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Infection dynamics following experimental challenge of pigs orally dosed with different stages of two archetypal genotypes of *Toxoplasma gondii*

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ABSTRACT

Toxoplasma gondii is a food-borne zoonotic parasite widespread in a variety of hosts, including humans. With a majority of infections in Europe estimated to be meat-borne, pork, as one of the most consumed meats worldwide, represents a potential risk for consumers. Therefore, we aimed to investigate the progress of *T. gondii* infection and tissue tropism in experimentally infected pigs, using different *T. gondii* isolates and infectious stages, i.e. tissue cysts or oocysts. Twenty-four pigs were allocated to treatment in four groups of six, with each group inoculated orally with an estimated low dose of either 400 oocysts or 10 tissue cysts of two European *T. gondii* isolates, a type II and a type III isolate. The majority of pigs seroconverted two weeks post-inoculation. Pigs infected with the type III isolate had significantly higher levels of anti-*T. gondii* antibodies compared to those infected with the type II isolate. Histopathological exams revealed reactive hyperplasia of the lymphatic tissue of all pigs. Additionally, a selected set of nine tissues was collected during necropsy at 50 dpi from each of the remaining 22 pigs for *T. gondii* DNA detection by quantitative real-time PCR. A positive result was obtained in 29.8 % (59/139) of tested tissues. The brain was identified as the most frequently positive tissue in 63.6 % (14/22) of the animals. In contrast, liver samples tested negative in all animals. The highest mean parasite load, calculated by interpolating the average Cq values on the standard curve made of ten-fold serial dilutions of the genomic DNA, corresponding to 10^0 to 10^4 tachyzoites/μL, was observed in shoulder musculature with an estimated concentration of 84.4 [0.0–442.5] parasites per gram of tissue. The study highlights the variability in clinical signs and tissue distribution of *T. gondii* in pigs based on the combination of parasite stages and strains, with type III isolates, particularly oocysts, causing a stronger antibody response and higher tissue parasite burden. These findings suggest the need for further investigation of type III isolates to better understand their potential risks to humans.

1. Introduction

Toxoplasma gondii is a zoonotic apicomplexan parasite capable of infecting essentially all warm-blooded animal species, and humans. Felids are the only known definitive hosts of the parasite, responsible for shedding oocysts into the environment with the faeces. The two main routes of infection for susceptible species are via accidental ingestion of sporulated oocysts from the environment (e.g., contaminated water,

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feed, or by soil intake) and via consumption of tissues of infected hosts containing viable *T. gondii* bradyzoites. Despite the existence of other transmission routes such as vertical transmission from mother to foetus, through an organ transplant or via blood transfusion, consumption of raw or undercooked meat of infected animals appears to be the main source of human *T. gondii* infections in Europe (Cook, 2000). Intermediate hosts may develop tissue cysts in their muscles and organs upon infection.

Although *T. gondii* infections are often subclinical or manifest with non-specific symptoms, an infection during pregnancy may cause abortion, stillbirth or congenital development disorders (Jones et al., 2001). Moreover, the infection may lead to serious cases of encephalitis and retinochoroiditis in particular (Luft and Remington, 1992; Maenz et al., 2014).

With the relatively high prevalence observed in various animal species across the world (Dubey, 2022) and one-third of the global human population estimated to be infected (Montoya and Liesenfeld, 2004), *T. gondii* is of a great importance to animal and human health. The major impact of this parasite on human health is reflected in the high placement of *T. gondii* among the top-priority food-borne in multicriteria-based ranking for risk management of food-borne parasites at both the global (4th out of 24 food-borne parasites) (Boireau, 2014) and European levels (2nd out of 25 food-borne parasites) (Bouwknegt et al., 2018).

The majority of human *T. gondii* infections are estimated to be meat-borne (Cook, 2000) and pork, as one of the most consumed meats in the world and the most consumed meat in Europe (OECD and Food and Agriculture Organization of the United Nations, 2022), plays an important role in public health safety. The European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ) identified *T. gondii* as one of the most relevant biological hazards in the context of meat inspection of swine (EFSA, 2011).

Pigs may represent an experimental animal model for the study of different aspects of human toxoplasmosis (Garcia et al., 2017; Nau et al., 2017). The factors responsible for the development of clinical toxoplasmosis in swine are poorly understood, but factors such as host age and immune status, coinfections with viral agents, parasite stage, inoculum, and genotype seem to play an important role (Basso et al., 2017a; Stelzer et al., 2019; Fernández-Escobar et al., 2020a).

In the last years, more attention is being paid to the genetic background of *T. gondii* isolates and their association with different virulence phenotypes. It seems that some genotypes may be more prone to cause clinical disease in some animal species than others. A variety of *T. gondii* strains has been identified but the majority in Europe and North America fall into three distinct clonal lineages (types I, II, and III) (Saeij et al., 2005). Isolates of type I tend to be lethal to mice regardless of the infective dose and are overall more aggressive than type II and type III (Dardé et al., 1992; Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Howe et al., 1996). Virulence is more variable for type II and type III strains; however, some type III isolates were proven to induce overall higher morbidity and mortality rates in animals compared to type II strains (Fernández-Escobar et al., 2020a, 2021; Largo-de la Torre et al., 2022). Effect of experimentally-induced *T. gondii* infection with type III isolates, arguably the least examined of the three lineages, and the comparison with type II isolates that dominate in Europe (Khan et al., 2007; Shwab et al., 2014; Lorenzi et al., 2016; Fernández-Escobar et al., 2022) helps to fill the knowledge gap in the development of a valid animal model for human *T. gondii* infections based on the effects of experimentally induced toxoplasmosis in pigs.

To shed some light on the factors that influence the clinical outcome in swine toxoplasmosis we evaluated the course of infection in pigs experimentally inoculated with a low dose of oocysts and tissue cysts of type II and type III *T. gondii* isolate, assessing the clinical signs, histopathological changes, and the evolution of the specific immunoglobulin G (IgG) humoral response in the different inoculation groups. Furthermore, we aimed to evaluate the tropism of *T. gondii* within the tested tissues of these experimentally infected pigs, according to the two infectious stages and isolates used, and rank the tissues based on the estimated parasite load per gram of tissue.

2. Materials and methods

2.1. *Toxoplasma gondii parasites*

For experimental infection of pigs, two archetypal *T. gondii* isolates were used for comparison. The first isolate ("CZ-Tiger") was obtained from the faeces of a Siberian tiger (*Panthera tigris altaica*), kept at the Dvůr Králové Zoo in the Czech Republic in February 2005 (Juránková et al., 2013). The oocysts of CZ-Tiger isolate used in this study were obtained from the fourth cat passage, two months prior to the inoculation of the pigs.

