Original Article

rROP2 from *Toxoplasma gondii* as a potential vaccine against oocyst shedding in domestic cats

rROP2 de *Toxoplasma gondii* como potencial vacina contra a eliminação de oocistos em gatos domésticos

Dauton Luiz Zulpo[1,2]; Michelle Igarashi[3]; Ana Sue Sammi[1]; Joeleni Rosa dos Santos[1]; João Pedro Sasse[1];
Ivo Alexandre Leme da Cunha[4]; Alessandra Taroda[1]; Luiz Daniel de Barros[1]; Jonatas Campos de Almeida[1];
Mark Christopher Jenkins[5]; Italmar Teodorico Navarro[1]; João Luis Garcia[1]*

[1] Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina – UEL, Londrina, PR, Brasil

[2] Faculdade de Medicina Veterinária, Pontifícia Universidade Católica do Paraná – PUCPR, Toledo, PR, Brasil

[3] Faculdade de Medicina, Universidade Federal de Mato Grosso – UFMT, Cuiabá, MT, Brasil

[4] Faculdade de Medicina Veterinária, Centro de Ciências Agrárias e Ambientais, Universidade Federal do Maranhão – UFMA,
São Luís, MA, Brasil

[5] Animal Parasitic Disease Laboratory, United States Department of Agriculture, Annapolis, MD, USA

Abstract

The aim of the present study was to evaluate oocyst shedding in cats immunized by nasal route with *T. gondii* proteins ROP2. Twelve short hair cats (*Felis catus*) were divided in three groups G1, G2 and G3 (n=4). Animals from G1 received 100 μg of rROP2 proteins plus 20 μg of Quil-A, G2 received 100 μg of BSA plus 20 μg of Quil-A, and the G3 only saline solution (control group). All treatments were done by intranasal route at days 0, 21, 42, and 63. The challenge was performed in all groups on day 70 with $\cong$ 800 tissue cysts of ME-49 strain by oral route. Animals from G1 shed less oocysts (86.7%) than control groups. ELISA was used to detect anti-rROP2 IgG and IgA, however, there were no correlation between number of oocyst shedding by either IgG or IgA antibody levels. In the present work, in spite of lesser oocysts production in immunized group than control groups, it was not possible to associate the use of rROP2 via nostrils with protection against oocyst shedding. For the future, the use of either other recombinant proteins or DNA vaccine, in combination with rROP2 could be tested to try improving the efficacy of this kind of vaccine.

Keywords: Definitive host, immunization, recombinant protein, toxoplasmosis.

Resumo

O objetivo do presente estudo foi avaliar a eliminação de oocistos de *Toxoplasma gondii* em gatos imunizados pela via nasal com proteínas ROP2 de *T. gondii*. Doze gatos sem raça definida (*Felis catus*) foram divididos em três grupos experimentais G1, G2 e G3 (n = 4). Os animais do G1 receberam 100 μg de proteínas de rROP2 mais 20 μg de Quil-A, G2 recebeu 100 μg de albumina de soro bovino (BSA) junto com 20 μg de Quil-A, e o G3 recebeu apenas solução salina (grupo de controle). Todos os tratamentos foram realizados pela via intranasal nos dias 0, 21, 42 e 63. O desafio foi realizado em todos os grupos no dia 70 com aproximadamente 800 cistos de tecido da cepa ME-49 por via oral. Os animais de todos os grupos tiveram as suas fezes examinadas e o número de oocistos foi determinado durante 20 dias após o desafio. Os animais de G1 eliminaram menos oocistos (86,7%) do que os grupos controles. O ELISA foi utilizado para detectar IgG e IgA anti-rROP2, no entanto, não houve correlação entre o número de eliminhação de oocistos com os níveis de anticorpos IgG ou IgA. No presente trabalho, apesar da menor produção de oocistos no grupo imunizado (G1) em relação aos grupos controles (G2 e G3), não foi possível associar o uso de rROP2 pela via nasal com proteção contra eliminação de oocistos de *T. gondii*. Para o futuro, a utilização de outras proteínas recombinantes, ou mesmo vacina de DNA, em combinação com rROP2 poderia ser utilizada para tentar melhorar a eficácia deste tipo de vacina.

Palavras-chave: Hospedeiro definitivo, imunização, proteína recombinante, toxoplasmose.

