UNIVERSIDADE ESTADUAL DE LONDRINA

DAUTON LUIZ ZULPO

Toxoplasma gondii E GATOS DOMÉSTICOS:

USO DE UMA VACINA PARA DIMINUIR A ELIMINAÇÃO DE OOCISTOS E AVALIAÇÃO DA REELIMINAÇÃO

Londrina
2014

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Tese apresentada ao Programa de Pós-graduação em Ciência Animal (área de concentração - Sanidade Animal) do Centro de Ciências Agrárias da Universidade Estadual de Londrina, como requisito para obtenção do título de Doutor.

Orientador: Prof. Dr. João Luis Garcia

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Londrina, abril de 2014.

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RESUMO

O presente estudo teve como objetivos, avaliar o uso de uma vacina de proteína recombinante (rROP2) em gatos domésticos para prevenir a eliminação de oocistos do Toxoplasma gondii, bem como, acompanhar a eliminação de oocistos em gatos infectados e re-infectados com diferentes cepas do parasita. Doze gatos foram utilizados no experimento para formar três grupos, G1, G2 e G3. O G1 foi imunizado com 100µg de rROP2 mais 20µg de Quil-A. O G2 recebeu 100µg de BSA mais 20µg de Quil-A. O G3 recebeu solução fisiológica, permanecendo como controle. Todos os tratamento de imunização foram administrados via nasal e realizados no dia 0, 21, 42, 63 do experimento. O desafio foi efetuado no dia 70, com 800 cistos da cepa ME-49. As fezes foram examinadas e o numero de oocistos por grama de fezes foi determinado. O G1 eliminou 86,7% e 34,1% menos oocistos que os grupos controles, solução fisiológica e BSA + Quil-A, respectivamente. Os anticorpos IgG e IgA foram avaliados pelo ELISA. Dois gatos apresentaram IgG e IgA antes do desafio, mas não houve relação com uma menor eliminação de oocistos. Todos os gatos soroconverteram após o desafio. Para avaliar a re-eliminação de oocistos em gatos, dez gatos soronegativos ao *T. gondii* foram utilizados para o estudo. Os gatos foram infectados com cistos da cepa ME-49. Após esta infecção os gatos foram divididos em três grupos, G1 (n: 2), G2 (n: 5), e G3 (n: 3). Os animais do G1 e G2 foram re-infectados após 12 meses com cistos da cepa VEG e PB-1, respectivamente. O G2 e G3 foram re-infectados novamente com 36 meses recebendo cistos da cepa VEG. As infecções foram realizadas com aproximadamente 800 cistos teciduais. Os gatos foram avaliados quanto a eliminação de oocistos e produção de anticorpos. Todos os gatos elimiram oocistos quando infectados com a cepa ME-49. Apenas um gato (1/2) do G1 quando re-infectados após 12 meses com a cepa VEG eliminou oocisto. O G2 não eliminou oocistos quando re-infectados após 12 meses com a cepa PB-1 e quanfo re-infectados novamente após 36 meses com a cepa VEG, três gatos (3/4) eliminaram oocistos. No G3, dois gatos (2/3) eliminaram oocistos quando re-infectado após 36 meses com a cepa VEG. Portanto, seis de nove gatos re-eliminaram oocistos quando re-infectados com cepa heteróloga. Todos os gatos foram IgM, IgG e IgA positivos após a primeira infecção com a cepa ME-49 e permaneceram até a ultima análise experimento.

Palavras-chave: Imunologia veterinária. Toxoplasma gondii. Vacina veterinária. Toxoplasmose em animais. Resposta imune. Gato.

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1.1 - INTRODUÇÃO

Toxoplasma gondii foi descrito por Splendore, em 1908, em um coelho de laboratório, em São Paulo, e inicialmente foi classificado como *T. cuniculli.* No mesmo ano, Nicolle e Manceaux (1908), no Instituto Pasteur de Túnis na Tunísia, também descreveram *o* parasita em um roedor (*Ctenodactylus gundi*), e posteriormente o classificaram como *T. gondii* (NICOLLE; MANCEAUX, 1909).