The second isolate ("CZ-Šimková") was derived from the faeces of a domestic cat (*Felis catus*) imported from the Balkan region in May 2015 to the Czech Republic (López-Ureña et al., 2023). The oocysts of CZ-Šimková used in this study were obtained from the second cat passage, five months prior to inoculation into pigs.

Both isolates pose a wild-type phenotype and were maintained by subsequent passages in Vero cells, outbred ICR (CD-1) mice, and domestic cats prior to this experiment. Sporulated oocysts of both above-mentioned isolates were purified from cat faeces as described by Wainwright et al. (2007), microscopically examined, and stored in PBS solution at 4 °C until further use. PCR-RFLP and microsatellite genotyping of the two *T. gondii* strains used for experimental infection in this study revealed or confirmed that both belonged to different clonal lineages, i.e. *T. gondii* types II and III (see 2.2).

Oocyst viability was confirmed by bioassay in outbred ICR mice six weeks before the inoculation of the pigs (see 2.4.).

For the production of *T. gondii* tissue cysts of both isolates, two groups of two mice each were orally inoculated with approximately 100 sporulated oocysts in 0.5 mL of water/mouse by gavage. After six weeks post-inoculation (wpi), the mice were euthanized, their brains were collected and gently homogenised in PBS solution supplemented with gentamicin using a mortar and pestle. The number of tissue cysts was determined by counting five 20 μL aliquots of the suspension in a cavity well microscope slide using a Nomarski interference contrast microscope (Olympus BX30, Japan). Obtained tissue cysts were diluted to 10 tissue cysts in 10 mL of PBS and used for the inoculation of pigs within 24 hours.

The experimental infection of mice was approved by the local ethical committee of the University of Veterinary Sciences Brno (UVS) under agreement number 19/2017.

2.2. *Genotyping of T. gondii strains*

Both *T. gondii* isolates (i.e., CZ-Tiger and CZ-Šimková) used for pig inoculation were genotyped by both PCR-RFLP and microsatellite typing methods, using DNA extracted from cell culture-derived tachyzoites. PCR-RFLP was carried out using eight chromosomal genetic markers (nSAG2, SAG3, BTUB, GRA6, c22–8, c29–2, L358 and PK1) and an Apicoplast marker as previously reported (Su et al., 2006; Herrmann et al., 2012). Microsatellite typing was performed using a multiplex PCR, which amplifies 15 different markers as previously described (Ajzenberg et al., 2010). These markers included 8 typing markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, XI.1) and 7 fingerprinting markers (M48, M102, N83, N82, AA, N61, N60) that display a high level of polymorphism within clonal lineages type I, type II or type III (Ajzenberg et al., 2010). The only divergence from the original methods was that in the case of M102, AA and N60 the fluorophore Atto550 was used instead of NED to label amplicons during multiplex PCR. Typing results were numerically adjusted using guidelines published recently (Joeres et al., 2023).

Genotyping by PCR-RFLP revealed type II variant (ToxoDB #3) in

case of CZ-Tiger confirming previous reports (Juránková et al., 2013; Koethe et al., 2015; Geuthner et al., 2019; Ramakrishnan et al., 2019; Fabian et al., 2020), and type III (ToxoDB #2) for CZ-Šimková. Microsatellite typing confirmed PCR-RFLP lineage typing and provided a detailed genotypic characterization by fingerprinting markers (Supplementary material 1).

2.3. Experimental inoculation of pigs with T. gondii

Twenty-seven weaned Danhybrid-LY gilts, weighing 35–40 kg, which tested seronegative to *T. gondii* by an *in-house* immunofluorescence test (data not shown), were purchased from an intensive pig production unit. The pigs were divided into four groups of six animals each (Groups 1–4), and one group of three animals (Group 5), with each group housed in a separate pen with slated floor and with water and feed accessible *ad libitum.*

One pig (pig No. 10) had to be euthanized before the start of the experiment and was replaced by pig No. 25 from Group 5.

The pigs of Group 1 (pigs No. 1–6) and Group 3 (pigs No. 14–18, and 25) were orally inoculated using a gastric tube with a suspension containing approximately 400 *T. gondii* oocysts of the isolates, respectively, in 10 mL of PBS solution. Subsequently, pigs of Group 2 (pigs No. 7–9 and 11–13) and Group 4 (pigs No. 19–24) were orally inoculated using a gastric tube with a suspension containing 10 *T. gondii* tissue cysts of the same isolates, respectively, in 10 mL of PBS solution (Table 1). The two remaining pigs of Group 5 (pigs No. 26 and 27) received 10 mL of PBS and served as negative, non-infected controls. Pig groups with corresponding administered inoculation doses are summarized in Table 1.

All pigs were clinically monitored twice a day. One week after inoculation, all boxes were thoroughly cleaned and disinfected with 3 % Neopredisan 135–1® (Menno Chemie, Germany) in order to eliminate all oocysts which might have passed non-excysted within the faeces, originating from the inoculum. No ancillary analyses were performed on the experimental animals

Experimental inoculation of pigs with *T. gondii* parasites for the purpose of this study was authorized by the Ministry of Education, Youth and Sports, Czech Republic (permission No. 55/2016) and was performed under the control of the Regional Veterinary Administration.

2.4. Mouse bioassay

The viability and virulence of *T. gondii* oocysts of both isolates used in the current study was tested by mouse bioassay in a harmonized mouse model. For each of the tested *T. gondii* isolates, two groups of six mice each were orally inoculated by gavage, with dilutions containing 10 and 100 oocysts in 0.5 mL water, respectively. All mice (n = 24) developed unspecific clinical signs (apathy, rough and shaggy hair) between 7 and 13 days after inoculation and were euthanized. Necropsies were performed and *T. gondii* tachyzoites were detected microscopically in peritoneal exudates and also visualized in touch imprint slides from lung tissues stained with Giemsa, confirming the viability of the parasites. The inoculation of mice was performed at the animal facility of the Parasitology Laboratory UVS Brno and was approved by the Ministry of Education, Youth and Sports, Czech Republic (permission No. 19/2017).

Table 1
Division of pigs into groups. Four experimentally inoculated groups were based on the different combinations of parasite stages and isolates, and one group comprised negative control pigs orally inoculated with PBS solution.

