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IFN-γ expression and infectivity of *Toxoplasma* infected tissues are associated with an antibody response against GRA7 in experimentally infected pigs

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ABSTRACT

Toxoplasma gondii, an obligate intracellular parasite, can be transmitted to humans via the consumption of infected meat. However, there are currently no veterinary diagnostic tests available for the screening of animals at slaughter. In the current work, we investigated whether cytokine responses in the blood, and antibody responses against recombinant *T. gondii* GRA1, GRA7, MIC3 proteins and a chimeric antigen EC2 encoding MIC2–MIC3–SAG1, are associated with the infectivity of porcine tissues after experimental infection with *T. gondii*. Two weeks after experimental infection of conventional 5-week-old seronegative pigs, an IFN-γ response was detected in the blood, with a kinetic profile that followed the magnitude of the GRA7 antibody response. Antibody responses to GRA1, MIC3 and EC2 were very weak or absent up to 6 weeks post infection. Antibodies against GRA7 occurred in all infected animals and were associated with the presence of the parasite in tissues at euthanasia a few months later, as demonstrated by quantitative real-time PCR and isolation by bio-assay. Remarkably, although brain and heart tissue remained infectious, musculus gastrocnemius and musculus longissimus dorsi were found clear of infectious parasites 6 months after experimental infection. Seropositive response in a GRA7 ELISA indicates a *Toxoplasma* infection in pigs and is predictive of the presence of infectious cysts in pig heart and brain. This new ELISA is a promising tool to study the prevalence of *Toxoplasma* infection in pigs. Clearance of the infection in certain pig tissues suggests that the risk assessment of pig meat for human health needs further evaluation.

1. Introduction

Toxoplasma gondii, an obligate intracellular parasite, is one of the most common parasitic zoonosis worldwide (Tenter et al., 2000). By estimation, one third of the world population has been infected (Montoya and Liesenfeld, 2004). *T. gondii* can be transmitted during all its life-cycle stages; by ingestion of sporulated oocysts that are secreted by cats in the environment, by tissue cysts via consumption of raw or undercooked meat from infected animals, or by tachyzoites via congenital transmission (Tenter et al., 2000; Kijlstra and Jongert, 2008). It is not known which of these routes is epidemiologically the most important. However,

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the consumption of raw or undercooked meat has been regarded as a major route of transmission to humans (Cook et al., 2000). Recent studies have shown that intensive farm management has considerably decreased the prevalence of *T. gondii* in meat-producing animals over the past 20 years, and in several European countries, prevalences of *T. gondii* in fattening pigs are now <1% (van Knapen et al., 1995; van der Giessen et al., 2007). Due to an increased concern about the welfare of pigs and due to consumer demands, more organic farms are being set up according to EU regulations (EU regulation, 2092/91). However, *T. gondii* infections are more prevalent when pigs are being raised in animal-friendly surroundings than on regular intensive farms (Kijlstra et al., 2004; van der Giessen et al., 2007), because they have outdoor access, straw bedding, and organic pig feed. This way, *T. gondii* is again a part of their habitat. With a rising number of organic farms and the increase of conventional free-range swine farming, the need is growing for the development of veterinary diagnostic techniques able to discriminate *T. gondii* infected from *T. gondii* free animals (Fosse et al., 2008; Kijlstra et al., 2004; van der Giessen et al., 2007).

Using experimental *T. gondii* infections in pigs, we investigated in the current study whether cytokine responses in the blood, and antibody responses against recombinant *T. gondii* dense granule proteins (GRA1, GRA7), microneme protein MIC3 and a chimeric antigen EC2 encoding microneme proteins (MIC2, MIC3) and surface antigen SAG1, could be associated with the infectivity of porcine tissues.

2. Materials and methods

2.1. Parasites

T. gondii strain IPB-G is routinely maintained by mouse passage. IPB-G is a type II strain (Vercammen et al., 1998), and was harvested from the brains of chronically infected Swiss mice. Mice were euthanized by cervical dislocation, and *T. gondii* brain cysts were counted under a microscope. For experimental infection of pigs, the mouse brain homogenates were diluted in PBS at a concentration of 300 cysts/ml.

2.2. Animals

For this study, ten indoor-born Belgian Landrace pigs, *Toxoplasma gondii* seronegative in an indirect immunofluorescence assay (IIFA), were weaned at an age of 4 weeks and housed in isolation units. Eight pigs were infected orally with 3000 tissue cysts of the *T. gondii* IPB-G strain at the age of 4 weeks. The 2 remaining pigs served as negative controls and were each given orally half a brain of a non-infected mouse. After infection, the pigs were divided into 2 groups. The first group was maintained for 6 weeks (5 infected pigs, 1 control pig) and the second group for 6 months (3 infected pigs, 1 control pig). All pigs were bled weekly until 6 weeks after infection, and the second group again at euthanasia. Euthanasia was performed by intravenous injection of an overdose natriumpentobarbital 20% (Kela, Hoogstraten, Belgium).