Corresponding author: João Luis Garcia. Departamento de Medicina
Veterinária Preventiva, Universidade Estadual de Londrina – UEL, CP 6001,
CEP 86057-970, Londrina, PR, Brasil. e-mail: joaoluisgarcia10@gmail.com

Introduction

Toxoplasma gondii is a protozoan parasite with worldwide distribution (DUBEY & THULLIEZ, 1993) which may be found in animals from the arctic (PRESTRUD et al., 2008) rain forest (CAÑÓN-FRANCO et al., 2013) arid zones (NIETO & MELÉNDEZ, 1998), and even in marine mammals (DUBEY et al., 1998). *Toxoplasma gondii* normally causes a subclinical infection in most animal species, however, a primary infection during pregnancy can cause foetal pathologies, as well as abortions in humans and some animal species (INNES et al., 2009; CARELLOS et al., 2014).

The main sources of infection by *T. gondii* for human beings include the consumption of either vegetables or water contaminated with sporulated *T. gondii* oocysts or undercooked meat infected with tissue cysts. The risk of becoming infected depends on culinary habits, including the regular consumption of raw or undercooked meat and living in an environment with a higher risk of oocyst contamination (NAVARRO et al., 1992; DIAS et al., 2005; JONES et al., 2006; SANTOS et al., 2010; SILVA & LANGONI, 2016). Cats play an important role in this process due to their close interactions with human beings where infected cats shed millions of oocysts in their feces that subsequently contaminate the environment (DUBEY, 1995; GARCIA et al., 2007).

In addition, Boyer et al. (2011) showed that environmental contamination by *T. gondii* oocysts substantially contributes to the acquisition of *T. gondii* and the subsequent development of disease in humans. They went on to suggest a systematic screening of pregnant women and the development of a vaccine with the potential to prevent the fetal diseases caused by the acquisition of *T. gondii* during gestation in North America. Thus, production of an effective vaccine against *T. gondii* in cats would be desirable to prevent infections of intermediate hosts and human beings (GARCIA, 2009).

A live mutant strain (T-263) was used to protect against *T. gondii* oocyst shed in cats with very good results (FRENKEL et al., 1991; FREYRE et al., 1993; MATEUS-PINILLA et al., 1999), however, no other studies were performed in the past years, probably because frozen difficulties of bradyzoites used for vaccination. Additionally, with the adverse effects and the difficulties to preserve live and attenuated vaccines, the recombinant proteins may surpass these problems (JENKINS, 2001).

Nakaar et al. (2003) showed that *T. gondii* proteins ROP2 are the major determinant of proper biogenesis and maintenance of rhoptry structure, which is responsible for parasite invasion, replication and parasite-host cell interaction.

Therefore, the aim of the present study was to determine the efficacy of rROP2 from *T. gondii* administered by the nasal route to protect domestic cats against oocyst shedding.

Materials and Methods

Ethics Committee

This study was approved by the Institutional Ethics Committee in Animal Use (CEUA, protocol number 51/07).

Toxoplasma gondii strains

The RH and ME-49 *T. gondii* strains were used in the present study. The RH strain (genotype I) was initially isolated in 1937 from a case of acquired toxoplasmosis in a six year old boy (SABIN, 1941). The ME49 strain (genotype II) was isolated from muscles of sheep in the 1960s by Lunde & Jacobs (1983). Those strains were used as following; RH for obtaining DNA to amplify *rop2* gene and ME-49 was used for tissue cyst production. RH strain has been propagated in Swiss mice by intra-peritoneal route weekly in our laboratory. For tissue cyst formation, oocysts of ME-49, which were obtained from other study (ZULPO et al., 2012), were used to infect ten mice with 50 sporulated oocysts each by the oral route and euthanatized 60 days after being infected, the burden of brain cysts were counted (IGARASHI et al., 2008) and prepared for challenge.

Construction of plasmids, expression and purification of rROP2

The protocols for obtaining of rROP2 were performed as previously described by Igarashi et al. (2010).

