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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

D. Verhelst[a,*,1], S. De Craeye[b,1], P. Dorny[c,d], V. Melkebeek[a], B. Goddeeris[a,e], E. Cox[a], E. Jongert[f]

[a] *Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820, Ghent, Belgium*
[b] *National Reference Laboratory for Toxoplasmosis, Operational Direction Communicable and Infectious Diseases, Scientific Institute of Public Health, Federal Public Service Public Health, Security of the Food Chain and Environment, Brussels, Belgium*
[c] *Department of Animal Health, Institute for Tropical Medicine, Antwerp, Belgium*
[d] *Laboratory of Parasitology, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium*
[e] *Department of Biosystems, Faculty of Bioscience Engineering, K.U. Leuven, Belgium*
[f] *Toxoplasma Research – Vaccinology, Laboratory for Toxoplasmosis, Pasteur Institute of Brussels, Belgium*

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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,

* Corresponding author. Tel.: +32 92647339.
E-mail address: Delfien.verhelst@ugent.be (D. Verhelst).
[1] These authors contributed equally to this work.

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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