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Original Article

Protection against *Toxoplasma gondii* cysts in pigs immunized with rROP2 plus Iscomatrix

Proteção contra cistos de *Toxoplasma gondii* em suínos imunizados com rROP2 associado à Iscomatrix

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Abstract

This study aimed to evaluate the humoral immune response in pigs immunized intranasally and intramuscularly with recombinant *Toxoplasma gondii* rROP2 protein in combination with the adjuvant Iscomatrix. Twelve mixed breed pigs divided into three groups (n=4) were used, G1 received recombinant ROP2 proteins (200 µg/dose) plus Iscomatrix, G2 received PBS plus Iscomatrix, and G3 as the control group. The intranasal (IN) and intramuscular (IM) routes were used. Animals were challenged orally with VEG strain oocysts and treated on day three after challenge. Fever, anorexia, and prostration were the clinical signs observed in all animals. All the G1 animals produced antibodies above the cut-off on the day of the challenge, while the G2 and G3 remained below the cut-off. Better partial protection against parasitemia and cyst tissue formation was observed in G1 than G3. The protection factors against tissue cyst formation were 40.0% and 6.1% for G1 and G2, respectively, compared to G3. In conclusion, there were not systemic antibody responses in pigs with IN immunization with rROP2+Iscomatrix; however, after IM immunization, those animals produced higher titers than animal controls. We associated these results with partial protection obtained against parasitemia and tissue cysts formation.

Keywords: Apicomplexa, rhoptry, organelles, vaccine, swine.

Resumo

O objetivo deste estudo foi avaliar a resposta imune humoral em suínos imunizados pelas vias intranasal e intramuscular com proteínas recombinantes rROP2 do *Toxoplasma gondii* associadas ao adjuvante Iscomatrix. Doze suínos cruzados divididos em 3 grupos (n=4) foram utilizados. O G1 recebeu proteína recombinante ROP2 (200mg/dose) associada ao adjuvante Iscomatrix; o G2 recebeu PBS associado ao Iscomatrix; e o G3 foi o grupo controle. As vias intranasal (IN) e intramuscular (IM) foram utilizadas. Os animais foram desafiados por via oral com a cepa VEG e tratados no dia três após o desafio. Febre, anorexia e prostração foram os sinais clínicos observados em todos os animais. Todos os animais do G1 produziram anticorpos acima do ponto de corte no dia do desafio, enquanto os animais do G2 e G3 permaneceram abaixo do ponto de corte no desafio. Proteção parcial contra parasitemia e formação de cistos teciduais foram observadas nos suínos do G1 comparados ao G3. Os fatores de proteção contra a formação de cistos teciduais foram 40,0% e 6,1% no G1 e G2, respectivamente, comparados com o G3. Como conclusão, não houve estimulação da resposta imune humoral sistêmica nos suínos após as imunizações IN com rROP2+Iscomatrix. Estes animais, porém, após a imunização IM, produziram títulos de anticorpos mais altos que os animais controles. Esses resultados foram associados a uma proteção parcial contra a parasitemia e formação de cistos teciduais.

Palavras-chave: Toxoplasmose, roptrias, organelas, vacina, suínos.

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Introduction

Toxoplasma gondii is a protozoan parasite that can infect all warm-blooded animals, including humans (Dubey, 1994). The worst consequence of infection is abortion, which is more frequent in goats, sheep, and humans. Meat from livestock when ingested raw or undercooked is an important source of infection for humans; the risk of acquiring *T. gondii* infection from pork was considered higher than from other meats (Dubey, 2010). Therefore, to decrease human cases of toxoplasmosis, it is necessary to control the cyst formation in these animals; this is especially important as cysts are not detected in the official inspection service of the carcass during the slaughter line.

Previous studies have used live vaccines and killed vaccines to protect pigs against *T. gondii* cyst formation (Dubey et al., 1991, 1994; Pinckney et al., 1994; Freire et al., 2003; Kringel et al., 2004; Garcia et al., 2005; Cunha et al., 2012; Burrells et al., 2015). However, live vaccines (RH and S48) carry the risk of reverting to virulence and becoming infective for humans; therefore, it is essential to produce a killed vaccine against *T. gondii* (Burrells et al., 2015).