Briefly, the DNA sequence of the gene encoding the rhoptry antigen ROP2 of *T. gondii* was obtained from Genbank database (Accession number: Z36906). Tachyzoites from *T. gondii* RH strain were used to isolate genomic DNA. This DNA was used as the template for amplification of gene *rop2* by using a standard PCR amplification protocol. The amplification product was analysed by electrophoresis on 0.8% agarose gel stained with ethidium bromide. The antigen ROP2 (nt 1022-2125) has a predicted molecular mass of 54 kDa. The ROP2 open reading frame was amplified using the primers ROP2F ($5'$ATCGAATTCACGGATCCTGGAGAC$3'$-introduced *EcoRI* recognition site, underline) and ROP2R ($5'$TGAAAGCTTTCATGCCGGTTCTCC$3'$ – introduced *HindIII* recognition site, underlined) by a PCR assay.

PCR product was obtained with 1103 pb size, this fragment was digested overnight with *EcoRI* and *HindIII* endonucleases and ligated into pTrcHis B (Invitrogen, life Technologies, USA) following manufacturer's recommendations. Sequence analysis of the DNA fragment cloned in plasmid was carried out using the DNA sequencer software.

Transformed *Escherichia coli* DH5-α bacteria were identified on LB/ampicillin agar plates by QIAprep Miniprep Kit (Qiagen) and colony-PCR with the same primers. Positive clones were confirmed by sequencing and then they were transferred into *E. coli* Rosetta (DE3). Soluble fraction was applied directly onto Ni-NTA Superflow resin (Qiagen) pre equilibrated with 20mM sodium phosphate, 500mM sodium chloride, pH 7.8 for soluble samples. The recombinant soluble antigen was eluted from resin by gravity flow with native elution buffer (200mM monobasic sodium phosphate and 5M NaCl pH 4.0), after 30 min incubation in elution buffer and gentle agitation at room temperature. *Escherichia coli* Rosetta 2 cells transformed with pTrcHis-TgROP2 showed high levels (~1 mg.mL−1) of recombinant protein after 4 hours of IPTG induction. A 54kDa protein was obtained and used for immunization of cats.

Immunization and challenge of cats

Twelve short hair domestic cats (*Felis catus*), of both sexes, between 3 and 6 months of age, were randomly allocated in individual cages; all cats received only commercially dry food and tap water *ad libitum*. Those animals were selected from kittens that were abandoned at Veterinary Hospital in University campus (Universidade Estadual de Londrina). The cats needed to be serum negative for *T. gondii* and free from *T. gondii* oocysts shedding. For this, sera and feces from all animals were sent to Parasitology laboratory from Londrina Veterinary Hospital to perform indirect immunofluorescence assay (cut-off of 16) and sucrose flotation, respectively.

The cats were divided into three groups, each group containing four animals: G1 animals received 100 μg of rROP2 plus Quil-A (20 μg); G2 received 100 μg of bovine serum albumin (BSA) plus Quil-A (20 μg) and G3 received only saline solution. The animals within treatment protocols were immunized nasally (100 μl of final solution was administrated in each animal per nostril). Intranasal vaccination was achieved by the introduction of an adapted stomach tube half-way through the nostrils of each cat. All inoculations were performed on days 0, 21, 42, and 63 of the experiment.

G1, G2 and G3 animals were challenged on day 70 with 800 tissue cysts of the ME-49 strain (contained in a volume of 2 mL) administered via stomach tube, after which they were injected with 5 mL of saline, at challenge day these animals were anesthetized with tiletamine plus zolazepam (Zoletil®, Virbac-Brazil, 3.15 mg/kg/IM).

Measurements of oocyst shedding by sucrose flotation

Feces from each cat were collected daily from 1st until 20th day after challenge and examined microscopically for oocysts as described by Garcia et al. (2007) and Zulpo et al. (2012). Briefly, feces obtained over a period of 24 h were diluted in a small volume of distilled water; 1 g of this material were admixed with 10 mL of sucrose solution (specific gravity, 1.18), filtered, and centrifuged (1200 x g for 10 min). One drop of solution, removed from the meniscus, was examined microscope. When oocysts were detected the supernatant was collected (approximated 9 mL) admixed with 40 mL of water in a 50 mL tube, and centrifuged (1200 x g) for 10 min. The supernatant was discarded and the sediment elevated to 1 mL with water. The number of oocysts was then determined in four white blood cells chambers of a hemocytometer (Neubauer Chamber).

