sciences, she was a member of the Doctoral Schools steering committee.

Malgorzata is an author and co-author of several publications in peer-reviewed international journals and she actively participated in national and international conferences, presenting her research.

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

Doctoral School courses

Scientific skills:

Introduction to biostatistics (VIB) (2011)
Effective Scientific Communication (Ugent) (2012)
Advanced Academic English - Conference Skills (Ugent) (2012)
Advanced Academic English - Writing Skills (Ugent) (2012)
Hands-on training in Systematic Reviews of animal studies (Syrcle, VUB) (2013)
Development and applications of monoclonal antibodies (VUB) (2013)
Applied Flow Cytometry: (pre)clinical haematological analyses (UZA-Ugent-VUB) (2014)
Clinical studies: study design, implementation and reporting (Ugent) (2015)

Teaching Skills:

Basic Assistants Training (Ugent) (2011)
Feedback Training (Ugent) (2012)
Seminarie: Uitdagende practica, een opstap naar het werkveld (Ugent, HoGent, Artevelde Hogeschool, Howest) (2013)
Universal Design for Learning Project (selected participant) SIHO) (2013-2014)

Managament Skills:

Meeting Skills (Ugent) (2011)
Communication Skills (Ugent) (2011)
Conflict Handling (Leadership & Efficiency) (Ugent) (2012)
Project Management (Career Management) (Ugent) (2013)

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how to drink the real stuff (=Starbucks coffie with whipped milk and caramel topping). **Jonas** and **Robert**, you are both very talented young researchers, make the immunology world unsafe! **Nicolas**, I did not have the opportunity to meet you yet but I am sure you will like it at Immunology like I did! Good luck boys!
Annelies, your positive and cheerful spirit brings everyone to his ease-level. Your kindness and contagious laughter was more than welcome when I was just about to start with teaching and doing a PhD on my own. Your motivating and supporting mails this year were so powerful and helped me to go on to the end! Please do not ever change! ☺ **Michaela**, you have been always a big example, when it comes to your efficiency and knowledge of molecular immunology. And apparently it is not only me who acknowledges you for these skills ☺ I wish you a succesful future at DeLaval, so nice you are staying within animal area! **Delfien**, thank you for introducing me to the of world of *T. gondii*! Your research made a good base to start mine. Thank you for sharing your experience. I hope you are enjoying your current job! **Sarah**, where shall I start: I admire you as a mum of three, being professional on every level and so sportive! It is a pity you left Immunology at the end, but I am happy for you to explore new areas and take on new challenges. I wish you a successful carrier and lots of joy in your personal life! You can be proud of such a wonderful family of yours! **Ann**, you have always been so helpful in every possible way, with all the last-minute orders, tracing lost products, printing and other requests, no matter how busy you were or how little time you had. Thank you!!!! I hope you find a solution or at least a good treatment for your back problem, because even in pain you were always there with a smile on your face!
I would like to thank **Mieke** and **Marijke** for all the administration work, **Gert** for the financial reports for my projects which were always perfect to finest detail, and **Dirk** for all the IT related issues and nice chats. I would like to mention colleagues who already left Immunology: **Kris, Eva, Tine, Maaike, Marina, Phillippe, Marjolein, Pieter, Manon, Karen, Kim, Nina, Celine, Thary, Kevin, Korneel, Christophe** and **Maria**. Thank you for many nice moments together and good luck with your professional and personal life!
Nicky and **Sabrina,** it was definitely a pleasure to collaborate with you and a big fun with the rabbits! I wish you all the best and wonderful moments with you families!
I would also like to mention my appreciation for the colleagues from WIV, Laboratory for Toxoplasmosis: **Ignacio, Isabelle, Maïté, Francesca** and **Jurgen**. **Ignacio**, we jumped on the same Toxo-train and will be finishing at almost the same time! So funny we both published the life-saving paper in the same week ☺ I really enjoyed working with you and I am very happy with your research results! I realize like no other which odds you had to be beat to be where you are now. Good job! And the degree and future career will follow shortly! **Isabelle**, je vous félicite pour vos trois beaux enfants. Merci beaucoup pour toute l'assistance technique dans toutes ces années. **Maïté**, mon article principal est basé sur vos excellentes techniques de laboratoire. Merci et bonne chance avec votre double bonheur! **Francisca**, votre sens de l'humour nous a aidé à