Group	Pig numbers	Isolate (type)	Stage	Dose
Group 1	1, 2, 3, 4, 5, 6	CZ-Tiger (II)	oocysts	400
Group 2	7, 8, 9, 11, 12, 13	CZ-Tiger (II)	tissue cysts	10
Group 3	14, 15, 16, 17, 18, 25	CZ-Šimková (III)	oocysts	400
Group 4	19, 20, 21, 22, 23, 24	CZ-Šimková (III)	tissue cysts	10
Group 5	26, 27	N/A	N/A	0

N/A = not applicable

2.5. Blood sampling and serological analyses of specific anti-T. gondii antibodies

Blood samples from all pigs were collected from the right jugular vein into sterile collection tubes for serological analyses on a weekly basis at 0, 1, 2, 3, 4, 5, and 6 wpi. Serum samples were obtained after coagulation for four hours at room temperature and centrifugation at 1500 x *g* for 10 minutes, aliquoted, and stored at −20 °C until further use.

Sera were tested for specific anti-*T. gondii* IgG using a commercial ELISA kit (PrioCHECK *Toxoplasma* Ab porcine, Prionics, Schlieren, Switzerland) at a dilution of 1:50 and results were expressed as the percentage of positivity (PP) relative to the reaction of the positive control: $PP_{sample} = [(OD_{sample} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control})] * 100$ as indicated by the manufacturer.

2.6. Histopathological examination of pig tissues

Necropsy was performed immediately following the euthanasia of all pigs at the slaughterhouse of the Faculty of Veterinary Hygiene and Ecology of the UVS Brno by professional butchers under veterinary supervision at 50 dpi. Tissue samples (brain, spleen, thymus, submandibular, cervical, mediastinal, inguinal, mesenteric, and splenic lymph nodes, tonsils, heart, liver, kidneys, stomach, and lungs) were collected from each pig and fixed in buffered 10 % neutral formalin and processed for a routine histopathological examination. Formalin-fixed tissues were embedded in paraffin, cut at 4 μm thick sections, mounted on glass slides, stained with haematoxylin and eosin (H&E), and screened for the presence of *T. gondii* stages and pathological changes under a microscope (Olympus BX30, Japan).

2.7. Tissue sampling for the tropism study

Nine tissues composed of three meat cuts ("shoulder" = *musculus triceps brachii, musculus supraspinatus, musculus infraspinatus*; "loin" = *musculus longissimus dorsi pars lumbalis*; "ham" = *musculus semimembranosus, musculus semitendinosus, musculus gluteus medialis*) and six organs (heart, brain, lungs, liver, spleen, and kidneys) were collected from each pig. A minimum of 200 g of each tissue, or the whole tissue in case of a smaller size of the tissue, were stored in labelled sterile plastic bags at −20 °C until further use.

2.8. Trypsin artificial digestion

Two hundred grams of each of the above mentioned nine tissues (i.e., shoulder, loin, ham, heart, brain, lungs, liver, spleen, and kidneys) from each of 22 pigs inoculated with *T. gondii* (n = 198 samples) were blended and incubated (90 minutes at 37 °C, 200 RPM on a shaking plate) in trypsin solution (Trypsin (1:250), powder, Gibco™, Scotland, final concentration of 4 g/L). The mixture was filtered through a double layer of gauze, transferred to 50 mL sterile centrifuge tubes, and centrifuged at 1800 x *g* for 10 minutes. The formed pellet was washed twice of leftover trypsin using a saline solution (0.9 % NaCl, Sigma-Aldrich, US). The weight of the final pellet was recorded and samples were stored at −20 °C until DNA extraction and subsequent qPCR analysis.

2.9. Molecular analyses of T. gondii

DNA was extracted from 80 mg of a digested pellet (see 0.2.8.), using NucleoSpin DNA RapidLyse, Mini kit for rapid DNA purification (Macherey-Nagel, France), according to the manufacturer's instructions. Extracted DNA was cleaned of PCR inhibitors and concentrated using ethanol precipitation protocol (Zeuigin and Hartley, 1985) and resuspended in 30 μL of Milli-Q water. The detection and quantification of

T. gondii DNA in each sample were performed in duplicate by amplification of a sequence within the 529 bp repetitive element, according to Opsteegh et al. (2010) with minor modifications (25 μL total reaction mixture volume, 2X Premix Ex Taq™ (TakaraBio, Japan) and 5 μL of DNA as template) and competitive internal amplification control (CIAC) probe modification by Deng et al., (2021), using LightCycler® 480 System 96-plate thermocycler (Roche, Germany).

Genomic DNA from a suspension of 10^6 cultured RH-strain (type I, ToxoDB Genotype #10) ("ToxoDB," n.d.) was extracted using NucleoSpin DNA RapidLyse, Mini kit (Macherey-Nagel, France), according to the manufacturer's instructions. A standard curve was prepared with ten-fold serial dilutions of the genomic DNA, corresponding to 10^0 to 10^4 tachyzoites/μL (Thomas et al., 2022). Positive and negative controls, distilled H_2O sample, and a standard curve were included in each qPCR run.

Only qPCR reactions with efficiency of 100 % ± 10 %, and the R2 correlation coefficient ≥ 0.99 were considered. Samples with a smooth amplification curve and a Cq value ≤ 40 in at least one of the duplicate reactions were considered positive.

2.10. Absolute quantification of *T. gondii* parasites

One μL of each serial dilution of the standard curve was included on each qPCR plate. The qPCR results were analysed using LightCycler® 480 System software, Version 1.2.9.11 (Roche, Switzerland). The starting quantity of parasites per qPCR reaction was estimated from the mean Cq values, obtained for duplicates of each sample, using a regression line from the ten-fold dilutions of the standard curve. Negative reactions were not considered in the calculation of the Cq mean. For the calculation of the final parasite load, the original weight of the sample, the final weight of the digested pellet, the proportion of the pellet used for DNA extraction, and the proportion of the DNA extract volume used in the subsequent qPCR reaction were taken into account. The final result was expressed as the number of parasites per gram of tissue (ppg).

2.11. Statistical analysis

For the statistical analysis of the serological data, a mixed linear model was applied to the data before ANOVA analysis of data to provide a more complete and accurate representation of the data by accounting for both fixed and random effects, thereby improving the accuracy and reliability of the ANOVA results.

The mixed linear model was fitted to the ELISA percentage positivity (PP)-values with an interaction between the fixed effect of the pig group (Groups 1–4) and blood sampling times in wpi (0–6 wpi) multiplied, and an individual animal kept as a random effect to test the differences in the data. The estimates from this analysis were used in a two-way repeated-measures ANOVA and post-hoc pairwise comparisons of all combinations of pig groups and blood sampling times, using Tukey's method for comparing a family of estimates. Outcomes with a *p*-value of less than 0.05 were considered statistically significant.