Iscomatrix is stable colloidal complexes with 40-100 nm open cage-like structures, composed of Quil-A, cholesterol, phospholipid, and an antigen, which are effective carriers of killed vaccine (Lövgren & Morein, 1988; Copland et al., 2000). Iscomatrix induces strong Th1, Th2, and cytotoxic T lymphocyte (CTL) responses (Cox & Coulter, 1997).

Toxoplasma gondii ROP2 is a 55 kDa protein located in the rhoptries and found in all stages of the parasite (El Hajj et al., 2006); the main function of the ROP2 is the formation of the parasitophorous vacuole with mitochondrial interactions (Nakaar et al., 2003). Additionally, ROP2 has been suggested as a candidate component for vaccines (Saavedra et al., 1996; Mishima et al., 2002; Igarashi et al., 2010).

This study aimed to immunize pigs with *T. gondii* rROP2 plus the Iscomatrix adjuvant nasally and intramuscularly and to evaluate its protection against cyst formation after challenge with a high number of oocysts.

Material and Methods

Toxoplasma gondii strain

RH and VEG *T. gondii* strains were used in the experiment. The RH strain was used for PCR, and antigens for the indirect fluorescent antibody test (IFAT), and the VEG strain was used for challenging the pigs. VEG strain oocysts were obtained from the feces of infected cats from a previous experimental study (Zulpo et al., 2018). Ninety days before challenging the pigs, three Swiss Webster mice were orally inoculated with 25 oocysts by gavage to evaluate the viability of them. The inoculated mice were monitored for 60, and after this period, they were euthanized for blood and brain collection. The blood samples were tested for the presence of antibodies anti-*T. gondii* by IFAT (Camargo, 1973), while the brain was used to detect tissue cysts by direct examination using smear slides and coverslips.

Construction of plasmids, culture conditions, and purification of rROP2

ROP2 recombinant protein (rROP2) was obtained, as described previously by Igarashi et al. (2008). Briefly, the DNA sequence of the gene encoding the rhoptry ROP2 antigen of *T. gondii* was obtained from the Genbank database (accession number: Z36906). The ROP2 antigen (nt. 1022-2125) has a predicted molecular mass of 54 kDa. The ROP2 open reading frame was amplified by a PCR assay using the primers ROP2 F (5'ATCGAATTCACGGATCCTGGAGAC3' – introduced EcoRI recognition site, underline) and ROP2R (5'-TGAAAGCTTTCATGCCGGTTCTCC-3'; introduced HindIII recognition site, underlined). The resulting PCR product was 1103 bp. This fragment was digested overnight with EcoRI and HindIII endonucleases and ligated into pTrcHis B (Invitrogen, Life Technologies, USA) following the manufacturer's recommendations.

Each PCR product was purified with a QIAquick PCR purification Kit (Qiagen) designed to purify single or double-stranded PCR DNA fragments. A specific PCR product was obtained, and after digestion, with the respective restriction enzymes, it was precipitated with 0.1mL of 100% ethanol and 0.01 mL of 3M sodium acetate and ligated into its respective vector using T4 DNA Ligase (Biolabs). Sequence analysis of the DNA fragment cloned in the plasmid was submitted to dideoxy sequencing methodology using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA), at the ABI3730xl Genetic Analyzer (Thermo Fisher Scientific, USA). Transformed *E. coli* DH5-α bacteria were identified on LB/ampicillin agar plates, QIAprep Miniprep Kit (Qiagen), and colony-PCR with the same

primers. Positive clones were confirmed by sequencing and transformed into *E. coli* Rosetta (DE3). The Rosetta strains (DE3) transformed with pTrcHis/ROP2 were grown with vigorous shaking at 37 °C in 50 mL LB supplemented with 100 µg/mL ampicillin and 100 µg/mL chloramphenicol to an optical density at 600 nm of 0.8. Protein production was then induced with isopropyl-D-thiogalactopyranoside (IPTG) at a final concentration of 1mM. The culture was incubated with shaking at 37 °C for 4 h. The cells were harvested by centrifugation (2,500 x g for 5 min), and the pellets were resuspended and lysed in 20 mM sodium phosphate and 500 mM sodium chloride pH 7.8 followed by three freezing-thaw cycles to obtain the soluble phase. The soluble fraction was applied directly onto a Ni-NTA Superflow resin (Qiagen) pre-equilibrated with 20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8. The recombinant soluble antigen was eluted from the resin by gravity flow with native elution buffer (200 mM monobasic sodium phosphate and 5 M NaCl pH 4.0); this included 30 min of incubation with the elution buffer along with gentle agitation at 25 °C.