survivre chaque fois avec le reste des cochons. Merci bien pour tout, profitez de vos filles et de votre vie! **Jurgen**, you will always remind me of Harvey Specter in a lab coat, rather than in a suit! Thank you for all your technical help and nice talks! I wish you many nice walks with your lovely dog and girlfriend!
Brecht, you are really a fantastic researcher and a very nice person to work with! I wish we collaborated more intense during my PhD. Thank you for your feedback on the manuscripts and your thoughtful but so kind remarks. I wish you, Bimala and your cute daughter Maya the continuation of the beautiful life together that you live now. I see a great future for you, to me you are as smart and humble as Einstein with all your statistical formula's that you play with. I hope we stay in touch even though my Toxo-story has come to an end.
My new **Colleagues from BD**, I am happy I could join the "Supporters" (read: "DIVAs" team), where I felt welcome from the very first day. This feeling has never left me since then and I am very happy I can now stay for good within the team or at least as long as flow cytometry will remain the core business of BD Biosciences! **Nadine** and **Paola**, thank you for your confidence in me and the opportunities I have been given. I will do my best to bring any troubleshooting to a good end and make any panel possible work! Paola, you are such a multitalented person: scientific wise, linguistic wise, management wise, sportive wise,…Probably I will still discover more of it! Thank you for giving me the warm welcome in your team and for keeping me in it ☺ I wish you a lot of challenges in your climbing and sailing adventures and a happy life with Paolo!
Luisa, your creativity and sparkling character make you so adorable! No dead silence or a boring moment when you are around! Thank you so much for all the cakes and love you share with us! I hope you get enough affection back because you are really such a generous and caring person! I love to have you around! I wish you the best of luck, love and happiness in life, wherever, with whomever and whenever, as long as it comes to you! **Vesna**, or Miss Multicolor, I am so happy we came across each other professionally again! I really enjoy working next to you (not only literally!) and learning from you. Thank you for sharing your knowledge, all the advices and the guidance, and the nice talks between the mails, phone calls, chats and the trainings ☺ The lunch breaks are always way to short when working out together in the fitness! I will always associate "Thanks God it's Friday!" and "What are we doing for lunch?" with you ☺ I hope Baldr will not be too naughty but hey, better a stallion with an attitude, right?
Sebastiaan, our charming Ryan Gosling lookalike! You can bring any Accuri to life with the ease you run a marathon! I wish you a bright future within BD and a wonderful life together with Ines. And if you need any advice or inspiration for the best circumstances to "pop up the question", you know where to ask!!! **Pieter**, I really admire your knowledge on clinical applications but even more the pursuit of adventurous life. I can only dream of it but you simply make it happen…Although I have known you for the shortest time of everyone, you are the reason I am here. So, even if it was indirectly: thank you! Just don't get too lyric…☺ **Heidi** and **Renée**, thank you for your kindness, handy tips about anything you might need within BD and the nice walks and talks.

Heidi, your organizational skills are legendary, I wish you a happy life with Karel and Asbn in your dream house! **Renée**, enjoy the last weeks before you get married and don't worry about the details, your big day will be just perfect because you are going to share it with the person you love the most! **Maritza**, I think your mantra for every day is "Always look at the bright side of life" or "Even if is bad today, it can still get worse tomorrow"…I have never heard your complaining, as overloaded as you can be with our requests…Your enthusiasm is contagious, I always feel better after having you on the phone. Keep up the good work at BD AS! You are really the leading lady between all the guys there! I would also like to thank the brilliant trainers **Joerg** (for the enjoyable lunch breaks) and **Marta** (for all the nice Polish moments with me, I really enjoy them a lot! dziękuje!), and the technical experts **Els, Stijn, Mounir** and **Ivan** for the nice atmosphere and their support (not only technical). **Els** and **Stijn**, when we start chatting about our kids, all the customers will probably hang up eventually ☺ I really enjoy sharing the experiences and funny stories with you! **Mounir**, enjoy the beautiful women in your life, they are treasures! **Ivan**, good luck with your career at BD! If you want to learn some Polish words to enrich your extended and multilanguage vocabulary, just ask ;-) **Morgane**, although you are always on the run between Europe, Russia, Middle East or Africa, it is always nice to have you around in Erembodegem. I think it is clear how brilliant and committed application specialist you are, keep up the good work! **Vanessa**, what a pity you left the team so soon and I did not have the opportunity to know you longer. But still we had a good chemistry and I hope to see you now and then. Keep up the stamina and the perfectly trained body we are all jealous about ☺ **Ariane**, my dear ex-colleague, so sad you left our team pursuing your dreams and hopes. I loved so much your warmth, generosity and the genuine kindness. I miss you (and your sometimes complicated life) every single day but I am happy for you that LOVE (or Zoli) finally has found you ☺ Have a blessed and happy life together! If you feel like having a drink and a chat in Erembodegem, I am in anytime!
I am enjoying so much this symphony of the nationalities, languages and cultures, which brings the best out of us every single day! The only thing I still need to say is: let's make BD "bigger, better and bolder" together! And win next Ekiden run from the Experts again !!! ☺

Tine, Ellen, Dominique, Tessa and **Bea,** thank you for the lovely moments during our reunions. I am very happy to see you building houses, starting a practice, getting married, having children or pets…I hope we can stay in touch for the coming years!
Ine, Nele, Josine and **Annelies**, thank you for supporting me all the way. **Ine**, we have known each other for more than a decade now and witnessed many heights and lows in each other's lives. I will never forget your help when I was still 'speachless' or shall I rather say 'Dutchless' in the very beginning. It was so hard for me and you were really a blessing for me! Finding the right words to express my thoughts was not easy but we clearly understood the common language of friendship. I looked up at you many times in the past and I am sure I will still do it in future as well ☺. Enjoy your beautiful family!