Animal experimentation was performed with the prior approval of the animal ethics committee of the faculties of Bioscience Engineering and Veterinary Medicine (EC, 2007/103).

2.3. Indirect immunofluorescence assay

The presence of IgM and IgG antibodies against *T. gondii* in pig sera was evaluated by an indirect immunofluorescence assay (IIFA). Fifty microliters 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-Meĭrieux, 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 isothio-cyanate (FITC) conjugated anti-pig IgM or anti-pig IgG (KPL, Maryland, USA). After washing and drying, the slides were read with a fluorescence microscope (Carl Zeiss). The cut-off read-out of the fluorescence test was established with *T. gondii* seronegative and seropositive porcine reference sera 1/50 diluted (Jongert et al., 2008a). For detection of seroconversion in the mouse bio-assay, sera from these mice were tested at a 1:25 dilution and a secondary Alex 488 anti-mouse IgG antibody (Invitrogen, Merelbeke) (1/500) was used as conjugate.

2.4. Purification of recombinant antigens

Recombinant GRA1, rGRA7 and rEC2 were purified as described previously (Bivas-Benita et al., 2003; Jongert et al., 2007, 2008b). The $MIC3_{234–307}$ fragment was amplified from pcEC2 with forward primer 5′ gcgcggatccctccccaggatgccatt 3′ and reverse primer 5′ gcgcggatccaggactggatgtcatgcc 3′. The amplicon was purified with the PCR purification kit (QIAGEN GmbH, Hilden, Germany) and digested overnight with *BamH*I and *Hind*III, and ligated into pQE80 expression vector (QIAGEN). A clone was identified by colony PCR using the same primers and sequencing confirmed the presence of $MIC3_{234–307}$. Expression of $rMIC3_{234–307}$ was confirmed by SDS–PAGE and Western blot with serum from infected mice. The his-tagged $rMIC3_{234–307}$ was produced at large scale and purified according to a protocol described previously by Bivas-Benita et al. (2003).

2.5. Antibody ELISA

To measure total antigen-specific IgG antibodies, Nunc immunoplates (Life Technologies) were coated for 2 h at 37 °C with recombinant GRA1, rGRA7, rEC2 or rMIC3 at a concentration of 10 μg ml^{-1} in bicarbonate coating buffer (pH 9.7) at 4 °C. In subsequent steps, plates were blocked overnight at 37 °C in PBS-0.2% Tween®80, incubated for 1 h at 37 °C with serum diluted 1/50 in PBS and for 1 h at 37 °C with horseradish peroxidase conjugated rabbit anti-porcine gamma heavy chain antibodies (Ig; 1/1000) (Serotec, Belgium). After this, an *o*-phenylenediamine dihydrochloride tablet (Sigma Fast; Sigma) in H_2O_2 solution was added. The reaction was stopped by addition of 2 M H_2SO_4. Absorbance was read at 450/692 nm in an iMARK

Table 1
Oligonucleotide primers used in cytokine qPCR.

Cytokine	Primers (5′–3′), S = sense AS = antisense
IL-10	(S) CCTGGGTTGCCAAGCCTT
	(AS) GCTTTGTAGACACCCCTCTCTT
IFN-γ	(S) GAGCCAAATTGTCTCCTTCTACT
	(AS) CTGACTTCTCTTCCGCTTTCT
GAPDH	(S) CCATCACTGCCACCCAGAA
	(AS) CAGGGATGACCTTGCCCA
β-actin	(S) GGCATCCTGACCCTCAAGTA
	(AS) GCCTCGGTCAGCAGCA
Ribosomal 18S	(S) GTTGATTAAGTCCCTGCCCTTT
	(AS) GATAGTCAAGTTCGACCGTCTT

Microplate reader (Biorad, Nazareth, Belgium). In between each step, plates were washed with PBS 0.2% Tween®20. The cut-off value was calculated from pre-immune sera from all pigs (day-0) at a 1/50 dilution, with cut-off value as mean OD_{450} + 3 × STDEV(OD_{450} pre-immune sera). Cut-off values for ELISA were as follows: rGRA1: 0.123; rGRA7: 0.146; $rMIC3_{234–307}$: 0.136; rEC2: 0.072.