Iscomatrix + rROP2 vaccine

Iscomatrix was prepared as described by Kawasaki et al. (2007). This method involves the hydration of dried phospholipid/cholesterol films using an aqueous solution with a ratio of 2:1:2 Quil A/cholesterol/PC. Phosphatidylcholine (8 mg) and cholesterol (4 mg) were dissolved in 0.5 mL chloroform and evaporated at 45 °C. The lipid film was hydrated in 3 mL of Tris buffer (pH 7.4) containing 8 mg of saponin Quil A at 25 °C for 2 h. The Iscomatrix/rROP2 vaccine was produced by the addition of the Iscomatrix adjuvant (1:1) to 200 µg of *T. gondii* rROP2 in a final volume of 1 mL.

Animal experiments

The maintenance and care of experimental animals complied with the Animal Ethics Committee of Londrina State University (CEUA 17/09). Twelve mixed breed pigs 25-30 days of age, including females and castrated males, were randomly allocated in cages (four animals each). The animals were left to acclimatize for 28 days before the experiment. They received food and water ad libitum. All pigs were serum negative (titer < 64) in the *T. gondii* IFA (Camargo, 1973). The pigs were divided into three groups, group 1 (G1) and group 2 (G2), and group 3 (G3) with 4 animals each. The G1 animals received Iscomatrix + rROP2 (200 µg) four times nasally and three times intramuscularly. The G2 animals received only Iscomatrix, and the G3 animals received saline in the same manner as the G1. The intranasal (IN) instillations were performed on days 28, 42, 56, and 72, and intramuscular (IM) injections on days 86, 93, and 100. The G1, G2, and G3 animals were challenged orally on day 110 with a high dose of VEG strain oocysts (~ 4×10^4 sporulated oocysts). The pigs were treated on day three after challenge with sulfadiazine (3 mg/kg) and trimethoprim (15 mg/kg) by the IM route (Garcia et al., 2005) to force encystation of parasites.

Sampling and measurements

Clinical signs and body temperature were recorded before and after the challenge. Serum samples were taken on days -6, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 86, 93, 97, 100, 103, 110, 113, 119, 126, 132, and at cull (145) and stored at -20 °C. Blood samples with EDTA were collected three days after challenge (d.a.c.) to detect parasitemia. At death, a pool of muscle (heart, tongue, diaphragm, and masseter) and brain samples were collected to investigate *T. gondii* cysts by mouse bioassay.

rROP2 ELISA

ELISA was performed as previously described (Garcia et al., 2005, 2006; Igarashi et al., 2008). Optimal dilutions of IgG, IgA, and IgM were established using checkerboard titrations with dilutions of sera, antigen, and conjugates. rRop2 was used as an antigen to coat the wells (5 µg/mL) of microtiter plates. 96-well flat-bottomed polystyrene microtitration plates (Nunc-Immuno Plate, MaxiSorp, Denmark) were coated with 0.1 mL of antigen (5 µg/mL) diluted in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 6 °C. The plates were washed 3 times with TBS-tween 20 (50 mM tris, pH 7.4, containing 150 mM sodium chloride and 0.05% tween 20), and non-specific immune sites were blocked with a buffer containing 8% nonfat powdered milk. The control and test sera were diluted 1:200 for IgG, IgM, and IgA, in PBS-tween 20 plus 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. After washing, anti-pig IgG, IgM, and IgA antibodies (Bethyl Laboratories Inc, Montgomery, TX, USA) were diluted 1:5000, 1:2500, and 1:5000, respectively, in

PBS-tween 20 plus 5% nonfat powdered milk, and 0.1 mL was added to each well and incubated for 1 h at 37 °C. After washing, the peroxidase activity was determined 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 H2O2), and the reaction was blocked by adding 0.05 mL of 1 N HCl. The optical density (OD) was read at 490 nm in an ELISA microplate reader. Mean absorbance values were measured, and the OD value was calculated as previously described (Garcia et al., 2006). The samples were considered positive when DO> [(DO negative control; n = 15) + 3 standard deviations (negative control; n = 15)]. Positive and negative control sera were included on every plate.