Este protozoário pertence ao filo Apicomplexa, classe Sporozoazida, sub-classe coccidiasina, ordem Eimeriorina, família Toxoplasmatidae, gênero e espécie *Toxoplasma gondii* (DUBEY, 2010). O protozoário é uma célula eucarionte e, estruturalmente, é formado por uma membrana externa, anéis polares, conóide, microtúbulos, grânulos densos, micronemas, roptrias, mitocondria, complexo de Golgi, ribossomos, retículo endoplasmático, microporo e um núcleo com parede bem definida (DUBEY, 1993; METSIS; PETERSEN, 1995).

Toxoplasma gondii normalmente causa uma infecção subclínica, porém, a infecção primária, durante a gestação, pode causar patologias fetais, bem como, abortos tanto nos animais como em humanos (DUBEY; BEATTIE, 1988; VERCAMMEN et al., 2000). É considerado um dos parasitas de melhor adaptação a seus hospedeiros, que incluem uma grande variedade de espécies, tais como, vertebrados aéreos, mamíferos marinhos, herbívoros e carnívoros terrestres (CARRUTHERS, 2002). A toxoplasmose tem sido encontrada em várias áreas geográficas do mundo, e o parasita foi descrito infectando cerca de um terço da população humana (DUBEY; BEATTIE, 1988; TENTER et al., 2000). Quando os fetos são infectados congenitamente eles podem apresentar hidrocefalia, coriorretinite e retardo mental (JONES et al., 2001). A infecção adquirida é mais grave em pacientes imunocomprometidos, que podem apresentar encefalite toxoplásmica, sendo que esta é considerada desde a década de 80, como a mais importante alteração neurológica em pacientes aidéticos (LUFT; CHUA, 2000).

Apesar de ter sido identificado no começo do século XX, apenas no início da década de 70 é que foram descritos os hospedeiros definitivos e intermediários do *T. gondii* (FRENKEL et al., 1970; MILLER et al., 1972). Há três estágios infectantes do *T. gondii*, taquizoitos, bradizoitos e esporozoitos (DUBEY, 2004). O parasita é intracelular obrigatório e possui a capacidade de invadir todas as células nucleadas, tecidos e hospedeiros, porém os gatos domésticos e felídeos selvagens são os

únicos hospedeiros definitivos (FRENKEL et al., 1970), ou seja, os únicos que eliminam oocistos pelas fezes, após o fase de multiplicação sexuada no intestino delgado. Os oocistos são eliminados não esporulados, no meio ambiente e dentro de cinco dias dependendo das condições de temperatura e umidade ocorre a esporulação, tornando-se então, infectantes ao homem e aos animais (DUBEY et al., 1998).

Os hospedeiros intermediários, incluindo os gatos, podem adquirir o *T. gondii* pela ingestão de tecidos de animais infectados com cistos, alimentos ou água contaminados com oocistos esporulados, ou por transmissão transplacentária. Após a ingestão de cistos teciduais ou oocistos, os bradizoitos ou esporozoitos, respectivamente, são liberados e invadem o tecido intestinal, transformam-se em taquizoitos, os quais multiplicam-se localmente e disseminam-se pela via hematógena ou linfática. Após o desenvolvimento da imunidade pelo hospedeiro a última geração de taquizoitos dará origem aos bradizoitos, que podem permanecer em forma de cistos teciduais muitas vezes pela vida toda do hospedeiro (DUBEY et al., 2004).

Assim sendo, a infecção no humano é favorecida pela alta prevalência do protozoário nos animais domésticos, principalmente ovinos e suínos (NAVARRO et al., 1992). E o risco de adquirir a infecção pelo consumo de carnes cruas ou mal cozidas, fato comum em várias regiões do Brasil, foi relatado por Vidotto et al. (1990).