The analyses of the parasite tropism and parasite load in the tissues were done in two steps. A generalized linear model with binomial distribution was fitted to estimate the frequency of *T. gondii*-positive tissues by qPCR. Parasite stage (oocyst or tissue cyst), isolate of the parasite (CZ-Tiger [type II] and CZ-Šimková [type III]) and tissue were set as fixed effects, with the pig kept as a random effect in the model. The predicted effects of the isolate, type and tissue itself on the frequency of positive tissues in pigs was expressed as the odds ratio (OR) with a 95 % confidential interval (CI).

Subsequently, a linear mixed model was used to estimate the effect of the stage and isolate of the parasite as well as tissue on the parasite load in positive tissues which were previously estimated in the analysis frequency of positive tissues. The relationship between the infection route and parasite burden in the tissues was expressed as a log10-transformed coefficient estimate with the standard error, with the random effect excluded from the prediction.

Results with a *p*-value of less than 0.05 were considered statistically significant. Statistical analyses were performed using RStudio (Team, 2009), R version 4.2.2 (2022–10–31 ucrt), using the following packages: ggplot2 (Wickham, 2016), tidyverse (Wickham et al., 2019), lmerTest (Kuznetsova et al., 2017), readxl (Wickham and Bryan, 2022), MuMIn (Barton, 2022), emmeans (Russell V. Lenth, 2023), scales (Wickham and Seidel, 2022), and lme4 (Bates et al., 2015).

3. Results

3.1. Clinical observation of experimentally infected pigs

All inoculated pigs manifested with intermittent fever of > 40.0 °C during 1–2 wpi (data not shown), accompanied by inappetence and lethargy with the occurrence of mild diarrhoea in some animals. These clinical signs persisted in all oocyst-infected pigs and four pigs infected with tissue cysts of type III (CZ-Šimková) isolate during the second wpi.

Two pigs from Group 3 (pigs No. 18 and 25), inoculated with type III strain oocysts, deceased due to serious health issues at 12 days post-inoculation. Tissues from the negative control pigs of Group 5 (pigs No. 26 and 27) and the two prematurely deceased pigs of Group 3 (pigs No. 18 and 25) were not included in the sampling for the subsequent tropism study.

3.2. Serological response

The development of the specific IgG anti-*T. gondii* antibodies in all sampled pigs in time is closely reported in Supplementary material 2. The statistical analyses of the effects of time (wpi), pig groups, and their combinations on anti-*T. gondii* antibodies development using ANOVA showed an overall significant effect on all three variables ($p < 0.001$ for all). The subsequent Tukey's pairwise comparison of the combinations of the effects of time and group are summarized in Supplementary material 3.

The comparisons of the serological response between the pig groups in time showed significant differences between some of the pig groups starting from 2 wpi, as detailed in Supplementary material 3. These differences were significant between Groups 1 and 2 ($p < 0.05$), Groups 1 and 3 ($p < 0.001$), Groups 1 and 4 ($p < 0.001$), Groups 2 and 3 ($p < 0.05$), and Groups 2 and 4 ($p < 0.001$). No difference was observed in the serological response between the type III-strain-inoculated Groups 3 and 4 ($p = 0.975$). At the same time Groups 3 and 4 showed the strongest serological response among the four groups, while the lowest response was observed in Group 1 inoculated with type II strain oocysts.

No specific anti-*T. gondii* IgGs were detected by ELISA in serum samples of the two negative control pigs during the monitored period. The evolution of specific IgG anti-*T. gondii* antibodies over time in the different inoculated pig groups is displayed in Fig. 1.

3.3. Histopathological examination

Reactive hyperplasia of lymphatic tissue (lymph nodes, spleen and thymus) (Supplementary material 4 sections A, B) and chronic interstitial pneumonia (Supplementary material 4 sections C, D), predominantly mild, were observed in all pigs. These histological changes were occasionally accompanied by hyperaemia in the tissues of some individuals. No *T. gondii*-related alterations were observed in the pigs of control Group 5.

At necropsy, pigs No. 18 and 25 displayed gross lesions, including pallor of skeletal muscles and subcutaneous tissue, cyanosis in mucosa, atrophy of nasal conchae, and purulent tonsillitis, while the vast majority of examined lymph nodes were enlarged and hyperaemic. Histopathological examination revealed splenomegaly and reactive hyperplasia of lymphatic follicles in the lymph nodes, along with focal