2.6. Real time quantitative PCR for porcine cytokines

Peripheral blood mononuclear cells (PBMC) and splenocytes were isolated by density gradient centrifugation using Lymphoprep (Nycomed, Brussels, Belgium). Lymphocytes were harvested and resuspended at a concentration of 5.10^6 cells ml^{-1} in RLT buffer (Qiagen, Nazareth, Belgium) and kept at −80 °C for later isolation of cytokine mRNA. mRNA was extracted using an RNAeasy 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 quantitative real time PCR (qPCR). The qPCR reactions were set up in 96-well optical microtiter plates with 25 μl mixture of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The oligonucleotide primers used for the detection of porcine IL-10 and IFN-γ the two house keeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin, and those for 18s rDNA are presented in Table 1. Each sample of cDNA was tested in duplicate, and nontemplate reactions were included in the runs as an internal control. For each sample, the target gene was amplified in parallel with the three house keeping 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 and 45 cycles of 2 steps: 95 °C for 15 s and 60 °C for 30 s. qPCR data were analysed 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 quantification was done relative to the geometric average of β-actin, GADPH and 18s rDNA genes, and data is represented as the relative normalised cytokine gene expression compared to the geometric mean number of housekeeping genes. The relative normalised expression of IL-10 and IFN-γ over the household genes in a control pig were 20205 and 3.68 respectively.

2.7. Detection of parasites by bio-assay and qPCR

After euthanasia of the pigs, 100 g of brain, heart, musculus gastrocnemius (mgastr) and musculus longissimus dorsi (mlongd) were collected from each pig in order to detect of parasites by bioassay and qPCR. The tissues were homogenised in 15 ml 0.9% NaCl, and the tissue suspensions were incubated 1–2 h in a 250 ml acidic pepsin solution (0.8 g l^{-1} pepsin and 7 ml l^{-1} HCl) at 37 °C. The suspension was filtered and centrifugated at 1180 × *g*, and the pellet was resuspended in 5 ml PBS with gentamicin. Per tissue sample, 85 μg was kept for qPCR quantification, and five mice were intraperitoneally inoculated with 1 ml tissue suspension each. Lungs and brains of mice that died from acute toxoplasmosis after inoculation were examined for *T. gondii* parasites by phase-contrast microscopy and qPCR. Serum was sampled from each mouse on day 45 post-inoculation and tested for *T. gondii* antibodies with IIFA as described above. The brains of all mice were examined in quantitative real-time PCR for *T. gondii* as described previously by Kijlstra et al. (2008) and Rosenberg et al. (2009). 85 μl of tissue suspension was used for DNA extraction with the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) 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-tachyzoites and one with a counted number of cultured swine kidney cells (SK-6) as described previously (Rosenberg et al., 2009). DNA was tested by duplex quantitative real-time Taqman PCR on a BioRad iCycler (Biorad, Hercules, CA) using the *T. gondii* repeat element (AF146527) as the first target (Homan et al., 2000). The second target is based on the ribosomal 18s rDNA of the host cells. The reaction was performed as described previously by Kijlstra et al. (2008).

3. Results

3.1. Humoral response after infection

In order to evaluate kinetics of the antibody response elicited after oral infection with *T. gondii* bradyzoites, sera were collected and tested in the Toxoplasma-specific IgM/IgG IIFA (Table 2), and in the rGRA1, rGRA7, $rMIC3_{234–307}$ and rEC2-specific IgG ELISAs (Fig. 1).

In IIFA, all infected pigs were seropositive two weeks post infection (PI) for both IgG and IgM, after which a gradual decline in IgM positive animals was observed. IgM disappeared by week 6 PI, while animals were still IgG positive. In ELISA, an early but weak response against

Table 2
Toxoplasma seroconversion rates in IIFA from pigs experimentally infected with IPB-G.

	Weeks post infection						
	0	1	2	3	4	5	6
IgM (positive/total number)	0/5	nd	5/5	4/5	3/5	2/5	0/5
IgG (positive/total number)	0/5	nd	5/5	3/5	4/5	5/5	5/5

nd = not determined.

