Humoral and cellular immune responses in pigs immunized intranasally with crude rhoptry proteins of *Toxoplasma gondii* plus Quil-A

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A B S T R A C T

We evaluated the humoral and cellular immune responses in pigs immunized intranasally with crude rhoptry proteins of *Toxoplasma gondii* plus Quil-A. The experiment used 13 mixed-breed pigs divided into the following three groups: G1 (vaccinated-challenged, $n = 6$), which received the rhoptry vaccine (200 (g/dose); G2 (adjuvant-challenged, $n = 4$), which received PBS plus Quil-A; and G3 (unvaccinated-challenged, $n = 3$), which was the control group. The treatments were performed intranasally at days 0, 21, and 42. Three pigs from G1 produced IgG and IgM antibody levels above the cut-off in the ELISA on the challenge day. Partial protection was observed in G1 at the chronic phase of infection when compared with G3. The preventable fractions were 41.6% and 6.5%, in G1 and G2, respectively. The results of this study suggest that rhoptry proteins plus Quil-A stimulated humoral, local, and systemic immune responses, which were able to partially protect the brain from cyst formation.

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1. Introduction

Toxoplasma gondii is a protozoan parasite that can infect humans and warm-blooded animals. Humans can become infected by ingesting raw or undercooked meat containing cysts (Garcia, 2009). The longevity of a tissue cyst in pork can last more than two years (Dubey et al., 1998), and pork is one of the most common sources of *T. gondii* infections in humans (Dubey et al., 1991). These results indicate that developing a vaccine against *T. gondii* in pigs would be desirable to reduce tissue cyst formation.

Live vaccines (RH, T263, and S48) have shown protection against toxoplasmosis (Frenkel et al., 1991; Dubey et al., 1991, 1994), but these carry the risk of reverting to virulence (Supply et al., 1999). A useful *T. gondii* vaccine for human beings and animals needs to be safe (non-infectious), to have a reasonable shelf life, to be usable in pregnant females without infecting the fetuses, to protect against transplacental infection, and to avoid oocyst shedding by cats and tissue cyst formation in animals (Garcia, 2009).

A vaccine study using crude *T. gondii* antigens incorporated into ISCOM subcutaneously in pigs, monitored through a mouse bioassay, did not isolate tissue cysts from vaccinated animals (Freire et al., 2003). Garcia et al. (2005) used rhoptry proteins incorporated into ISCOM to prevent tissue cyst formation in pigs challenged with sporulated

oocysts of the VEG strain. The results indicated that the rhoptry vaccine conferred partial protection during the chronic phase of the disease.

Smith et al. (1998) showed that oral administration of free Quil-A together with OVA reproduced most of the local and systemic immune responses obtained with ISCOM and OVA. The saponin adjuvant Quil-A is obtained from the bark of a tree, *Quillaja saponaria*. Quil-A is a widely used veterinary adjuvant that is inexpensive, simple to formulate, and generally safe (Cox and Coulter, 1997). Additionally, QS21, a purified fraction from Quil-A, was used for control of *Plasmodium falciparum* in humans, and the authors described an enhanced in immunogenicity of peptide vaccine (Kashala et al., 2002).

The most common infection route of *T. gondii* is oral ingestion. Therefore, stimulation of a mucosal immune response will be desirable in controlling oral toxoplasmosis (Chardés and Bout, 1993). The intranasal route has been evaluated as an immunization route in pigs, however, these studies tested virus and bacterial immune responses (Yokomizo et al., 2002; Zhang et al., 2007; Neumann et al., 2009). Yokomizo et al. (2002) showed that intranasal immunization is more efficient for inducing local and systemic immunity than oral immunization in pigs.

In the present study, we evaluated humoral and cellular immune responses in pigs immunized intranasally with crude rhoptry proteins of *T. gondii* plus Quil-A.

2. Materials and methods

2.1. Toxoplasma gondii strain

LIV-5 and VEG *T. gondii* strains were used in the experiment. LIV-5 strain was used to obtain rhoptries and VEG strain was used for the pigs challenge. Oocysts from VEG strain were obtained from feces of recently infected cats. After sporulation the oocysts had their virulence tested by mouse infection before pig infection.