1.2 - ELIMINAÇÃO DE OOCISTOS

Experimentalmente, a maioria dos gatos soroconvertem durante a segunda e terceira semana após infecção pela via oral de cistos teciduais e geralmente após terem eliminado oocistos (DUBEY; FRENKEL, 1972; DUBEY; THULLIEZ, 1989; DUBEY et al., 1995). Portanto, quando os gatos são soropositivos, normalmente já eliminaram milhões de oocistos pelas fezes, contaminando o meio ambiente (DUBEY; BEATTIE, 1988). Desta forma, os dados de soroprevalência são importantes, pois podem indicar indiretamente a contaminação ambiental, uma vez que a detecção de oocistos nas fezes de gatos é relativamente raro. A prevalência de anticorpos contra *T. gondii* em gatos domésticos estudada no mundo, revelam

100% de positividade, ou seja, em todos os estudos foram identificados gatos soropositivos (DUBEY, 2010).

Por outro lado, a detecção de oocistos nas fezes pode ser observada em aproximadamente 1% dos animais estudados, isto se baseia no fato de que a maioria dos gatos eliminam oocistos por um período curto, ou seja, de uma a duas semanas (DUBEY, 1995). Existem descrições de filhotes em amamentação (DUBEY; CARPENTER, 1993), bem como, animais idosos (com 13 anos de idade) eliminando oocistos de *T. gondii* (SCHARES et al., 2008).

Um dos problemas para o diagnóstico de oocistos nas fezes dos felinos é o limiar de detecção dos exames de microscopia, que giram em torno de aproximadamente 1.000 oocistos por grama de fezes, abaixo dessa quantidade a sensibilidade do teste fica comprometida (DUBEY, 1995). Assim, o uso do bioensaio em camundongos, bem como, das técnicas moleculares, ajudam na detecção de baixa concentração de oocistos nas fezes (DUBEY et al., 1995; FRENKEL et al., 1995). Poucos estudos foram realizados a fim de estimar a quantidade de oocistos eliminados nas fezes de gatos naturalmente infectados (DUBEY, 2010).

Dubey (1995) mostrou que quatro de nove gatos re-infectados pela via oral com cistos teciduais após 77 meses da primeira infecção com *T. gondii* re-eliminaram oocistos, o número de oocistos eliminados durante a segunda infecção foi menor, porém, dois dos quatro animais eliminaram alta quantidade. No entanto, o potencial de gatos naturalmente infectados em re-eliminar oocistos ainda é desconhecido (DUBEY; FRENKEL, 1974).

1.3 - RESPOSTA IMUNE

A infecção pelo *T. gondii* raramente apresenta sinais clínicos no hospedeiro, no entanto, a severidade da doença, quando ocorre, esta relacionada à espécie, idade do hospedeiro, hormônios sexuais, prenhez, estado imunológico, condição nutricional, estágio e cepa do parasita e infecções concomitantes (LUFT; REMINGTON, 1992; DUBEY, 1994; DUBEY et al., 1994; LIESENFELD et al., 2001; DUBEY; JONES 2008). Os mecanismos envolvidos na proteção do hospedeiro contra a infecção toxoplásmica são a resposta imune humoral e celular (GARCIA, 2009).

O número de hospedeiros acometidos pelo *T. gondii* é muito grande, contudo têm-se demonstrado que algumas espécies (ratos e galinhas) exibem um alto grau de resistência natural. A idade é outro fator importante para a resistência natural, animais jovens, nas diferentes espécies, apresentam-se mais susceptíveis à infecção (DUBEY; BEATTIE, 1988).

A resposta do hospedeiro ao *T. gondii* está relacionada à proteção inata e adquirida. As diferenças de virulência entre as cepas do parasita são importantes na resistência do hospedeiro sendo que a base molecular dessas diferenças permanece desconhecida. Três linhagens clonais do *T. gondii* foram descritas e correlacionadas com a virulência do parasita (HOWE; SIBLEY, 1995). A linhagem tipo I está associada à virulência na fase aguda em camundongos, a linhagem tipo II na indução da fase crônica e a linhagem tipo III é a menos virulenta (ALEXANDER; HUNTER, 1998).

Após a fase aguda, o hospedeiro desenvolve uma boa imunidade que normalmente é duradoura e protetora contra re-infecções (DUBEY, 1994). Altos títulos de anticorpos específicos na presença de complemento, bem como citotoxicidade celular dependente de anticorpos, podem lisar os parasitas extracelulares e bloquear a invasão na célula do hospedeiro, uma vez que a produção máxima de anticorpos coincide com o desaparecimento de taquizoitos viáveis (FRENKEL, 1990; WASTUNG et al., 1995).