Genotyping of tissue cysts and oocysts

Sample of tissue cysts of challenge and pool of feces from each group was collected to perform the genetic characterization of *T. gondii*. Tissue cysts and feces underwent to DNA extraction using a commercial kit following the manufacturer's instructions (NucleoSpin® Tissue, Macherey-Nagel, Germany). Genotyping was performed using multilocus PCR-RFLP with 11 genetic markers (SAG1, 5'-3'SAG2, alt.SAG2, SAG3, BTUB, GRA6,

c22-8, c29-2, L358, PK1 and Apico) as previously described (SU et al., 2010). DNA from strains GT1, PTG, CTG, TgCgCa1, MAS, TgCatBr5, TgCatBr64 and TgRsCr1 were used as positive controls. All digested PCR product were analyzed by agarose gel electrophoresis, stained with Sybr Safe (Invitrogen®, USA), visualized under UV light and photographed using a gel documentation system (Safe imager, Invitrogen®, USA).

Enzyme-linked immunosorbent assay (ELISA)-IgG and IgA

Blood was collected from jugular vein on days 0, 21, 42, 63, 70, 85, 100, 115 and 130 of the experiment to evaluate antibody levels. Sera were obtained and kept at -20°C. Optimal dilutions were established by using checkerboard titrations with dilutions of sera and conjugates. Proteins from rROP2 of *T. gondii* were used as antigens to coat the flat-bottom 96-well polystyrene microtitration plates (Nunc-Immuno Plate, MaxiSorp, Denmark) with 0.1 mL of the antigens (5 μg/mL) diluted in 0.1 M carbonate buffer (pH 9.6) by incubation overnight at 6 ℃. The plates were rinsed thrice 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 were blocked by incubation for 1 h at 37 ° C with carbonate buffer and 8% nonfat dry milk. Control and sample sera were diluted (1:100 for IgG and 1:20 for IgA) in PBS-tween 20 and 5% nonfat dry milk and 0.1 mL of this mixture was added to the wells of microtitre plates in duplicate. Further, the plates were incubated for 1 h at 37 °C for IgG and overnight at 6 ℃ for IgA detection. After rinsing, the conjugate for IgG detection (HRP anti-cat IgG antibodies, Bethyl Lab, Montgomery, TX, USA) were diluted 1:10,000 in PBS-tween 20 and 5% nonfat dry milk, after which 0.1 mL of the mixture was added to each well and incubated for 1 h at 37 °C. For IgA detection, an additional step was added, a goat anti-cat IgA antibody were diluted 1:2,000 and left for 2 h at 37 °C, three rinses were performed and HRP rabbit anti-goat IgG antibodies were diluted 1:1,000 and incubated for 1 h at 37 °C. After rinsing, the peroxidase activity was revealed by adding 0.1 mL of orthophenylenediamine 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 of HCl. The optical density (OD) was read at 490 nm in an ELISA microplate reader. Positive and negative control sera were included in every plate and a corrected OD value was calculated according to the formula described before (GARCIA et al., 2007). A serum was considered to be positive when ODcorr > [OD mean (from negative control sera, n = 11) + 2SD (standard deviation from negative control sera)].

Statistical comparisons

Wilcoxon-Mann-Whitney U test was used to determine statistical differences in oocysts shedding and antibodies response. A $p ≤ 0.05$ was considered as statistically significant. Protection against oocysts elimination in cats was evaluated by estimating the preventable fraction (PF) as previously described (GARCIA et al., 2007) with modifications: PF= (P2-P1)/P2; where P2 = mean of

oocysts shedding among group unvaccinated (G3) and P1 = mean of oocysts shedding among group vaccinated (G1 or G2).

Results

According with antibodies production (Figure 1), only cats from G1 demonstrated IgG and IgA levels above cut-off at the time of challenge (day 70). The antibody OD averages showed by G1, before challenge, was $IgG_{63}=0.197\pm0.141$ and $IgG_{70}=0.294\pm0.283$, and $IgA_{63}=0.230\pm0.289$ and $IgA_{70}=0.349\pm0.340$ at days 63 and 70, respectively. Additionally, after challenge, cats from G1 produced comparatively more IgA ($IgA_{85}=0.561\pm0.358$) at day 85 than cats from G2 ($IgA_{85}=0.325\pm0.013$) and G3 ($IgA_{85}=0.040\pm0.056$). Cats from all groups seroconverted after challenge (Figure 1).