2.2. Rhoptries purification

Tachyzoites of LIV-5 strain were obtained from peritoneal fluid of infected Swiss mice. The material was passed three times through a 26 gauge needle to purification and washed twice with 10 mM phosphate buffered saline (PBS, pH 7.5). The pellet was resuspended and washed twice in homogenization medium (HM: 250 mM sucrose; 1 mM EDTA; 5 mM triethanolamine–HCl; pH 7.5), after washed, tachyzoites were prepared at a concentration of 10^9 tachyzoites/mL. Cell suspension was disrupted in a French pressure cell at 50 kg/cm^3. Unbroken cells were sedimented by a 10 min centrifugation at $750 \times g$. Supernatant was centrifuged at $12,000 \times g$ for 10 min to sediment the crude organellar. Final pellet was fractionated by isopynic sucrose density gradient centrifugation to rhoptries isolation following Garcia et al. (2004). Briefly, the final pellet was resuspended in 4 mL of HM, layered onto a 6 mL preformed continuous 1.0–1.6 M sucrose gradient and centrifuged overnight at $72,000 \times g$. Sub-fractions were collected from each visible gradient fraction, suspended in 10 mL of HM, and pelleted by centrifugation $120,000 \times g$

for 1 h. Fraction 3 (1.4 M and a density of 1.17 g/cm^3) showed rhoptries (Garcia et al., 2004) and was used to vaccinated the animals. From one hundred mice infected with *T. gondii* LIV-5 we collected 300 mL of peritoneal fluid with 3×10^7 tachyzoites/mL and obtained 4600 µg of rhoptry proteins in a total volume of 2 mL. Protein concentration in each sub-fraction was determined using the bicinchoninic acid technique (BCA Protein Assay Reagent, Pierce).

2.3. Vaccination and challenge of pigs

2.3.1. Animals

The maintenance and care of experimental animals complied with the Animal Ethic Committee from Universidade Estadual de Londrina (CEEA 17/09). Thirteen mixed breed pigs between 6.5- and 7.5-week-old, including females and castrated males, were randomly allocated in separate stables (2 for 2). The animals were left to acclimatize for 6 days before we began the experiment. They received food and water ad libitum. All pigs were serum negative (titre < 64) in the *T. gondii* indirect immunofluorescence assay (IFA).

2.3.2. Vaccination and challenge

The pigs were divided into 3 groups, group 1 (G1, $n = 6$), group 2 (G2, $n = 4$) and group 3 (G3, $n = 3$). The G1 received 200 µg of rhoptry proteins plus Quil-A (50 µg) by nasal route (NA) at days 0, 21, and 42 of the experiment. The G2 and G3 received just Quil-A (50 µg) and PBS by nasal route, respectively. At challenge day (day 49) two animals from G1 were euthanized for lymphocytes proliferation assay, and the other animals from G1 ($n = 4$), G2 ($n = 4$) and G3 ($n = 3$) were challenged with 10^3 oocysts of VEG strain by oral route.

2.3.3. Sampling and measurements

Clinical signs and body temperatures were recovered before and after challenge. Serum samples were obtained at days −6, 0, 21, 42, 49, 64, 79 and at slaughter (day 94) and stored at −20 °C. At death, brain samples were collected to investigate *T. gondii* tissue cysts by mouse bioassay.

2.3.4. Immunofluorescence assay (IFA)

The presence of antibodies against *T. gondii* in serum samples of pigs (before of experiment to select the animals) and mice were measured by indirect immunofluorescence assay (Camargo, 1974) considering as positive pigs with titre ≥ 64 and mice with titre ≥ 16 (Garcia et al., 2005).

2.4. Enzyme-linked immunosorbent assay (ELISA) for IgG, and IgM

Flat-bottom 96 well polystyrene microtitration plates (Nunc-Immuno Plate, MaxiSorp, Denmark) were coated with 0.1 mL of the rhoptry antigens (5 µg/well) diluted in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at +6 °C as described by Garcia et al. (2005). The plates were washed 3 times with PBS-tween 20 (50 mM tris, pH 7.4, containing 150 mM sodium chloride and 0.05% tween 20) and non-specific immune sites blocked by incubation for

1 h at 37 °C with carbonate buffer −8% nonfat dry milk. The control sera and test sera were diluted 1:200 in PBS-tween 20–5% nonfat dry milk and added to the microtitre plates in duplicate, 0.1 mL in each well, and incubated for 1 h at 37 °C. The positive and negative control sera were included in each plate. After washing, peroxidase-labeled anti-pig IgG and IgM antibody (Bethyl Laboratories Inc., diluted 1:2500 in PBS-tween 20–5% nonfat dry milk) was added 0.1 mL in each well and incubated for 1 h at 37 °C. After washing, the peroxidase activity was revealed by adding 0.1 mL of ortho-phenylenediamine solution (40 mg ortho-phenylenediamine/100 mL of 0.1 M phosphate citrate buffer, pH 6.0, and 40 μL of H_2O_2), and the reaction was stopped by adding 0.05 mL of 1 N HCL, and the optical density (OD) was read at 490 nm in an ELISA microplate reader. For control of plate-to-plate variation, the same positive and negative control sera were included on every plate and a corrected OD value was calculated for each sample as described previously by Garcia et al. (2006). A serum was considered to be positive when OD testes serum > [OD mean (from negative sera obtained from all plates, $n = 15$) + 2 SD (standard deviation from negative serum from all plates)].