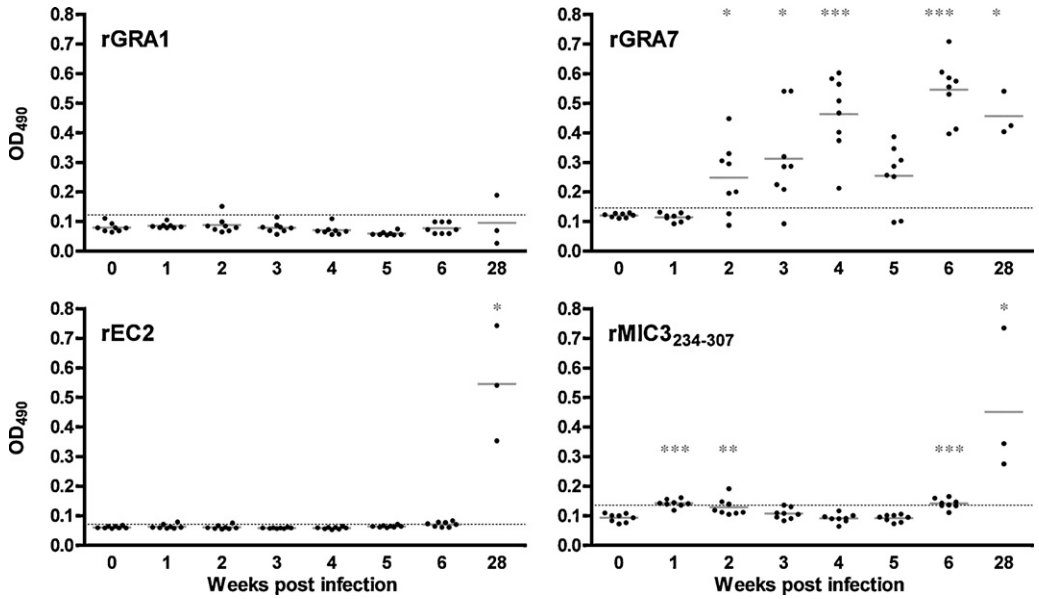

Fig. 1. Kinetics of the antibody responses against rGRA1, rGRA7, rEC2 and rMIC3$_{234–307}$ after oral infection of pigs with *T. gondii* IPB-G presented as OD-values for the different animals. The lines present the mean OD-value at each time point, The dashed lines indicate the cut-off values defined with pre-immune sera. *, **, *** present significances at $p < 0.05$, 0.01 and 0.001, respectively, in comparison with pre-immune sera.

rMIC3$_{234–307}$ was seen in 7 out of 8 pigs (week 1 PI). This seropositivity waned during the first five weeks, increased again at 6 weeks PI and at 28 weeks PI all 3 remaining animals showed a strong humoral response against this antigen. Antibody responses against rEC2 were absent during the first week after infection, but at 28 weeks PI all pigs had mounted a strong antibody response against this antigen. The recombinant dense granule protein rGRA7 was detected quickly by the majority of infected animals at 2 weeks PI. These initially moderate antibody responses increased in magnitude over time. At the same time, the number of seropositive animals increased so that all pigs had seroconverted against rGRA7 with a strong antibody response at 4 weeks PI. Thereafter all pigs remained seropositive for GRA7. Against rGRA1, only one of the 8 infected pigs showed a temporary response at two weeks PI, and only one of three pigs euthanized at 28 weeks PI was weakly seropositive. Control pigs orally infected with *T. gondii* negative brain tissue did not become seropositive in IIFA or the ELISAs at any time (data not shown).

In further experiments, combinations of proteins were tested in ELISA, but compared to the rGRA7 ELISA, no added value with regards to seroconversion rates or OD$_{450}$ values was observed (data not shown).

3.2. Interferon-γ and IL-10 cytokine production in the blood from pigs infected with T. gondii IPB-G

In order to evaluate whether an acute *T. gondii* infection could increase IFN-γ and/or IL-10 levels in the blood of infected pigs, PBMCs were isolated on a weekly basis until 5 weeks PI. Relative normalised IFN-γ and IL-10 mRNA levels were determined by qPCR, and compared with baseline levels from non-infected pigs. While there was no increase in IL-10 mRNA expression, except for one pig at 5 weeks PI (Fig. 2A), marked increases in IFN-γ expression were observed (Fig. 2B). At 2 weeks PI, 2 out of 8 IIFA-seropositive animals showed elevated IFN-γ expression levels, which had further increased in both animals by the third week PI. At 4 weeks PI, 5 out of 8 seropositive animals and one week later, even 6 out of 8 animals were producing IFN-γ.

3.3. Isolation of parasites by bio-assay and detection of bradyzoites in the tissues of infected animals

Evaluation of tissue infectivity was carried out by bio-assay on tissues obtained after euthanasia of the animals. The live parasite was isolated by bio-assay from all tested tissues from all the inoculated animals six weeks after infection, except from the musculus gastrocnemius

Table 3
Infectivity of tissues from pigs orally infected with IPB-G, determined by bio-assay and detection of bradyzoites by qPCR.

Tissues	Bio-assay (positive/total number)				qPCR (positive/total number)			
	Brain	Heart	Mgastr	Mlongd	Brain	Heart	Mgastr	Mlongd
6 weeks pi	5/5	5/5	4/5	5/5	5/5	5/5	3/5	3/5
6 months pi	3/3	3/3	0/3	1/3	3/3	3/3	0/3	0/3

pi = post infection, mgastr = musculus gastrocnemius, mlongd = musculus longissimus dorsi.