Josine, you were an example for me in the last intense months, when I was questioning myself whether it was all worth the efforts and nights spent working instead of sleeping. But you made it too, while having another job and that really pushed me through the most difficult moments. Thank you for being my inspiration! **Nele** and **Annelies,** I still remember our nice daily walks with the Beagles during the lunch breaks between the classes. I really admire your courage of running a practice on your own! Congratulations and at the same time I wish you many future customers to come!

Ellen, thank you for the many cozy real-life talks and Whatsapp messages we exchanged as our children were growing. You are such a warm and kind person, supporting me for so long. I have really neglected you in the past months…I am sorry for that and I promise I will catch up more often from now on! I am looking forward to the moments we can share together!
Inge, my BFF! I am so glad you, Nina and Tom are in our life! You have always been there for me during all these years. Your listening skills, your genuine friendship and endless support meant (and mean!) so much to me. I really appreciate you never got bored of the news about my experiments, deadlines, submissions and so much more. You have always been an example for me as a person and I cannot imagine a better godmother for Amelie! You are like a fairy for her with all your magical kindness and love. And if she becomes like Nina later, I will be more than proud! I am sorry for neglecting you due to my busy life and if I did not offer you back what I got from you, but I am looking forward to the moments together, whether it will be learning French, jogging, doing yoga or anything else as long as it is with you!

My family in law or better to say: my second family! Thank you for all the help and warm acceptance from the beginning. These were maybe not really the most expected circumstances, but life is full of surprises, I already learned it on my own! Thank you all for taking me as I was, for supporting me in every way and for believing in us. **Ria and Guy, Lia and Georges, Gaby and Jean, Nancy and Jan, Athina and Koen, Elyne and Dirk, Sylvie and Joeri, Valerie**: I got so much love and support from you in all these years! I hope I can ever give it back in the same amount! I would also like to thank **Annie, Patrick and Irith** for the kind support and interest in my research. I enjoy meeting you on different occasions and really admire your professional careers.
Mamo i **Tato,** bardzo dziekuje za kazdy rodzaj wsparcia, jaki od Was orzymalam od tylu lat, a zwlaszcza w tych bardzo decydujacych momentach studiow i doktoratu. Nigdy nie udaloby mi sie polaczyc wszystkich obowiazkow, zarowno w pracy jak i w domu, gdybym nie brala z Ciebie przykladu Mamo! Dziekuje! Mam nadzieje, ze Amelka pojdzie w nasze slady ☺ **Asia,** you have been always my example, regardless of my age and circumstances. I was always looking up to you as my big sister and even now, watching you still studying for another medical specialization, I could put my own efforts in a better perspective. I hope, once we both finish studying, we will make more time for each other and our families. **Darek,** the life turned to be very harsh for you

lately…So unexpectedly tearing the plans and future in pieces…And still, as hopeless and scary the reality was, your determination to recover replaced the worst-case-scenario by a more acceptable alternative. The future will show where you can eventually get but I have learned from your experience that we should not put limits on our plans but dream big and aim high. I am supporting you all the way!

Simon, Mathias and Amelie, my three youngest supporters, I am sure you are as happy as I am I finally got to this point. You have never seen me doing anything else than studying or doing a PhD, what actually felt like exactly the same thing for you in all these years. I am very sorry I have never been the type of mum waiting for you every day at the school's gate. But I am very grateful that you do not expect it from me either, because you know it is not the type of person your Mum really is. Maybe it is hard for you to understand that all I have done, I have done for you and because of you. I hope you are proud of me today as I am of you every single day. And I promise I will make up all the evenings and weekends I had to choose my work above spending time with you. I am excited and happy about all the fun and joyful moments to come!!! PS.: Mummy loves you to the moon and back but please choose another job, Mummy will not handle going through all of it again with you ;-) ♥

Krisie, I really hardly find words to thank you for everything we have been through together since my adventure with veterinary medicine and research started. You have been the most supportive, helpful and loving husband in the world, having an endless faith in me and my capabilities from the very beginning, even if I saw it differently sometimes. Your enthusiasm and motivation pushed me to the end and it is not an exaggeration if I say that I would not be here if you have not been there for me all these years. I am sorry for all the stressful/tearful/drama/no-end-story moments, I am glad I finally made it and I promise from the depth of my heart that I will not do this to you any more ☺ I just want to say I have not just done my PhD along you but together with you and you fully deserve the co-authorship of this work. I think it is a good compromise to put at least your family name on it ☺ You have never known me differently than studying, but this will change now, our 'normal life' can get started and I am so looking forward to it! ☺ The only thing we need is time for each other and the kids. "Looks like we made it , look how far we've come my baby, I am glad we didn't listen, look at what we would be missing…". Even if we do not have it all together, together we have it all! ♥

And tonight, with all the support, friendship and love around me I dare to say:
I really have it all!

Thank you all! Dziekuje wszystkim! Bedankt iedereen!

Gosia
3rd of October 2017
Merelbeke, Belgium