Acreditava-se que a imunidade para o *T. gondii* fosse dependente da infecção crônica, pela persistência dos cistos teciduais, que, portanto, estimulariam constantemente o sistema imunológico. Contudo estudos com a cepa TS-4 (não cistogênica) demonstraram imunidade estéril, sem a presença da infecção crônica por cistos teciduais (FRENKEL, 1990), o que é estimulante para estudos com vacinas inativadas. Vários autores têm demonstrado a importância da resposta celular na imunidade ao agente (FRENKEL, 1990; SHER; COFFMAN, 1992; BUXTON; 1993; IGARASHI et al., 2010; CUNHA et al., 2012; ZULPO et al., 2012), esta sendo importante pela localização intracelular do protozoário e isoladamente, a resposta humoral não é suficiente para promover proteção no hospedeiro, provavelmente devido à localização do parasita no hospedeiro (GARCIA, 2009).

Os antígenos secretados-excretados (roptrias, grânulos densos, e micronemas) do *T. gondii* têm uma função importante no estímulo da resposta imune, tanto na infecção aguda quanto crônica (SAAVEDRA et al., 1996). Antígenos

que estimulam células CD4 específicas estão envolvidos na imunidade duradoura em indivíduos saudáveis cronicamente infectados (SAAVEDRA et al., 1991; SAAVEDRA et al., 1996).

A principal porta de entrada do *T. gondii* é a via oral, desta forma, a imunidade local via linfócitos e IgA são de fundamental importância na proteção contra o parasita (BOURGUIN et al. 1993; VELGE-ROUSSEL et al., 2000). Chardés et al. (1990) descreveram que a infecção toxoplásmica induz secreção de IgA no intestino, que pode iniciar a função protetora contra o parasita. A imunidade de mucosa tem revelado proteção em outros coccídeos intestinais pela inibição da penetração intestinal, portanto um estímulo eficiente da resposta imune de mucosa será de grande valor para o controle da infecção (CHARDÉS; BOUT, 1993).

1.4 - VACINAS

Os gatos são considerados a chave no controle do *T. gondii*, devido ao fato desses animais serem os hospedeiros definitivos e eliminarem milhares de oocistos pelas fezes, sendo responsáveis pela contaminação do meio ambiente, assim, uma vacina nesta espécie deveria evitar ou diminuir a eliminação de oocistos (GARCIA, 2009). Nas últimas décadas houve um grande aumento no número de técnicas e pesquisas para a produção de novos imunógenos, contudo, existem poucos estudos com vacinas para o controle da eliminação de oocistos (DUBEY, 1996; GARCIA, 2009).

A única vacina de uso comercial existente é a Toxovax®, licenciada para uso em ovinos e caprinos, disponível apenas na Grã Bretanha, Nova Zelândia, França, e Irlanda. Essa vacina utiliza taquizoitos vivos da cepa S-48, e a dose para as fêmeas deve ser $\geq 10^5$ taquizoitos contidos em 2 mL, pela via intramuscular, pelo menos três semanas antes do acasalamento. A imunidade induzida pela vacina é de aproximadamente 18 meses caso não ocorra desafio natural. O aumento de temperatura corporal pode ser observado após a vacinação e a carne dos animais não deve ser consumida por, pelo menos, 42 dias após a imunização. O potencial da Toxovax® na prevenção da formação de cistos teciduais em ovelhas e cabras é desconhecido (BUXTON et al., 1993).

A via nasal foi descrita como uma rota capaz de estimular a imunidade celular local e sistêmica, além do mais, esta via requer menos antígenos para promover a imunidade do que a via oral, pois a atividade proteolítica na via nasal é muito menor do que na via oral (VELGE-ROUSSEL et al., 2000). Considerando que a porta de entrada natural do *T. gondii* é a superfície da mucosa do intestino e que a infecção adquirida naturalmente gera uma imunidade protetora persistente, isso aponta à importância do desenvolvimento de uma vacina que estimule a proteção de mucosa (BONENFANT et al., 2001).