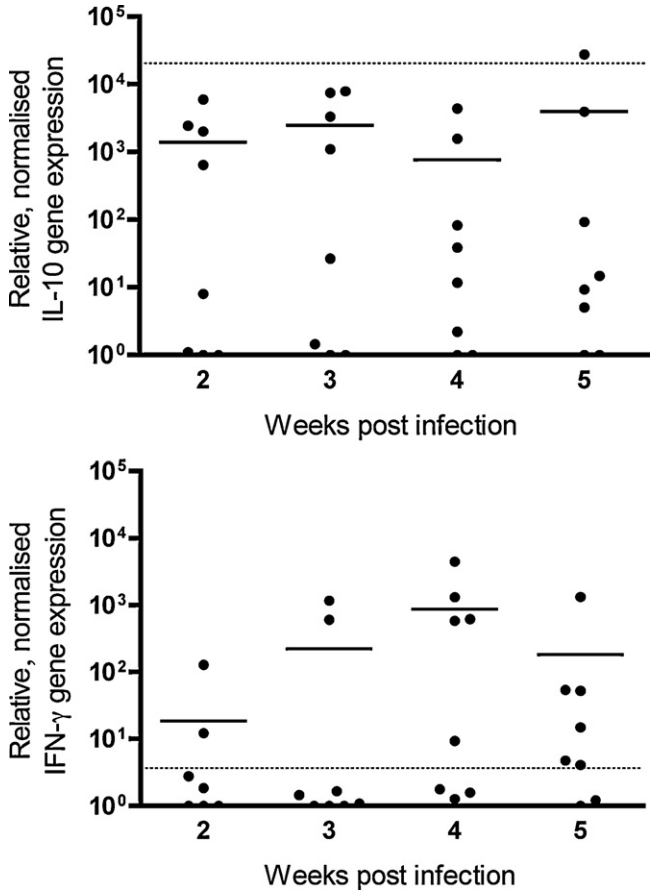

Fig. 2. Relative normalised IFN-γ and IL-10 cytokine expression in PBMCs from pigs orally infected with *T. gondii* IPB-G. Relative normalized cytokine levels are presented in scatter dot plots with a line presenting the mean of 8 pigs. Cytokine levels in the blood one week pi were not tested. The dotted line presents the relative normalized cytokine baseline expression level determined in a non-infected pig.

(mgastr) of one pig (Table 3). These results were confirmed by qPCR for brain and heart, but detection of parasite DNA was less sensitive for mgastr and musculus longissimus dorsi (mlongd) as 2 animals were found negative for both tissues. Six months PI, live *T. gondii* parasites could be isolated from heart and brain from all animals (Table 3). Surprisingly, all mgastr samples and 2 out of the 3 mlongd samples were negative in bio-assay. Results of the bio-assay were confirmed by qPCR for brain, heart, mgastr and 2 out of the 3 mlongd. The mlongd of one pig, positive in bio-assay, could not be confirmed by qPCR (negative result). Both control animals remained *T. gondii* free as demonstrated by bio-assay and qPCR (data not shown).

3.4. Quantification of the parasite load in tissues at 6 weeks and 6 months after infection

In order to determine whether parasite load changed over time, the numbers of bradyzoites were determined by qPCR in brain, heart, mgastr and mlongd samples at 6 weeks and 6 months PI. In pigs euthanised 6 weeks PI, brain, heart and mlongd contained 80.8 ± 51.3 (SEM), 64.1 ± 36.0 (SEM) and 112.7 ± 79.9 (SEM) bradyzoites per 10^8 porcine cells, respectively (Fig. 3A). The parasites were less prominent in the mgastr, with 12.0 ± 6.2 (SEM) bradyzoites per 10^8 cells. Six months after infection, all tissues were free of *T. gondii* parasites except the brains (18391 ± 18391 bradyzoites per 10^8 porcine cells), and the hearts (4.6 ± 4.2 bradyzoites per 10^8 porcine cells) (Fig. 3B). The negative control animals remained *T. gondii*-free in all tissues (data not shown).

4. Discussion

Because direct detection of the infectious *T. gondii* parasite in bio-assay is tedious and requires large numbers of experimental animals, we have aimed to develop a rapid, sensitive and *T. gondii*-specific serological test for veterinary use, that is associated with the infectivity of the animal tissue. Different recombinant *T. gondii* proteins have previously been tested for their application in *T. gondii*-specific ELISA's in swine, sheep and goat (Tenter et al., 1992; Coughlan et al., 1995; Andrews et al., 1997; Lind et al., 1997; Gamble et al., 2000; Garcia et al., 2006b, 2008; Jiang et al., 2008; Velmurugan et al., 2008). However, none of these studies addressed the relationship between a positive ELISA-result and the infectivity of the animals' tissues.