2.5. Lymphocyte proliferation

The proliferation assays of peripheral blood mononuclear cells (PBMC) and lymphocytes from mesenteric lymph nodes (LMLN) were performed as previously described (Solano-Aguilar et al., 2000). Two pigs from G1 were euthanized at challenge day. Whole blood was obtained by venipuncture in EDTA vacutainers and mixed 1:2 with phosphate-buffered saline (PBS), and MLN were collected from ileum and jejunum into 50-mL conical tubes and kept in collection media (CM) at 4 °C. Cells from MLN were release by sharp scissors into a petri dish containing RPMI 1640 medium (GIBCO), and then the cell suspension were filtered. PBMC were isolated by density gradient centrifugation by using lymphocyte separation media (LSM). The isolated cells were washed twice in RPMI 1640 medium. The cell suspensions from PBMC and MLN were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% bovine fetal serum, L-glutamine (2 mM; BioWhittaker), sodium pyruvate (1 mM; Sigma), and penicillin–streptomycin (1 mM; Sigma). The suspensions were then seeded in triplicate at 10^6 cells per well into flat-bottomed 96-well microtiter plates (Costar) with 200 μL of culture medium that contained 5, 10, and 15 μg of rhoptry proteins per mL. Phytohemagglutinin-M (Sigma) at 10 μg/per mL was then added to the culture medium and served as positive control for proliferation; while medium without additives were used as the negative controls. The plates were incubated in 5% CO_2 at 37 °C for 64 h. The viability of the cells was higher than 90% as determined by trypan blue exclusion. The cellular proliferation was determined by Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes). The absorbance determination at 570 nm was realized by using a microplate reader; proliferation was expressed as stimulation indices (SI = ratio of the mean proliferation of the cells after stimulation relative to the proliferation of unstimulated control cells from the same animal).

2.6. Bioassay of pig brains for T. gondii

Brain samples (50 g) from each pig were used to evaluate the presence of *T. gondii* cysts as described previously (Dubey, 1998). Briefly, each sample was homogenized in a blender for 30 s in 250 mL of saline solution (0.14 M NaCl). After homogenization 250 mL of pepsin solution (50 g) was added and incubated at 37 °C for 1 h. The homogenate was filtered through 2 layers gauze and centrifuged at $1180 \times g$ for 10 min. The supernatant was discarded and the sediment was resuspended in 20 mL PBS (pH 7.2) and 15 mL 1.2% sodium bicarbonate (pH 8.3) was added and centrifuged at $1180 \times g$ for 10 min. The supernatant was discarded and the sediment was resuspended in 5 mL of antibiotic saline solution (1000 U penicillin and 100 μL of streptomycin/mL of saline solution) and inoculated subcutaneously into 3 mice (1 mL/mouse).

2.7. Examination of mice

Impression smears of lung from the mice that died were fixed in methanol, stained with Giemsa, and examined microscopically. Blood samples were drawn from the mice that survived 45 days after post-inoculation, and the brain of each mouse was examined microscopically for *T. gondii* tissue cysts by squashing a portion of brain between a coverslip and a glass slide. Serum from each mouse was diluted at 1:16 and 1:64 and examined for *T. gondii* antibodies, using IFA.

2.8. Statistical analyses

Qui-square was used to show statistical difference in mice bioassay. Protection against tissue cysts formation in pigs was evaluated by estimating preventable fraction (PF) as previously described (Siev, 1994) with some adjustments; PF = $(p2 − p1)/p2$, where $p2$ = % of positive mice from pigs from G3 and $p1$ = % of positive mice from pigs from G1 or G2. The statistical evaluation of the lymphocyte proliferation data was performed using the Student's t-test.

3. Results

There were no clinical symptoms of toxoplasmosis in pigs after the challenge (ac), except for a fever on days 6–8 ac (>40.0 °C, Fig. 1).