The efficacy of protection against oocyst shedding was evaluated by counting of oocyst excreted during 20 days of the experiment. Thus, the total average oocyst shedding/g of feces showed that animals from G1 (OOPG: 2.49×10^5) shed fewer oocysts than the animals from G2 (OOPG: 3.79×10^5) and G3 (OOPG: 1.88×10^6); however, there was no statistically significant differences (p>0.05) (Table 1 and Figure 2). Additionally, the averages for pre patent period (PPP), patent period (PP), and peak oocyst shedding are shown in Table 1. The preventable fractions (PF) of groups were
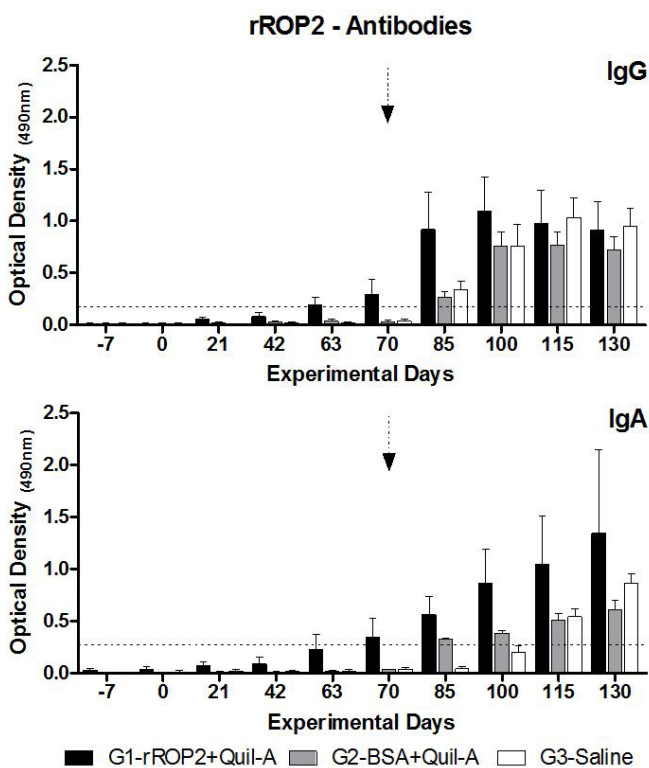
$PF_{G1 \times G3}= 86.7\%$ and $PF_{G1 \times G2}= 23.9\%$, and $PFG2 \times G3 =82.5$ (Table 1). All samples genotyped were characterized as type II.

Discussion

In the present study, we observed that cats that were immunized with rROP2 by the nasal route shed less oocysts 23,9 and 86.7% than control groups G2 and G3, respectively, however, there was no statistical differences. This result could be considered remarkable considering ROP2 is only one protein among over 20,000 from *T. gondii* (WASMUTH et al., 2009); and immunization with additional recombinant proteins could improve the efficacy of this type of vaccine. A vaccine that decreases oocyst shedding in cats is very desirable. This vaccine would decrease environmental contamination by oocysts and consequently reduce the risk of infection in animals and humans. Contact with the ground and oocyst infection were described as major risks for acquiring *T. gondii* during pregnancy (DECAVALAS et al., 1990; LOPES-MORI et al., 2013). It is estimated that cats infected for the first time with *T. gondii* are able to produce millions of oocysts with an annual environmental contamination of approximately 94 to 4,671 oocysts/m² (DABRITZ et al., 2007). These data demonstrated the need to control oocyst shedding in cats, however, few studies have been conducted with this aim (GARCIA, 2009; VERMA & KHANNA, 2013; CORNELISSEN et al., 2014; GARCIA et al., 2014).

Previously studies (GARCIA et al., 2007; ZULPO et al., 2012) with immunization in cats by the nasal route against *T. gondii* oocyst shedding have reported 66.6% and 98.6% protection, respectively. However, these authors immunized the animals with crude rhoptry antigens and challenged cats with VEG (600 cysts) and ME-49 (800 cysts) strains, respectively. The dynamics of oocyst shedding that were observed in the present study were similar to those that were described previously. Furthermore, ME-49 is the most frequently used *T. gondii* strain for challenging cats (DUBEY & THULLIEZ, 1989; LAPPIN et al., 1994; BURNEY et al., 1995; DUBEY, 1995; DUBEY et al., 1995; ZULPO et al., 2012), and it is able to produce a larger amount of oocysts compared with the VEG strain (DUBEY, 1995).