In our study, a strong antibody response shortly after *T. gondii* infection was observed against rGRA7, while vir-

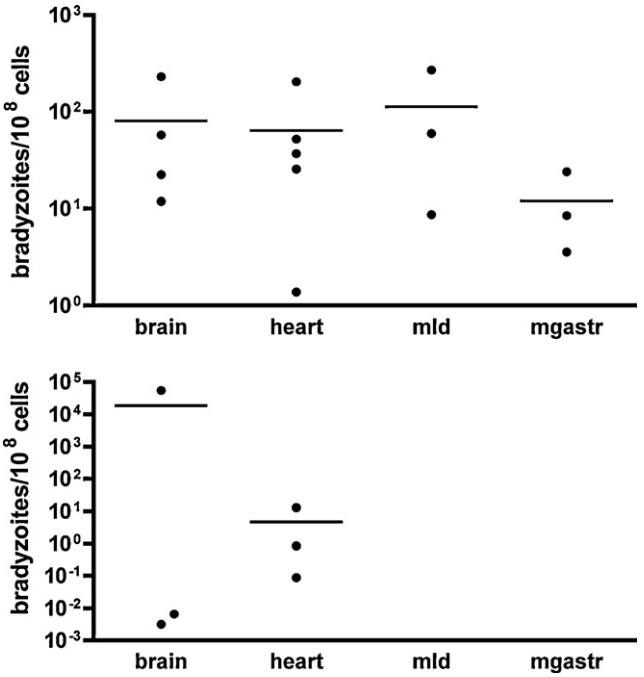

Fig. 3. Bradyzoite load in tissues from pigs orally infected with *T. gondii* IPB-G, determined by qPCR. The number of bradyzoites per 10^8 porcine cells are presented in dot plots. A line presents the mean number of 5 pigs in each tissue at 6 weeks pi (A), or from 3 pigs at 6 months pi (B). mgastr = musculus gastrocnemius. mld = musculus longissimus dorsi.

tually no response was obtained against rGRA1 or rEC2 at the early stage of infection. The rGRA7-specific antibodies were detected in the majority of infected animals as soon as 2 weeks PI and corresponded to the result obtained in IgG IIFA. GRA7-specific antibody levels increased over time and all animals were GRA7 seropositive at 6 weeks PI. The majority of animals showed a weak antibody response against $rMIC3_{234–307}$ at 7 days PI, which then waned. Similarly, a rMIC3-based latex-agglutination test detected *T. gondii* specific antibodies as soon as 8 days PI, yet could not detect *T. gondii* antibodies later than 42 days after infection (Jiang et al., 2008). In another study described by Lind et al. (1997), responses against SAG1 were shown to be independent of the life stage of the parasite inoculum used in the experimental infection of pigs, and were detectable as soon as 2 weeks PI and persisted for three to four months. In goats, both rGRA7 and rSAG1 were recognized in ELISA-using serum from IIFA positive animals (Velmurugan et al., 2008). In our study, a chimeric antigen encoding MIC2–MIC3–SAG1 could not reveal a humoral response elicited in the first week after *T. gondii* infection.

Six weeks after the experimental infection, parasites were detected by bio-assay and qPCR in the pigs' heart, brain, m. longissimus dorsi and m. gastrocnemius of the majority of animals. Surprisingly, six months after infection no parasites could be detected in the m. gastrocnemius of all and the m. longissimus dorsi of 2 out of the 3 pigs, while brain and heart remained infectious. All of the animals remained seropositive in rGRA7 ELISA, and had seroconverted against rEC2 and $rMIC3_{234–307}$, but not against rGRA1. Previously, antibody responses were shown to be linked to infectivity of experimentally (Dubey et al., 1997) and naturally infected pigs (Gamble et al., 2005), using an ELISA based on whole tachyzoites. A good association was found between tissue infectivity and a positive result in ELISA against a total tachyzoite lysate, and this ELISA was also shown to work with meat juice samples as well (Wingstrand et al., 1997). Furthermore, the serologic response and meat infectivity at 4 months PI were independent of the type of parasite inoculum; such as tissue cysts or oocysts (Wingstrand et al., 1997). Persistence of *T. gondii* was demonstrated in 31% of animals in commercial cuts of meat 875 days after experimental infection of the pigs with *T. gondii* oocysts, while parasites could be isolated from brain and heart in 75% and 69% of the animals respectively (Dubey, 1988).

Our serological results suggest that rGRA7 antibody responses may be independent of the parasite burden in commercial cuts of meat. A similar observation has been made in pigs naturally infected with *T. gondii* (Hill et al., 2006).

We cannot exclude that the clearance of parasite infection in commercial cuts of meat, as observed in our bio-assay and qPCR, is a phenomenon specifically associated with the IPB-G strain. Other type II strains are able to establish a persistent infection in commercial pig meat cuts (Velmurugan et al., 2009; de Sousa et al., 2006). Furthermore, we observed that the qPCR was less sensitive than bio-assay on muscle tissue, most probably because a smaller amount of digested tissue is used in this test. Other studies have indicated that DNA from pig tissues interfered with PCR sensitivity, and that mouse bioassay was better than PCR in detecting *T. gondii* in pig tissues (Garcia et al., 2006a; Hill et al., 2006). In contrast, a posi-

tive rGRA7 ELISA result was always correlated to *T. gondii* infection and infectivity of heart and brain, and a preceding *T. gondii* infection. This opens possibilities for the development of pre-slaughter serological monitoring of *T. gondii* infection in meat-producing animals. However, clearance of the infection in certain pig tissues suggests that the risk assessment of pig meat for human health needs further evaluation.