Mouse bioassay

A 50 g brain sample and a pool of muscle samples with 12.5 g of tongue, masseter, diaphragm, and heart from each pig were used to check for the presence of *T. gondii* cysts as described previously (Dubey, 1998). Briefly, each sample was homogenized in a blender for 30 seconds 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 x g for 10 min. The supernatant was discarded, and the sediment was resuspended in 20 mL PBS (pH 7.2), 15 mL 1.2% sodium bicarbonate (pH 8.3) was added to the solution, and the solution was centrifuged at 1180 x g for 10 min. The supernatant was discarded, and the sediment was resuspended in 5 mL of antibiotic saline solution (1,000 U penicillin and 100 µL of streptomycin/mL of saline solution) and inoculated subcutaneously into three mice (1 mL/mouse).

Examination of mice

Animals that showed clinical signs of infection were euthanized, and impression smears of the lungs were fixed in methanol, stained with Giemsa, and examined microscopically. Blood samples were drawn from the mice that survived 60 days post-inoculation, and the brain of each mouse was examined microscopically for *T. gondii* cysts by squashing a portion of the brain between a coverslip and a glass slide. Serum from each mouse was diluted 1:16 and 1:64 and examined for *T. gondii* antibodies using IFA (Camargo, 1973). Mice were considered positive if animals had either tachyzoites, tissue cysts detected at samples microscopely, or IFA with titer ≥ 16.

Toxoplasma gondii parasitemia

Toxoplasma gondii parasitemia followed the methodology describe by Costa et al. (1977) with some adjustments. Briefly, 5 mL of pig whole blood was collected and mixed with EDTA on day three after challenge and centrifuged at 2,000 x g to separate the layers. The buffy coat was collected, and 0.5 mL was inoculated intraperitoneally into three mice for each sample. Mice were observed daily and euthanatized after 45 days to detect cysts and performing serology as described above.

Real-time quantitative PCR on 529-bp repeat element

Brain tissues from pigs were homogenized, and 25 mg of each tissue was subjected to DNA extraction by using a commercial kit (PureLink Genomic DNA Mini Kit; Invitrogen, USA) according to the manufacturer's instructions. The DNA samples were stored at −20 °C until molecular analysis.

PCR amplification was performed in 96-wells plates using a StepOne ™ Plus (Life Technologies, EUA) as described by Reischl et al. (2003) to amplify a fragment of 162bp from 529bp repetitive fragment. Each 25 µl reaction consisted 12,5µl of TaqMan®Universal PCR MasterMix (Life Technologies, EUA), 0.7 µM of each primer (Tox-9F and Tox-11R), 0.05 µM of Tox-TP1, BSA (10 µg / ml), 2 µL of DNA template and ultrapure water. The reaction mixture was initially incubated at 95 °C for 10 min followed by 45 amplification cycles that consisted of a denaturation step at 95 °C for 1s, an annealing step at 58 °C for the 20s, and an extension step at 72 °C for 20s. Fluorescence at 530 nm (Tox-TP1) was measured at the end of each extension step. The negative control consisted of water samples without *T. gondii* and was included in each assay. The positive controls consisted of different concentrations of *T. gondii* DNA, 5fg, 50fg, 500fg, 5pg, 50pg, and 500pg, which also were used for standard curve calculation.

Statistical analysis

ANOVA and Kruskal–Wallis were used to show statistical differences in antibody serology responses, and Student's t-test was used to compare the averages. A comparison between mouse bioassay used the chi-square test. Protection against cyst formation in pigs was evaluated by estimating the preventable fraction (PF) as previously described (Siev, 1994) with some adjustments; PF = p2 - p1/ p2, where p2 = % of positive mice from the non-vaccinated pigs (G3) and p1 = % of positive mice from vaccinated pigs (G1 or G2). P-value < 0.05 was considered statistically significant.