In contrast to our study, (MISHIMA et al., 2002) used a DNA vaccine expressing ROP2, and they did not reduce oocyst shedding in cats. This could be related to the fact that DNA vaccines are usually administered via a systemic route, whereas we used an intranasal route. This fact was reported by Frenkel & Smith (1982), who observed that only 25% of cats that developed systemic antibodies did not shed oocysts when challenged with *T. gondii*. Frenkel et al. (1991) found that the primary focus of immunity against oocyst shedding is the intestinal epithelium of kittens. The local protection of secretory IgA in the intestine mucosa against *T. gondii* was described previously (MCLEOD et al., 1988; BONENFANT et al., 2001; DIMIER-POISSON et al., 2006).

Animals from G2, which received BSA plus Quil-A, showed less oocyst shedding than control group (G3). This could be associated with immune modulation of Quil-A. This adjuvant was described as being capable of inducing strong Th1 and Th2 responses and moderate CTL responses (COX & COULTER,

Figure 1. rROP2-serum IgG and IgA antibody responses of cats evaluated by the indirect enzyme-linked immunosorbent assay (MEAN ± SD). G1, vaccinated with rROP2 plus Quil-A, G2 received bovine serum albumin (BSA) plus Quil-A and G3 received only saline, by nasal route. Treatments were made at days 0, 21, 42, and 63. All groups received a challenge of 800 cysts from the ME-49 strain on day 70 (dashed arrow). Dashed line indicates positive cut-off.

Toxoplasma gondii - Oocyst Shedding

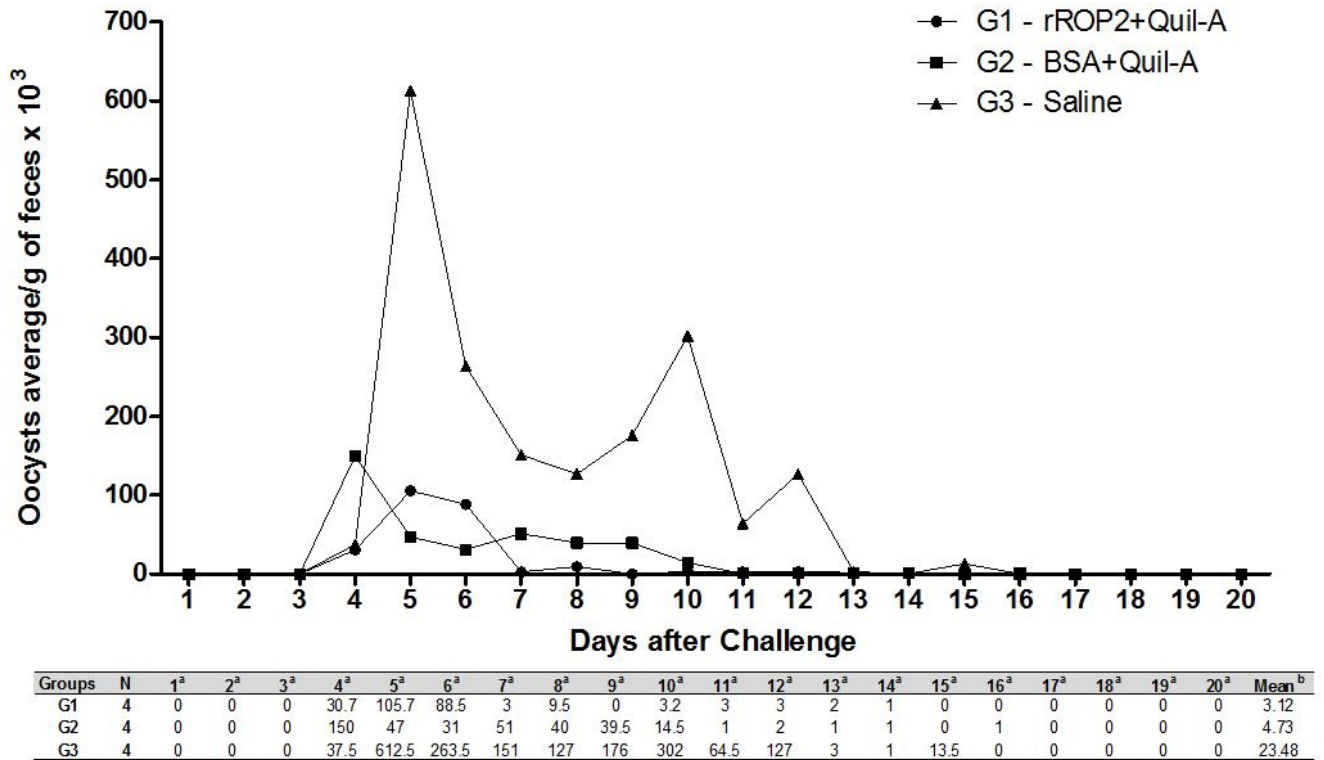| Groups	N	1[a]	2[a]	3[a]	4[a]	5[a]	6[a]	7[a]	8[a]	9[a]	10[a]	11[a]	12[a]	13[a]	14[a]	15[a]	16[a]	17[a]	18[a]	19[a]	20[a]	Mean[b]
G1	4	0	0	0	30.7	105.7	88.5	3	9.5	0	3.2	3	3	2	1	0	0	0	0	0	0	3.12
G2	4	0	0	0	150	47	31	51	40	39.5	14.5	1	2	1	1	0	1	0	0	0	0	4.73
G3	4	0	0	0	37.5	612.5	263.5	151	127	176	302	64.5	127	3	1	13.5	0	0	0	0	0	23.48