IgG and IgM antibody results are shown in Fig. 2. The average antibody levels at the challenge in the immunized group (G1) were IgG OD mean = 0.222 ± 0.229 and IgM OD mean = 0.445 ± 0.215. Three animals from G1 had IgG and IgM antibody levels above the cut-off on the challenge day (ODIgG = 0.195, IgM OD mean = 0.376). Animals from G2 and G3 remained IgG and IgM negative before the challenge. All pigs from G1, G2, and G3 seroconverted after the challenge. The IgM antibody levels had a downward tendency after the challenge day, and some animals were negative.
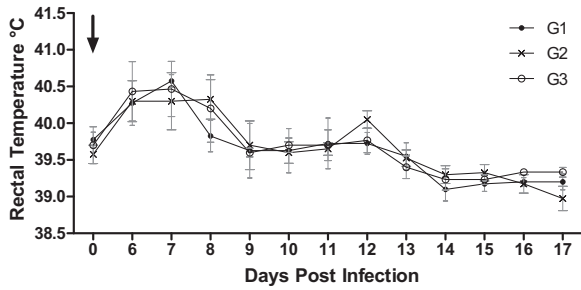
Fig. 1. Rectal temperatures of the pigs from G1, G2 and G3. Temperatures are expressed as mean ± SEM. On the day 0 (day 49 of the experiment) all animals were challenged (black arrow) with 10^3 oocysts of the *T. gondii* VEG strain.

The proliferation assays of lymphocytes from peripheral blood mononuclear cells (PBMCs), and mesenteric lymph nodes (LMLNs) elicited lymphocyte proliferation response (Fig. 3), however, the proliferation was higher in LMLNs than PBMCs.

The results of the bioassay are summarized in Table 1. Mouse bioassays of pig brains in G1, G2, and G3 animals were positive: 5/11 (45.4%), 8/11 (72.7%), and 7/9 (77.8%). The preventable fraction (PF) was 41.6% and 6.5% in G1 and G2, respectively. One pig from G1 did not have brain cysts detected through the mouse bioassay; however, all pigs from G2 and G3 did have brain cysts.

4. Discussion

In the present study, we observed that intranasal immunization with rhoptry proteins of *T. gondii* did not stimulate all pigs to produce serum IgG and IgM antibodies, however, the animals from G1 showed a partial protection against tissue cyst burden. This is a particularly significant finding, as it indicates that subunit vaccine for *T. gondii* could be differentiated from natural infection (Garcia, 2009). This could be important for future vaccination protocol for prevent *T. gondii* in negative dams, including pregnant women. Additionally, the lack of correlation between circulating antibodies and intestinal immunity was described previously (Dubey and Frenkel, 1972). Frenkel and Smith (1982)
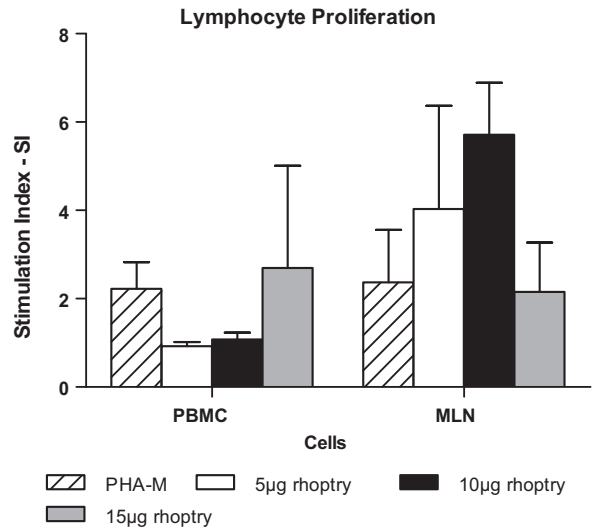

Fig. 3. Lymphocyte proliferation response from peripheral blood mononuclear cells (PBMCs) and mesenteric lymph nodes (MLNs) from G1 (two animals) at challenge day (day 49). The lymphocyte stimulation was performed using 5, 10 and 15 μg of *T. gondii* rhoptries and the positive control stimulated with 1% of Phitohemaglutinin (PHA-M).

observed that immunity in the absence of antibodies was reached, especially in cats treated with monesin (2/7) and sulfadiazine (3/4), and the central focus of immunity to oocyst shedding appears to be intestinal epithelium of kittens (Frenkel et al., 1991).