Results

Humoral immune and clinical signal responses

The rROP2 protein used as antigen showed a band about 54 kDa in SDS-PAGE only in the soluble fraction. All pigs in G1, G2, and G3 showed clinical signals beginning three d.a.c. The animals presented with mean rectal temperatures rising from 40.0 °C to 41.0±0,569 from 3 to 5 d.a.c., anorexia, and prostration at 5 d.a.c. when they were treated as described above. The animals from all groups recovered completely by day 8 post-challenge.

IgG, IgM, and IgA anti-*T. gondii*-rROP2 antibody results are shown in Figure 1. Cut-offs were determined for each antibody, IgGCO = 0.177; IgMCO = 0.237; IgACO = 0.305. None of the antibody levels were above cut-off after nasal immunization. The IgG levels in G1 began to increase on day 93 and rose until 126 ($p = 0.00033$, t-test) before declining to the end of the experiment. The G2, and G3 groups had IgG levels just above the cut-off on day 126; however, the levels were lower than those seen in G1. The IgM and IgA responses in all groups were very similar to those of IgG. On the day of the challenge (day 110), all animals from G1 showed an average OD above the cut-off, IgG (OD = 0.893 ± 0.132; $p < 0.0001$, t-test), IgM (OD = 0.778 ± 0.544; $p < 0.05$) and IgA (OD = 0.895 ± 0.368; $p = 0.0096$, t-test). After the challenge, all animals were serum converted.

Cyst detection

The mouse bioassay on blood and meat, and qPCR results are summarized in Table 1. *Toxoplasma gondii* parasitemia was not detected in G1 animals (0%, 0/12), but was found in G2 (41.6%, 5/12) mice, and G3 animals (16.6%, 2/12). Mouse muscle bioassay found 6/12 (50%), 4/12 (33.3%), and 10/24 (58.3%) positive mice in G1, G2, and G3, respectively. PF for the bioassay was G1 x G3 = 40%, and G2 x G3 = 10.1%.

Analysis of qPCR on brain tissue detected one positive animal in G1 (pig 2 = 6 femtogram DNA equivalents (fg)), three in G2 (pig 5 = 1023 fg, 6 = 571fg, and 7 = 364), and finally two in G3 (pig 9 = 22fg, and 12 = 6fg).

Discussion

In the present study, we observed pigs immunized with rROP2 + Iscomatrix by IN did not show systemic antibody response; however, after IM inoculation, they raised all immunoglobulins, which we associated to partial protection against the high dose of *T. gondii* oocyst challenge. Although pigs from our study did not show a humoral response after intranasal immunization, previous studies have demonstrated pigs and cats produced IgG and IgM antibodies after vaccination with crude and recombinant ROP2 proteins of *T. gondii* (Cunha et al., 2012; Zulpo et al., 2012, 2017). Kringel et al. (2004) used tachyzoites from the RH strain plus CpG-oligodeoxynucleotides to infect pigs and observed 10 times more anti-*T. gondii* IgG from the immunized pigs. Freire et al. (2003) showed that an Iscom + *T. gondii* surface antigens (SAgs) vaccine for pigs was capable of stimulating a humoral immune response. Jongert et al. (2008) immunized pigs with a DNA vaccine expressing *T. gondii* GRA1 and GRA7, and they produced high levels of IgG antibodies. Additionally, crude rhoptries were used by Garcia et al. (2005) and Cunha et al. (2012) to immunize pigs, and they also observed partial protection against oocysts challenge, which was similar to the present study. Pork is one of the main transmission sources for humans, and considering that the major source of *T. gondii* infections in pregnant women was undercooked or raw meat (Cook et al., 2000), these results assume a high significance.