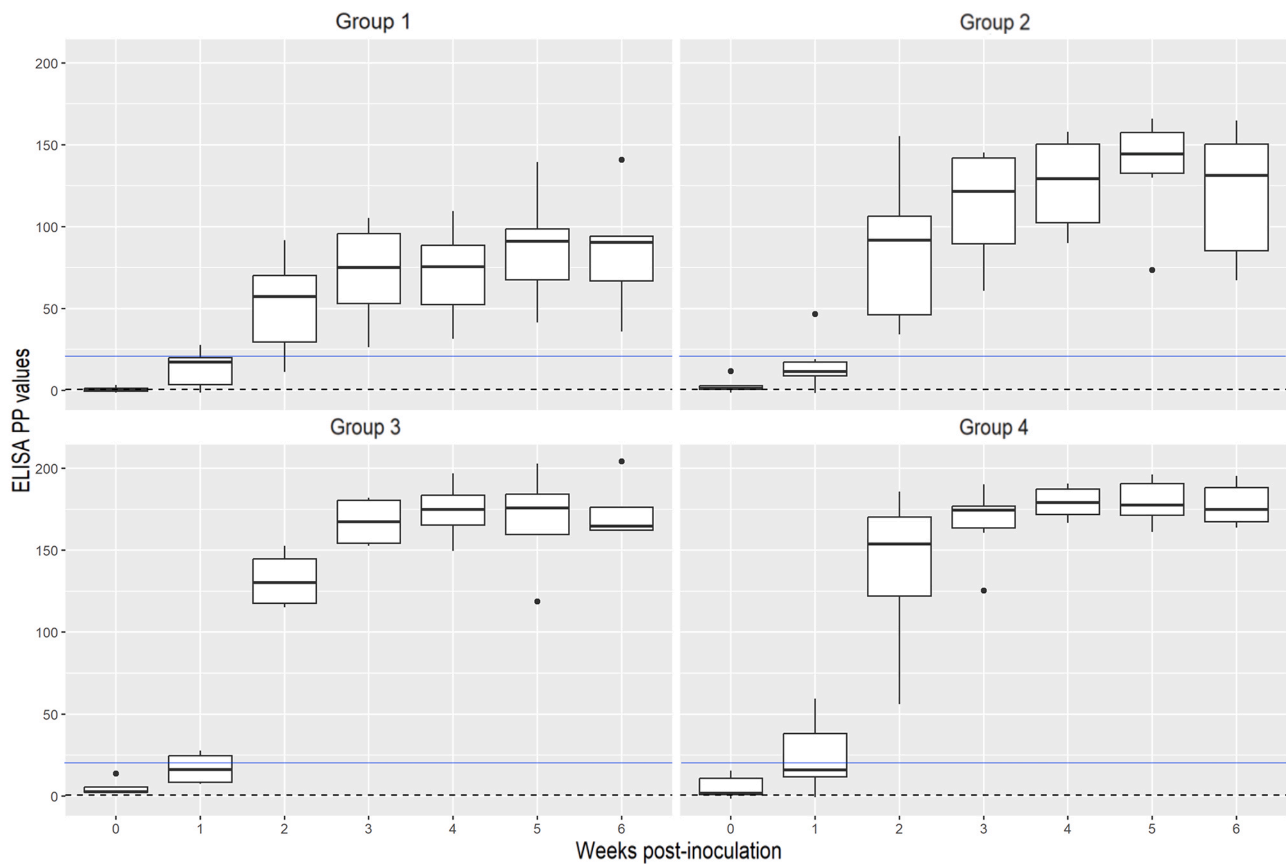

Fig. 1. Visualization of the seroconversion of pigs. The time of serum collection is shown on the x-axis and observed ELISA PP values are shown on the y-axis. The results are visualised per groups of pigs - Group 1 (type II oocysts), Group 2 (type II tissue cysts), Group 3 (type III oocysts), and Group 4 (type III tissue cysts). Boxes represent the main body of the boxplot showing the quartiles and the median's confidence intervals, with a median value represented by a horizontal line, vertical lines ("whiskers") represent the spread of sample parasite burdens within the interquartile range (IQR = third quartile – first quartile). Black dots represent values outside of the 1.5 IQR. Black dashed horizontal line signalizes the mean ELISA negative control PP value. Blue horizontal line represents manufacturer's recommended cut-off of PP = 20.

haemorrhages and neutrophilic inflammatory infiltrate. The tonsillar tissue showed marked neutrophilic inflammatory infiltrate and the presence of bacterial colonies. Similarly, lung histopathology confirmed bacterial presence in the exudate, accompanied by alveolar oedema, fibrinous inflammation, and focal coagulation necrosis.

3.4. Detection of *T. gondii* DNA in tissues

A total of 198 tissue samples (9 tissues x 22 pigs) were analysed by qPCR, resulting in 29.8 % (59/198) positive tissues (Fig. 2, Supplementary material 5). Overall, the most frequently positive tissue was the

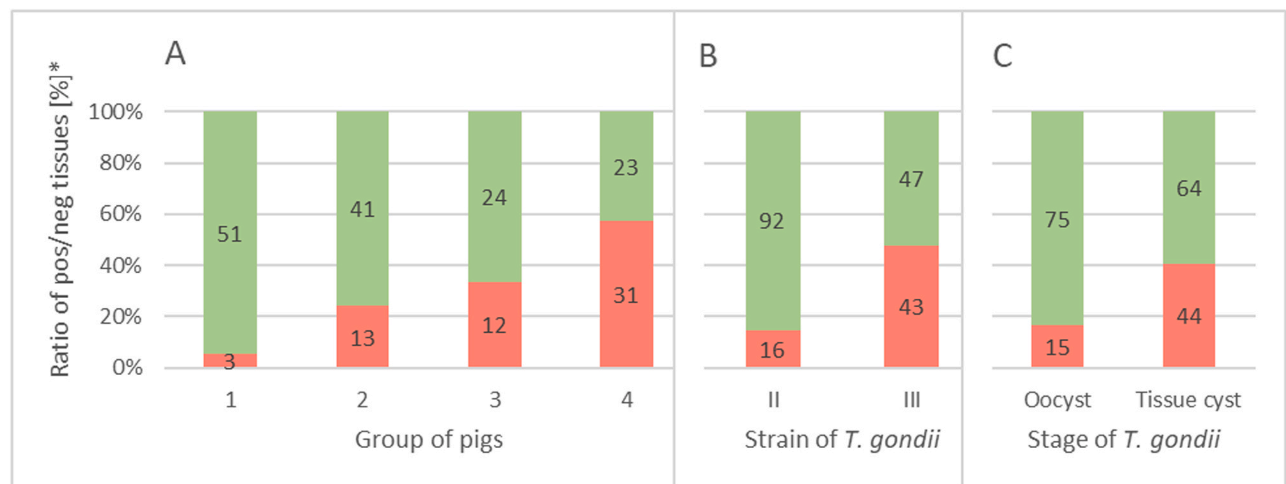

Fig. 2. 1 Proportions of positive and negative tissues. Proportion of positive (red) and negative (green) tissues per Group (A), per strain (B), and per stage (C) of the parasite. The number of tissues positive and negative for T. gondii DNA by qPCR is provided for the group, strain/isolate and stage of the parasite and their proportions are visualised in red for positive and green for negative tissue samples. *the results are scaled proportionally to each other.

brain at 63.6 % (14/22), while no liver samples were found to be positive (Supplementary material 5).

Tissue samples of pigs infected with the type II strain (Groups 1 and 2) were positive in 14.8 % (16/108) of cases, whereas tissues of pigs infected with the type III strain (Groups 3 and 4) were positive in 47.8 % (43/90) of cases (Fig. 2B, Supplementary material 5). Moreover, the pigs infected with *T. gondii* oocysts (Groups 1 and 3) and tissue cysts (Groups 2 and 4), resulted in 16.7 % (15/90) and 40.7 % (44/108) positive tissues respectively (Fig. 2C, Supplementary material 5).

Multivariate binomial regression model showed statistically significant effects of the parasite stage, isolate and tissue. Infection with *T. gondii* tissue cysts in the pigs showed a significantly higher frequency of positive tissues by qPCR (OR 17.41, 95 % CI: 3.03–99.88) compared to the oocyst infection. Similarly, type III strain CZ-Šimková caused a significantly higher frequency of positive tissues (OR 45.20, 95 % CI: 7.07 – 288.80) in comparison with type II strain CZ-Tiger (Table 2). The tissues of the heart, kidney, lung and spleen contained *T. gondii* significantly less frequently than the brains of the tested pigs, as detailed in Table 2. The outcomes of the statistical analysis comparing the predicted values and the actual frequencies of positive samples per group and tissue are visualized in Supplementary material 6.

3.5. Parasite burden in tissues

Detectable *T. gondii* DNA was found in 59 sampled tissues, ranging from an equivalent of 0.1–697.5 parasites per gram of tissue (ppg). The highest mean parasitic burden was found in type III-inoculated pigs, especially those of Group 4 and the lowest in pigs of Group 1 inoculated with type II oocysts.