The immune response against *T. gondii* in mice is strongly correlated with a T-helper-1 profile, characterized by the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells, while IL-10 has been shown to down regulate acute hyper-inflammatory responses against this intracellular parasite (Denkers, 1999). In our study, compared to the baseline IFN-γ level from a non-infected pig, a gradual increase in IFN-γ expression by PBMCs was observed in infected pigs from week 2 to 5, with a kinetic profile somewhat similar to the magnitude of antibody responses against rGRA7 during the same follow-up period. In contrast, IL-10 responses were not increased and slightly lower than IL-10 levels from a non-infected animal. In the present study, the increase in IFN-γ mRNA production by blood lymphocytes was linked to the experimental infection, but we are aware that in reared pigs, increased blood IFN-γ levels can be reminiscent of other infections. Nevertheless, our observation of a Th1 response during acute toxoplasmosis is in line with a study from Solano Aguilar et al. (2001) who reported a similar PBMC IFN-γ profile during acute infection and somewhat reduced IL-10 levels in experimentally infected miniature swine.

In conclusion, the results reported here show that antibodies against GRA7 can be used to demonstrate infection of pigs with *T. gondii*. However, in this experimental model they are not reflective of the infectivity of pig tissues, since experimentally infected pigs seems to clear the IPB-G parasite from certain tissues. A rGRA7 based ELISA demonstrates previous infection with *T. gondii*, but further analysis is needed to determine infection risk of meat from seropositive pigs after natural infection.

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References

Andrews, C.D., Dubey, J.P., Tenter, A.M., Webert, D.W., 1997. *Toxoplasma gondii* recombinant antigens H4 and H11: use in ELISAs for detection of toxoplasmosis in swine. Vet. Parasitol. 70, 1–11.

Bivas-Benita, M., Laloup, M., Versteyhe, S., Dewit, J., De Braekeleer, J., Jongert, E., Borchard, G., 2003. Generation of *Toxoplasma gondii* GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary in vivo studies. Int. J. Pharm. 266, 17–27.

Cook, A.J., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *Toxoplasma* infection in pregnant women: European multicentre case–control study. European research network on congenital toxoplasmosis. BMJ 321, 142–147.

Coughlan, S.N., Saman, E., Jacobs, D., Mercier, C., Cesbron-Delauw, M.F., Trees, A.J., 1995. Cellular and humoral immune responses to recombinant antigens in sheep infected with *Toxoplasma gondii*. Parasite Immunol. 17, 465–468.

de Sousa, S., Ajzenberg, D., Canada, N., Freire, L., da Costa, J.M., Dardé, M.L., Thulliez, P., Dubey, J.P., 2006. Biologic and molecular characterization of *Toxoplasma gondii* isolates from pigs from Portugal. Vet. Parasitol. 135, 133–136.

Denkers, E.Y., 1999. T lymphocyte-dependent effector mechanisms of immunity to *Toxoplasma gondii*. Microbes Infect. 1, 699–708.

Dubey, J.P., 1988. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. Am. J. Vet. Res. 49, 910–913.

Dubey, J.P., Andrews, C.D., Thulliez, P., Lind, P., Kwok, O.C., 1997. Long-term humoral antibody responses by various serologic tests in pigs orally inoculated with oocysts of four strains of *Toxoplasma gondii*. Vet. Parasitol. 68, 41–50.

Fosse, J., Seegers, H., Magras, C., 2008. Foodborne zoonoses due to meat: a quantitative approach for a comparative risk assessment applied to pig slaughtering in Europe. Vet. Res. 39, 1.

Gamble, H.R., Andrews, C.D., Dubey, J.P., Webert, D.W., Parmley, S.F., 2000. Use of recombinant antigens for detection of *Toxoplasma gondii* infection in swine. J. parasitol. 86, 459–462.

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

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

Garcia, J.L., Navarro, I.T., Vidotto, O., Gennari, S.M., Machado, R.Z., da Luz Pereira, A.B., Sinhorini, I.L., 2006b. *Toxoplasma gondii*: comparison of a rhoptry-ELISA with IFAT and MAT for antibody detection in sera of experimentally infected pigs. Exp. Parasitol. 113, 100–105.

Garcia, J.L., Gennari, S.M., Navarro, I.T., Machado, R.Z., Headley, S.A., Vidotto, O., da Silva Guimaraes Jr., J., Bugni, F.M., Igarashi, M., 2008. Evaluation of IFA, MAT, ELISAs and immunoblotting for the detection of anti-*Toxoplasma gondii* antibodies in paired serum and aqueous humour samples from experimentally infected pigs. Res. Vet. Sci. 84, 237–242.