Herein, lymphocyte responses were observed in the blood and intestine of pigs (G1), but the proliferation was higher in LMLNs than PBMCs. This could explain why G1 animals showed a higher PF (41.6%) than did G2 animals (6.4%), including one G1 animal that did not show brain cysts. Pork is considered the most common infection source of *T. gondii* for human beings in the USA (Dubey, 2009); therefore, a *T. gondii* vaccine for pigs should prohibit the formation of tissue cysts (Garcia, 2009). Most of studies about the immune response to *T. gondii* were done in murine models, however, studies using pigs as *T. gondii* model are important because there are some differences
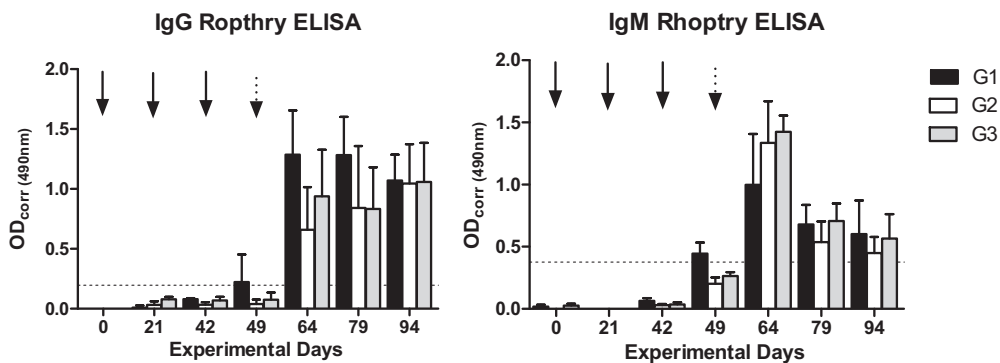

Fig. 2. IgG and IgM antibody responses measure by the indirect enzyme-linked immunosorbent assay (ELISA) from pigs of the G1, G2 and G3 (bars = OD mean from animals, and error bars = standard deviation). The G1 was vaccinated with *T. gondii* rhoptry vaccine, G2 received PBS plus Quil-A, and G3 received only saline. The treatments were performed by nasal route at days 0, 21 and 42 (black arrows). At day 49 all groups received a challenge route of the 10^3 oocysts of the *T. gondii* VEG strain (dashed arrow) and at day 94 all animals were slaughtered. Dashed line indicates positive cut-off.

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Table 1
Outcome of *Toxoplasma gondii* mouse bioassay performed from pig brains after treatments and challenged with oocysts of VEG strain.

Group/pig no.	Sex[a]	Immunization PID[b]			Challenge PID	Bioassay of brain PID	
		0	21	42	49	Results[d]	Pig negative (%)/mice negative (%)/PF (%)[e] 94
G1							
1	M		Rhoptry (200 μg)			0(1)/3	
2	F		+		10^3 oocysts	2/2[g]	25/54.5/41.6
3	F		Quil-A (50 μg)		VEG	0(0)/3	
4	M		NA[c]			2(0)/3	
G2							
5	F					3/3	
6	F		Quil-A (50 μg)		10^3 oocysts	0(1)/3	0/27.2/6.5
7	F		NA		VEG	0(2)/3	
8	M					2/2[g]	
G3							
9	F					0(2)/3	
10	F		Saline		10^3 oocysts	3/3	0/22.2/NE[f]
11	F		NA		VEG	0(2)/3	

[a] M: male; F: female.

[b] PID: post inoculation day.

[c] NA: nasal route.

[d] Results are expressed as number of mice positive for *T. gondii* from three mice inoculated with pig brains. Numbers in parenthesis indicate the number of mice with antibody titers 16 (IFA), but in which tachyzoites or cysts were not seen in their lung, peritoneal liquid, and brain, respectively; tachyzoites or tissue cysts were seen in the other mice.

[e] Preventable fraction (PF) was calculated from each group.

[f] Not evaluated.

[g] One mice died due to bacterial contamination.

in susceptibility, and immune mechanisms when compare to mice (Dawson et al., 2005).

Clinical signs after *T. gondii* infection in pigs depend on the breed and age of the animals, stage of the parasite, method of administration of parasite, and the number of infective parasites (Jungersen et al., 1999). No clinical symptoms were observed, except for a rise in temperature occurring 5–8 days after challenge. Using the same strain (VEG) and number of oocysts to infect swine, Solano Aguilar et al. (2001) observed death caused by severe fibronecrotic enteritis in 3 animals 10, 11, and 12 days after infection, however, these authors used miniature swine.

More recently, pigs were immunized intradermally with a DNA vaccine cocktail that encodes GRA1 and GRA7 dense granule proteins (Jongert et al., 2008). The authors found that this vaccine was able to elicit strong humoral and Type 1 cellular immune responses in those animals. The results evaluating tissue cyst showed that two out of three pigs vaccinated did not have the parasite detected in heart.

In conclusion, we observed that nasal immunization with crude rhoptry proteins of *T. gondii* in pigs, using Quil-A as an adjuvant, was able to stimulate a strong response in LMLNs and partially protect animals from brain cyst formation.

Acknowledgements

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