In the last decades, many countries have observed a decline in the prevalence of toxoplasmosis in pigs. In the US, it has decreased from 10.05% to 2.7% (Zimmerman et al., 1990; Hill et al., 2010), and in Brazil from 24% to 7.2% (Garcia et al., 1999; Silva et al., 2008). This decreasing prevalence is due to the introduction of modern management

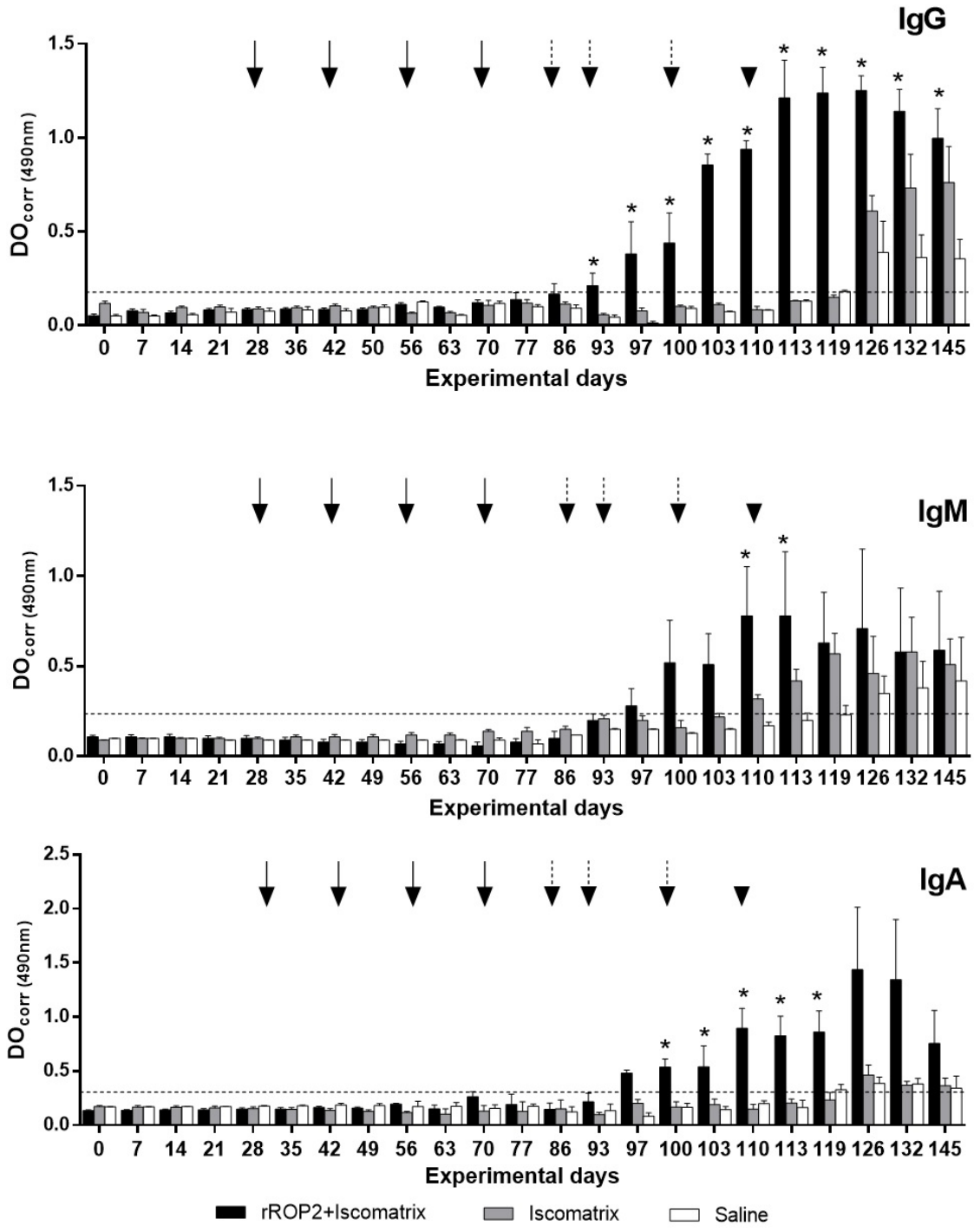

Figure 1. IgG, IgM, and IgA serological responses in pigs immunized with *Toxoplasma gondii* rROP2 by indirect ELISA. Black arrows, and dashed arrows, are days were nasal and intramuscular immunization were done, respectively. Arrowhead represents oocyst challenge. Data represent means ± standard error of the mean (SEM). *statistical difference ($p < 0.05$) (ANOVA followed by t-test). DO = optical density.

systems. However, even this lower prevalence constitutes a considerable risk for human consumers. Hill et al. (2010) described that 820,000,000 individual 113 g servings of *Toxoplasma* infected pork could be available for consumption in the US each year, considering the prevalence of 2.7%.