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

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

Jiang, T., Gong, D., Ma, L.A., Nie, H., Zhou, Y., Yao, B., Zhao, J., 2008. Evaluation of a recombinant MIC3 based latex agglutination test for the rapid serodiagnosis of *Toxoplasma gondii* infection in swines. Vet. Parasitol. 158, 51–56.

Jongert, E., de Craeye, S., Dewit, J., Huygen, K., 2007. GRA7 provides protective immunity in cocktail DNA vaccines against *Toxoplasma gondii*. Parasite Immunol. 29, 445–453.

Jongert, E., Melkebeek, V., De Craeye, S., Dewit, J., Verhelst, D., Cox, E., 2008a. An enhanced GRA1–GRA7 cocktail DNA vaccine primes anti-*Toxoplasma* immune responses in pigs. Vaccine 26, 1025–1031.

Jongert, E., Verhelst, D., Abady, M., Petersen, E., Gargano, N., 2008b. Protective Th1 immune responses against chronic toxoplasmosis induced by a protein–protein vaccine combination but not by its DNA–protein counterpart. Vaccine 26, 5289–5295.

Kijlstra, A., Jongert, E., 2008. Control of the risk of human toxoplasmosis transmitted by meat. Int. J. Parasitol. 38, 1359–1370.

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

Kijlstra, A., Meerburg, B., Cornelissen, J., De Craeye, S., Vereijken, P., Jongert, E., 2008. The role of rodents and shrews in the transmission of *Toxoplasma gondii* to pigs. Vet. Parasitol. 156, 183–190.

Lind, P., Haugegaard, J., Wingstrand, A., Henriksen, S.A., 1997. The time course of the specific antibody response by various ELISAs in pigs experimentally infected with *Toxoplasma gondii*. Vet. Parasitol. 71, 1–15.

Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet 363, 1965–1976.

Rosenberg, C., De Craeye, S., Jongert, E., Gargano, N., Beghetto, E., Del Porto, P., Vorup-Jensen, T., Petersen, E., 2009. Induction of partial protection against infection with *Toxoplasma gondii* genotype II by DNA vacci-

nation with recombinant chimeric tachyzoite antigens. Vaccine 27, 2489–2498.
Solano Aguilar, G.I., Beshah, E., Vengroski, K.G., Zarlenga, D., Jauregui, L., Cosio, M., Douglass, L.W., Dubey, J.P., Lunney, J.K., 2001. Cytokine and lymphocyte profiles in miniature swine after oral infection with *Toxoplasma gondii* oocysts. Int. J. Parasitol. 31, 187–195.
Tenter, A.M., Vietmeyer, C., Johnson, A.M., 1992. Development of ELISAs based on recombinant antigens for the detection of *Toxoplasma gondii*-specific antibodies in sheep and cats. Vet. Parasitol. 43, 189–201.
Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30, 1217–1258.
van der Giessen, J., Fonville, M., Bouwknegt, M., Langelaar, M., Vollema, A., 2007. Seroprevalence of *Trichinella spiralis* and *Toxoplasma gondii* in pigs from different housing systems in The Netherlands. Vet. Parasitol. 148, 371–374.
van Knapen, F., Kremers, A.F., Franchimont, J.H., Narucka, U., 1995. Prevalence of antibodies to *Toxoplasma gondii* in cattle and swine in The Netherlands: towards an integrated control of livestock production. Vet. Q. 17, 87–91.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, RESEARCH0034.
Velmurugan, G.V., Tewari, A.K., Rao, J.R., Baidya, S., Kumar, M.U., Mishra, A.K., 2008. High-level expression of SAG1 and GRA7 gene of *Toxoplasma gondii* (Izatnagar isolate) and their application in serodiagnosis of goat toxoplasmosis. Vet. Parasitol. 154, 185–192.
Velmurugan, G.V., Su, C., Dubey, J.P., 2009. Isolate designation and characterization of *Toxoplasma gondii* isolates from pigs in the United States. J. parasitol. 95, 95–99.
Vercammen, M., Dardé, M.L., El Bouhdidi, A., Ben Messaoud, A., De Meuter, F., Bazin, H., Dubremetz, J.F., Carlier, Y., 1998. Fc receptor activity of *Toxoplasma gondii* tachyzoites: immunoglobulin binding by *T. gondii* strains with different isoenzyme patterns, and by SAG1 and SAG2 deficient mutants. In: Isao Tada, S.K.a.M.T. (Ed.), 9th International Congress of Parasitology. Monduzzi Editore, Italy, pp. 1027–1032.
Wingstrand, A., Lind, P., Haugegaard, J., Henriksen, S.A., Bille-Hansen, V., Sorensen, V., 1997. Clinical observations, pathology, bioassay in mice and serological response at slaughter in pigs experimentally infected with *Toxoplasma gondii*. Vet. Parasitol. 72, 129–140.