Figure 2. *Toxoplasma gondii* oocyst shedding in cats immunized with rROP2 (G1), G2 were adjuvant control and G3 were saline control. Challenged was performed at day 70 with 800 tissue cysts of ME-49 strain of *T. gondii* (**a:** Total of oocysts shedding / g of feces x 10³; **b:** Mean of oocyst shed by four animals).

Table 1. The dynamics of *Toxoplasma gondii* oocyst shedding after challenge with ME-49 strain. Cats from G1 were vaccinated with rROP2 plus Quil-A, G2 received bovine serum albumin (BSA) plus Quil-A and G3 received only saline, by nasal route. All groups received a challenge of 800 cysts from the ME-49 strain on day 70.

Groups	No. of cats	Dose of tissue cysts/ animal	Mean pre patent period[1]	Mean patent period[1]	Mean peak of oocysts shedding[1]	Total oocysts shedding[2] x10³	Preventable fraction (%)	
G1	4	800	4.2	9.25±1.7	5.33	249.6	G1xG3=86.7	G1xG2=23.9
G2	4	800	4.7	9.00±2.6	6.25	328	G2xG3=82.5	
G3	4	800	5	9.75±1.7	5.66	1,878.5		

[1]Period in days. [2]oocysts per gram of feces (OOPG).

1997; PAEPENMÜLLER & MÜLLER-GOYMANN, 2014). Additionally, the Quil-A adjuvant is widely used in animals, has a low cost and a simple design, and is generally safe (COX & COULTER, 1997; GARCIA et al., 2007; IGARASHI et al., 2010; CUNHA et al., 2012).

Herein, ROP2 protein was chosen because it is the major protein involved in the biogenesis and maintenance of rhoptry structure, parasite invasion, replication and host cell interaction (NAKAAR et al., 2003); it has been observed in all subgroups (I, II, and III) and stages of the life of the parasite (FISCHER et al., 1996). Additionally, ROP2 was recognized by a human T-cell clone isolated from an immune donor specifically for the parasite and produced high levels of IFN-γ (SAAVEDRA et al., 1991).

For all of these reasons, ROP2 has been used for vaccine studies (UGGLA et al., 1988; VERCAMMEN et al., 2000; LEYVA et al., 2001; DZIADEK et al., 2009; IGARASHI et al., 2010; DZIADEK & BRZOSTEK, 2012).

According to genotyping, all oocyst samples were characterized as type II. This was expected because the challenge was performed with ME49, which is a type II clonal strain. The type II strain is the most common strain associated with human toxoplasmosis in patients with AIDS, as well as in congenital infections in animals and humans (HOWE & SIBLEY, 1995). A vaccine that decreases oocyst shedding of the type II strain could prevent environmental contamination and may reduce the incidence of new cases of toxoplasmosis in livestock and humans.

In summary, despite of lesser oocysts production in immunized group than control groups, it was not possible to associate the use of rROP2 via nasal route with protection against *T. gondii* oocyst shedding. We are now working to produce more recombinant proteins (ROP18, GRA5, GRA7) from *T. gondii* to use in combination with rROP2 what could improve the efficacy of this type of vaccine. Further studies should be conducted to evaluate the effect of Quil-A on the immune system of cats against *T. gondii*.

Acknowledgements

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