The overall highest parasite burden in tested tissues was observed in the shoulder at 84.4 (0.0 – 442.5) ppg, closely followed by the other tested meat cuts, loin and ham, at 82.2 (0.0 – 697.5) and 61.0 (0.0 – 675.4) ppg. No *T. gondii* parasites were detected in the liver of sampled pigs (Table 3).

Tissue samples from pigs of Group 1 carried the lowest parasite load, in contrast to the highest load observed in pigs of Group 4. Means of 5.1 ppg and 60.7 ppg were found in pigs infected with type II and type III isolates respectively. The tested samples of oocyst-infected pigs (Groups 1 and 3) contained a mean parasite load of 6.4 ppg, compared to a mean of 50.3 ppg in tissue-cyst-infected pigs (Group 2 and 4) (Table 3, Supplementary material 5).

The statistical analysis of the parasite load in tissues showed a significant effect of the parasite stage and tissue. In contrast, no statistically significant effect of the isolate strain of *T. gondii* was noted. In animals infected with tissue cysts, the tissues contained on average approximately 1.8 times (0.59 log10-units) higher parasite load than the tissues of the oocyst-infected pigs. Although not statistically significant, the tissues of pigs infected with the type III strain contained approximately 1.4 times (0.35 log10-units) more parasites than the tissues of the pigs infected with the type II strain (Table 4). The parasite tropism was expressed as an overall ranking of tissues based on their parasite burden, rather than a type- or stage-related comparison. Significantly higher parasite burden was observed in shoulder muscles, with an approximately 1.65 (0.51 log10-units) times higher parasite load, when compared to the brains, a frequent predilection site of similar studies. This is in contrast with the significantly lower parasite burden found in the hearts of the pigs, approximately 1.8 (0.58 log10-units) times lower compared to the brains (Table 4). The comparisons of the parasite load of the remaining tissues can be found in Table 4. The log10-transformed parasite burden per gram of tissue and split by parasite stage inoculated is summarized in Supplementary material 7.

Table 2
Odds ratios for the parasite stage, the strain of the isolate and tissue itself as a risk factor for presence/absence of *T. gondii*-positive tissues. Binomial regression model outcomes are presented as odds ratios with 95 % confidential intervals (CI), with effects in bold being statistically significant ($p < 0.05$) at this level.

Variable	Value	Odds ratio (95 % CI)	*p*-value
	intercept	0.12 (0.02–0.78)	**0.026***
Parasite stage	oocyst (reference)		
	tissue cyst	17.41 (3.03–99.88)	**0.001****
Isolate (strain)	CZ-Tiger (type II, reference)		
	CZ-Šimková (type III)	45.20 (7.07 – 288.80)	< **0.001*****
Tissue	brain (reference)		
	ham	0.46 (0.08 – 2.76)	0.395
	heart	0.04 (0.00–0.31)	**0.002****
	kidneys	0.00 (0.00 – 0.06)	< **0.001*****
	liver	0.00 (0.00 – 0.00)	0.729
	loin	0.46 (0.08 – 1.95^{e}+29)	0.395
	lungs	0.01 (0.00 – 0.09)	< **0.001*****
	shoulder	0.31 (0.05 – 1.88)	0.202
	spleen	0.00 (0.00 – 0.03)	< **0.001*****

Significance codes: < 0.001***, < 0.01**, < 0.05*

4. Discussion

In order to exclude differences in the outcome of the infection due to the number of parasites inoculated, pigs were administered an equal dose of oocysts (∼ 400) of both isolates. Storage of the oocysts used in this experiment of two and five months for the type II and III isolates respectively, should not have affected the infectivity of the oocysts which are capable of surviving for months even under harsh environmental conditions (Dubey et al., 2011; Shapiro et al., 2019). Accordingly, we aimed to inoculate an approximately similar number of bradyzoites in tissue cysts as sporozoites contained in oocysts, considering that 400 oocysts should contain 3200 sporozoites in case of perfect sporulation rate and assuming that 10 tissue cysts harbour approximately 3500 bradyzoites (Watts et al., 2015). Since the infective dose of bradyzoites used in the current study was based on an average number of bradyzoites per cyst instead of counting the content of each of these tissue cysts, a potential deviation of the true numbers from the expected numbers is possible. Type II *T. gondii* isolates have been previously shown to produce larger and more numerous cysts in mice models compared with type III isolates (Fux et al., 2007); however, it should still be considered that heterogeneity amongst bradyzoites and tissue cysts may still outweigh potential inter-strain variability (Watts et al., 2015). In contrast to most studies using thousands of oocysts or tissue cysts for experimental infection, an intentionally lower dose was chosen in this experiment, considering doses as low as a single oocyst were proven to cause infections in pigs (Dubey et al., 1996). Moreover, a lower inoculation dose does not necessarily result in a lower parasite load in tissues (Jennes et al., 2017), suggesting a space for further investigation of the dose-effect on the progress of *T. gondii* infections in intermediate hosts.

The seroconversion of pigs in this study, manifested by a significant rise of IgG specific anti-*T. gondii* antibodies, occurred between 1 and 2 wpi in most animals. The lack of significant difference in the immunological response observed between pigs of Groups 3 and 4 may suggest a similar immunogenicity of the CZ-Šimková type III isolate regardless of the parasite stage inoculated. The level of the measured IgG antibody response in pigs of Group 1 was overall lower compared to the rest of the pigs. Combined with the overall lower levels of measured antibodies in the type II-inoculated Groups 1 and 2 compared to the levels in the type III-inoculated Groups 3 and 4 (Fig. 1), this result may suggest a lower immunogenicity of CZ-Tiger type II strain in pigs compared to the CZ-Šimková type III strain, and oocysts infections compared to those induced by tissue cysts. A similar strain effect to the one described in the current study was observed in a previous comparison of type II and III strains from Spain in pigs (Largo-de la Torre et al., 2022). Interpretation

Table 3
Ranking of tissues according to their parasite burden estimates. Detectable parasite burden (arithmetic mean) of T. gondii in nine tissues composing of three meat cuts ("shoulder" = musculus triceps brachii, musculus supraspinatus, musculus infraspinatus; "loin" = musculus longissimus dorsi pars lumbalis; and "ham" = musculus semitendinosus, musculus semimembranosusnosus, musculus gluteus medialis) and six organs (heart, brain, lungs, liver, spleen, and kidneys). The results are presented in parasites per gram of tissue and ranked from high to low. Arithmetic mean with the range of observed minimal and maximal mean of the corresponding group is provided for individual pigs (column on the right) and for single tissue (line at the bottom).