Para o desenvolvimento da imunidade por meio de uma vacina deve-se associar o local correto da imunização, bem como o uso de proteínas do parasita que promovam proteção adequada, considerando que o parasita apresenta três estágios diferentes (esporozoito, taquizoito e bradizoito) com diferenças antigênicas entre eles (SPEER et al., 1995; GARCIA et al., 2004).

Frenkel e Smith (1982) estudaram a eliminação de oocistos e a imunidade em 75 felinos inoculados com os três estágios do *T. gondii.* Estes autores realizaram uma primeira infecção e depois re-infectaram estes gatos, como resultado, obtiveram uma proteção contra a eliminação de oocistos de 93% nos gatos que tinham eliminado oocistos na primeira infecção, 25% de proteção nos gatos que desenvolvem somente anticorpos durante a primeira infecção, e nenhuma proteção nos gatos que não eliminaram oocistos e não apresentaram anticorpos na primeira infecção. No mesmo estudo, estes autores utilizaram taquizoitos inativados, e apenas um (1/24) gato não eliminou oocisto.

Frenkel et al. (1991) verificaram uma proteção de 100% utilizando uma vacina viva contendo bradizoitos de uma cepa mutante do *T. gondii*, a T-263 (não forma oocistos em gatos). Durante as imunizações não houve a eliminação de oocistos, e quando estes gatos foram desafiados com um isolado normal do *T. gondi*, os autores observaram que nenhum gato eliminou oocisto. O mesmo resultado foi obtido por Freyre et al. (1993) que utilizaram cistos teciduais intactos e bradizoitos livres da cepa T-263 como imunizante, observando uma imunidade de 100% frente a um desafio heterólogo. Outro estudo para determinar a eficácia desta cepa T-263, em felinos, foi realizado a campo e reduziu a exposição de suínos ao *T. gondii* (MATEUS-PINILLA et al., 1999). A desvantagem dessa vacina é a utilização da cepa viva do *T. gondii* (DUBEY, 1995).

Omata et al. (1996) testaram uma vacina em gatos com taquizoitos da cepa Beverley irradiados com ^{60}Co, esta imunização impediu parcialmente a eliminação de oocistos, onde 43% não eliminaram oocistos quando desafiados com cistos cerebrais da mesma cepa. No mesmo estudo outro grupo foi vacinado com taquizoitos irradiados, porém da cepa RH e quando desafiados com a cepa Beverley, todos eliminaram oocistos.

Alternativamente, uma vacina de DNA que expressa a proteína ROP2 foi testada em felinos. A imunização induziu a produção de IgG sérica e reduziu a quantidade de cistos no cérebro dos gatos, porém, não apresentou eficácia na diminuição da eliminação de oocistos nas fezes (MISHIMA et al., 2002).

Garcia et al. (2007) testaram uma vacina de roptrias incorporadas ao Quil-A pela via nasal para imunizar felinos com objetivo de diminuir a eliminação de oocistos, obtendo uma eficácia de 67%, onde dois de três gatos vacinados não eliminaram oocistos nas fezes. Da mesma forma, Zulpo et al. (2012) testaram a mesma vacina em felinos, porém em um número maior de animais (n=5) e obtiveram uma redução do número de oocistos eliminados de 98% quando comparados ao grupo controle.

1.5 - CONSIDERAÇÕES FINAIS

As medidas preventivas e as estratégias para reduzir a transmissão do *T. gondii* incluem práticas de manejo e educação sanitária. Essas medidas são efetivas principalmente quando adotadas individualmente pelas pessoas, principalmente por gestantes e pacientes imunocomprometidos, porém, ainda permanece o potencial dos gatos domésticos e errantes na contaminação ambiental, uma vez que esses animais mantém o hábito de caça, podendo se infectar ainda jovens. Desta forma, estudos para tentar diminuir a eliminação de oocistos por esses animais devem ser estimulados. Uma vacina para isso seria de grande interesse na saúde humana e animal.