Additionally, consumers have a heightened interest in animal welfare, with an increasing market for organic meat. Furthermore, pork is labeled either as “free-range” or “outdoor bred and reared” in UK supermarkets. It means that since the environment is a potential source for toxoplasmosis, there is a possible increase in the risk of infection and, consequently, more cysts in pork. Therefore, based on the fact that cysts can remain in pork for

Table 1. Detection of *Toxoplasma gondii* by mouse bioassay using blood and tissue form pigs immunized with recombinant *T. gondii* ROP2 proteins after challenge with VEG oocysts.

Pig No.	Mouse Bioassay			qPCR* brain
	Blood	Pool of muscles	Total	
G1				
1	0/3	2/3	2/6	0
2	0/3	3/3	3/6	6
3	0/3	0/3	0/6	0
4	0/3	1/3	1/6	0
Total			**6/24 (25%)**	
G2				
5	2/3	3/3	5/6	1023
6	1/3	0/3	1/6	571
7	1/3	1/3	2/6	364
8	1/3	0/3	1/6	0
Total			**9/24 (37.5%)**	
G3				
9	0/3	1/3	1/6	22
10	0/3	1/3	1/6	0
11	0/3	3/3	3/6	0
12	2/3	3/3	5/6	6
Total			**10/24 (41.7%)**	

*femtogram DNA equivalents (fg).

more than two years (Dubey et al., 1998), and that pork is one of the most important sources of human *T. gondii* infections (Dubey et al., 1991), a vaccine against *T. gondii* in pigs would be very desirable, and should be focused on reducing tissue cysts.

Garcia et al. (2006) standardized an IgG-ELISA with crude rhoptries of *T. gondii* for pigs, and it was able to detect serum conversion in all animals on day 14 post-infection. Garcia et al. (2005), using an ELISA with *T. gondii* rhoptry antigens, detected seroconversion in pigs nine days after challenged with 4 x 10^4 oocyst of VEG strain. Here, we observed IgG antibody levels above the cut-off, in non-immunized pigs, nine days post-infection (day 119), and seroconversion in all animals on day 126, what could be related to the use of rROP2.

Mouse bioassay is considered the gold standard test for *T. gondii* cyst detection (Pinckney et al., 1994; Dubey et al., 1998). Here, mouse bioassay was used to detect tachyzoites from blood during the acute phase and bradyzoites from a pool of muscles after challenge to the pigs. Considering the results of all bioassays, we showed higher protection in vaccinated animals (G1) than in the adjuvant control (G2) and negative control animals (G3). As a highlight, one animal (n.3) from G1 did not have any parasite detected in either blood, muscles, or brain. Similarly, in two previous studies, we observed better protection in vaccinated animals using crude rhoptries delivered subcutaneously and nasally (Garcia et al., 2005; Cunha et al., 2012). Additionally, Dubey et al. (1991) observed fewer positive mice in bioassays on pigs immunized with a live RH strain than in the negative controls. Garcia et al. (2017) used the same qPCR and methodology and was able to detect 50 fg of DNA equivalent; however, using retina tissues. Here, less than 20 fg was detected, which could show that the brain area a better tissue to detect parasite DNA.

Clinical signs of *T. gondii* in pigs depend on breed, age, nutrition, strain characteristic, method of inoculation, and several infecting parasites (Dubey, 1994; Dubey et al., 1994). The animals from our experiment infected with a high dose of oocysts showed similar signs as the study by Garcia et al. (2005), who also used a high dose of oocysts. This high dose was used and combined with treatment to ensure the formation of a high number of tissue cysts.

Conclusion

The IN immunization of Iscomatrix/rROP2 was not able to stimulate a systemic humoral immune response; however, when IM injection was used, these animals presented higher antibody titers than the other groups. Additionally, the immunized animals did not have any detectable parasites in their blood three days after infection, and they had fewer cysts and parasites in the brain than the controls. In conclusion, partial protection was observed.

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