Type	Stage	Group	Pig number	Shoulder	Loin	Ham	Brain	Heart	Lungs	Kidneys	Spleen	Liver	Arithm. mean [min - max]
III	TC	4	22	442.5	697.5	675.4	20.8	58.8	26.6	10.7	ND	ND	214.7 [0.0–675.4]
III	TC	4	19	336.4	310.1	223.1	295.5	23.7	ND	ND	ND	ND	132.1 [0.0–336.4]
III	TC	4	24	356.3	479.6	137.3	116.3	ND	33.0	ND	ND	ND	124.7 [0.0–479.6]
III	OOC	3	14	295.5	17.3	24.1	66.2	ND	ND	ND	ND	ND	44.8 [0.0–295.5]
II	TC	2	13	58.7	109.5	54.3	46.7	ND	ND	ND	ND	ND	29.9 [0.0–109.5]
III	TC	4	23	47.6	72.9	71.9	56.3	0.1	ND	ND	ND	ND	27.6 [0.0–72.9]
III	TC	4	21	70.9	34.1	39.1	23.7	12.3	39.1	23.9	ND	ND	27.0 [0.0–70.9]
III	TC	4	20	99.2	26.6	63.0	6.2	ND	ND	ND	ND	ND	21.7 [0.0–99.2]
II	TC	2	7	57.3	18.3	23.0	ND	14.7	ND	ND	ND	ND	12.6 [0.0–57.3]
III	OOC	3	16	37.2	19.1	ND	8.6	ND	ND	ND	ND	ND	7.2 [0.0–37.2]
II	TC	2	8	55.69	ND	ND	ND	ND	ND	ND	ND	ND	6.2 [0.0–55.7]
II	TC	2	9	ND	ND	1.9	46.7	ND	ND	ND	1.3	ND	5.5 [0.0–46.7]
II	OOC	1	5	ND	ND	27.1	0.9	13.5	ND	ND	ND	ND	4.6 [0.0–27.1]
III	OOC	3	17	ND	11.6	0.8	22.2	ND	ND	ND	ND	ND	3.8 [0.0–22.2]
III	OOC	3	15	ND	11.5	ND	17.6	ND	ND	ND	ND	ND	3.2 [0.0–17.6]
II	TC	2	12	ND	ND	ND	18.2	ND	ND	ND	ND	ND	2.0 [0.0–18.2]
II	TC	2	11	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0–0.0]
II	OOC	1	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0–0.0]
II	OOC	1	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0–0.0]
II	OOC	1	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0–0.0]
II	OOC	1	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0–0.0]
II	OOC	1	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0–0.0]
Arithmetic mean				84.4	82.2	61.0	33.9	5.6	4.5	1.6	0.1	0.0	

ND = not detected in the sample, TC = tissue cyst, OOC = oocyst

Table 4
Coefficient estimates with standard errors for each of the infection routes. The estimates for the different infection routes (natural or experimental, via ingestion of oocysts or tissue cysts) are presented in log-transformed parasite load in the T. gondii-positive tissues. Bold are significant effects at the $p < 0.05$ level.

Variable	Fixed effects	Estimate (log10-transformed)	CI 95 %	Standard error	*p*-value
	intercept	0.77	0.27–1.27	0.28	**0.011***
Parasite stage	oocyst (reference)				
	tissue cyst	0.59	0.17–1.00	0.22	**0.020***
Isolate (strain)	CZ-Tiger (type II, reference)				
	CZ-Šimková (type III)	0.35	-0.05–0.77	0.22	0.131
Tissue	brain (reference)				
	ham	0.09	-0.29–0.48	0.21	0.657
	heart	-0.58	-1.07- −0.09	0.27	**0.034***
	kidneys	-0.58	-1.34–0.20	0.42	0.173
	liver	ND	ND	ND	ND
	loin	0.26	-0.12–0.65	0.21	0.217
	lungs	-0.32	-0.96–0.35	0.35	0.369
	shoulder	0.51	0.11–0.92	0.22	**0.024***
	spleen	-1.11	-2.23- −0.03	0.59	0.068

Significance codes: < 0.001***, < 0.01**, < 0.05*

of the effect of the parasite stage might be confined only to the isolates used in this study, CZ-Tiger and CZ-Šimková, as the overall effect of the parasite stage on immunogenicity is impossible to assess due to the lack of studies comparing infections induced by oocysts and tissue cysts of the same strain and dose. Regarding the time until seroconversion, the results observed in the remaining Groups 2–4 are in line with most experimental studies in pigs, which reported seroconversion between two and four wpi (Verhelst et al., 2011; Juránková et al., 2014; Basso et al., 2017b; Garcia et al., 2017; Genchi et al., 2017; Jennes et al., 2017).

Organs and muscular tissue may be microscopically altered during the acute phase of the infection due to the pathogenic effect of the parasite. Reactive interstitial hyperplasia of the lymphatic tissue and interstitial pneumonia was found in all pigs of the present study. Almost identical findings of lymphoplasmacytic interstitial pneumonia lymphadenomegaly, and non-collapsed lungs were observed in pigs infected with an atypical *T. gondii* genotype (Piva et al., 2022). Likewise, chronic interstitial pneumonia was also linked with toxoplasmosis in piglets (Thiptara et al., 2006). The affinity of *T. gondii* towards lung tissue, resulting in a high concentration of the parasite within, was observed in previous experimental infections of pigs (Juránková et al., 2014; Algaba et al., 2018).

The mostly non-specific observations found during the necropsy and results of the histopathological examination of the tissues of the two prematurely deceased pigs introduced various co-morbidities as probable causes of their grave condition. Cases of purulent necrotic splenitis, such as the one found in pig No. 18, are predominantly of bacterial origin, although the involvement of *T. gondii* cannot be excluded, as splenitis may be linked with *T. gondii* infections in animals (Oz, 2014; Campbell et al., 2022; Rodrigues Oliveira et al., 2022). Confirmation by additional molecular of immunohistochemical analyses would have been beneficial in order to firmly establish a direct link to *T. gondii* infection in this case.

Since the pathogenicity of *T. gondii* strains in mice varies from other animal species, and humans as an extension, the mice model was contested as the most suitable animal model prevising the pathogenicity in

human toxoplasmosis (Miranda et al., 2015; Calero-Bernal et al., 2022), possibly in the prospect of pigs becoming a promising replacement due to anatomical, physiological, immunological and dietary similarities to humans (Perleberg et al., 2018; Mukhopadhyay et al., 2020). The anatomical similarity of pigs and humans enabled us to observe reactive hyperplasia in tonsil tissue which would be otherwise completely missed in mice due to a lack of this tissue (Liebler-Tenorio and Pabst, 2006). Similarly, minor lesions of chronic interstitial pneumonia observed in all pigs could be undetected in mice because of known differences in lung disease development (Rogers et al., 2008). In this study, the obvious advantage of the pig model was observed in the possibility of repeated weekly blood collection for seroconversion monitoring which in these volumes would not be possible in mice models. The pig model is also deemed to be a more appropriate model for human immunological studies compared to mice (Dawson, 2011). Moreover, due to the longevity of pigs compared with mice, the possibility to study long-time effects of toxoplasmosis arises.

Type II strains were frequently identified in the Northern Hemisphere (Shwab et al., 2014; Fernández-Escobar et al., 2022), hence the effects of this lineage have been relatively well-described through experimental pig infections with type I and II isolates (Dubey et al., 1996; Algaba et al., 2018). The hereby presented comparison of type II and type III strains, although using isolates different to ours, was partially explored in pigs only recently (Largo-de la Torre et al., 2022). Significant variability in virulence of the different *T. gondii* strains was shown across animal species (Dubey et al., 2012; Calero-Bernal et al., 2022). Since laboratory-adapted strains were used in the current study, it is also important to note that the virulence and cyst formation in various laboratory-adapted *T. gondii* strains may be influenced in each strain to a different extent, as was shown recently using type II and type III strains (Colos-Arango et al., 2023). The current study presents an opportunity to fill the knowledge gap with a more complete view of the strain effect on the course of toxoplasmosis in animals.

Previous studies exploring inter-strain differences in the disease outcome between type I and II isolates in swine reported earlier antibody production in infections with type II isolate (Jennes et al., 2017) and a higher parasite load in tissues (Algaba et al., 2018). An infection with the CZ-Šimková type III isolate in this study resulted in significantly more positive tissues by qPCR when compared to an infection with the CZ-Tiger type II isolate (Table 2). However, no significant difference between the isolates was observed regarding the parasite load in tissues, despite the overall higher parasite load in pigs infected with the type III strain compared to the type II strain. This is in agreement with the previously observed higher parasite loads in tissues of animals infected with type III strains (Fernández-Escobar et al., 2020b; Largo-de la Torre et al., 2022).

Among other factors, *T. gondii* infections can be influenced by the infective stage of the parasite (Dubey et al., 2020). By involving all four possible combinations of infection with type II (CZ-Tiger) or type III (CZ-Šimková) isolates and the two infective stages, oocysts and tissue cysts, we present a complete picture of the effects of these variables on *T. gondii* experimental infection in pigs while demonstrating that pigs can be successfully infected with either *T. gondii* stage. An infection with tissue cysts in this study resulted in more than twice more positive tissues by qPCR when compared to infection with oocysts (Table 2). The fact that the parasite burden in tissues of the oocyst-infected pigs in the current study was observed to be lower than in the tissue-cyst-infected counterparts suggests that clinical signs in pigs are independent of the number of tissue cysts developed in the tissues of the pig. However, the severity of clinical signs in the infection with type III oocysts in this study, in addition to the premature euthanasia of two pigs from this group, may suggest that clinical signs are parasite-stage-dependent. This observation is in agreement with the previously published results where oocyst-induced infections in animals were more often associated with more severe clinical signs (Dubey and Beattie, 1988).

Comparison of the most frequently infected pig tissues and parasite burdens within is complicated by the variability of reported doses, isolates of different strains, and stages of *T. gondii*, which all influence the outcome. Parasites could be recovered from a variety of tissues and the most frequently infected groups of pigs in this study contained the highest observable parasite load at the same time. Brain and heart were repeatedly mentioned as tissues with higher *T. gondii* parasite load (Opsteegh et al., 2010; Verhelst et al., 2011; Juránková et al., 2014; Algaba et al., 2018), with lungs carrying higher loads of the parasite, especially in the early stages of the infection (Juránková et al., 2013; Algaba et al., 2018; Rahman et al., 2020). An interesting distribution pattern was observed in the tested organs of pigs included in this study which often tested negative for the presence of *T. gondii* DNA by qPCR, with the exception of the brain. A similar outcome, was presented by Juránková et al. (2014) who identified the brain as a predilection site by MC-qPCR using the same type II (CZ-Tiger) isolate, however, we did not observe high parasite loads in lungs, perhaps due to the lower inoculation dose we used. Alternatively, this might have been a result of the method used, as MC-qPCR was previously described as a more sensitive alternative to qPCR (Opsteegh et al., 2010; Juránková et al., 2013; Gisbert Algaba et al., 2017). In contrast to the results of this study, high numbers of parasites were described also in the hearts of pigs experimentally infected with different type II and type III isolates (Largo-de la Torre et al., 2022) as well as with isolates of other lineages (Verhelst et al., 2011; Jennes et al., 2017; Algaba et al., 2018; Xia et al., 2020). Low positivity rates reported for the liver and spleen are in concordance with the result presented in the current study. However, the relatively low prevalence and parasite load found in the hearts of tested pigs does not correlate with the 100 % positivity rates reported for the same isolate of type II (CZ-Tiger) (Juránková et al., 2014). Moreover, the liver and spleen were reported to carry fewer parasites which might be due to their unique structure rather than a shortcoming of the method, considering the two different methodological approaches applied, qPCR (used in the current study) and MC-qPCR. Overall, the tissues of the pigs included in this study that were infected with tissue cysts were significantly more frequently positive and contained on average significantly more parasites than tissues of animals infected with *T. gondii* oocysts. A different outcome observed by Algaba et al. (2018) in pigs, may have been caused by the different *T. gondii* isolate used for the inoculation, by the potential variability in virulence due to strain adaptation in laboratory conditions, or by the notably higher number of tissue cysts compared to oocysts. The result of this study correlates with the previously observed higher parasite burdens in tissue-cyst-infected definitive hosts (Dubey, 2022).

5. Conclusion

The current study presents an animal model highlighting the differences in the severity of clinical signs and distribution of the parasite within tissues of pigs experimentally infected with *T. gondii*, according to the combination of stages and strains of the parasite inoculated. Experimental infection with the type III isolate, and oocysts of this isolate in particular, caused a stronger antibody response in the pigs while leading to a higher parasite burden in the tissues, especially in pigs infected with tissue cysts. The observed variability in the virulence of *T. gondii* strains in pigs, a model species for human infections, and the significant effect of type III (CZ-Šimková) isolate in this study, suggest the need for further investigation of type III isolates to better understand the potential risks to humans.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2024.110222.

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