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OBJETIVOS

Geral

Estudar experimentalmente a eliminação de oocistos do *Toxoplasma gondii* em gatos domésticos após vacinação e re-infecção.

Específicos

Avaliar a eliminação de oocistos em gatos domésticos imunizados pela via nasal com proteína recombinante (rROP2) do *T. gondii*.

Acompanhar a resposta imune humoral em gatos imunizados com rROP2.

Estudar a re-eliminação de oocistos do *T. gondii* em gatos infectados com cistos teciduais de cepas heterólogas.

Analisar a resposta imune humoral em gatos infectados com diferentes cepas do *T. gondii*.

CAPÍTULO 02

(Este capítulo está nas normas da revista Veterinary Parasitology)

Toxoplasma gondii and domestic cats: the use of recombinant protein (rROP2) as a vaccine against oocyst shedding

ABSTRACT

Toxoplasma gondii is a protozoan parasite that able to infect birds, mammals including humans. Domestic cats are major definitive hosts and they can shed millions of oocysts by feces, which may contaminate the environment. The aim of the present study was to evaluate oocysts shedding in cats immunized by nasal route with rROP2 of *T. gondii*. Twelve short hair cats were used and divided in three groups G1, G2 and G3 (n=4). Animals from G1, G2, and G3 received 100µg of rROP2 proteins plus 20µg of Quil-A, 100µg of bovine serum albumin (BSA) plus 20µg of Quil-A and saline solution (control group), respectively. All treatments were done by intranasal route at days 0, 21, 42, 63. The challenge was performed in all groups on day 70 with ≅800 cysts of ME-49 strain by oral route. Animals from groups had their feces examined and the number of oocysts determined from challenge to 20 days. Animals from G1 excreted lesser oocysts and demonstrated a higher preventable fraction of PF=86.7% when compared with G3, and when the G1 were compared with G2 the PF was 34.1%. ELISA was used to detect anti-*T. gondii* IgG and IgA against rROP2, however, there were no correlation between number of oocyst shedding and antibody levels. The present work showed that rROP2 was able to reduce oocyst shedding in domestic cats immunized by nasal route.

Key words: definitive host, immunization and toxoplasmosis.

1- INTRODUCTION

Felids are the definitive host of *Toxoplasma gondii* (Frenkel et al., 1970; Hutchison et al., 1971), which is a protozoan parasite able to infect all warm blood animals, including human being. Cats are important in the life cycle of this parasite because of the strict contact with humans, which after the primary infection with *T. gondii* might shed millions of oocysts by feces (Dubey and Beattie, 1988; Garcia, 2009), and consequently contaminate the environment, what are transmission source for animals and humans (Innes et al., 2009).

The domestic cat is a very popular pet in many countries and the number of cats has increased quickly and great importance is given to the zoonotic potential of domestic felines, chiefly focusing on the transmission of *T. gondii* to humans (Dabritz and Conrad, 2010). There are some studies based on vaccines against oocyst shedding in cats, and with exception of the use of a live T-263 strain (Frenkel et al., 1991; Freyre et al., 1993), this vaccine used bradyzoites of the mutant strain to protect cats against oocyst shedding and they found 100% of protection after challenge with heterologous strain, but the disadvantages of this vaccine are that the vaccine contains live *T. gondii* bradyzoites and the risk of human contamination is high (Dubey, 1995). Other studies observed partial protection (Omata et al., 1996; Garcia et al., 2007; Zulpo et al., 2012).

Nakkar et al., 2003 showed that *T. gondii* proteins ROP2 are the major determinant of proper biogenesis and maintenance of rhoptry structure, due to be responsible for parasite invasion, replication and parasite-host cell interaction. The crude rhoptry, recombinant ROP2 and ROP4 was used in some studies with cats, mice and pigs and showed immune response humoral and cellular in this animals (Garcia, et al., 2005, Garcia, et al., 2007, Igarashi, et al., 2008, Dziadek, et al., 2009, Cunha, et al., 2012, Dziadek, et al., 2012, Zulpo, et al., 2012). This has stimulated our study, therefore, the aim of the present study was to immunize cats with recombinant proteins (ROP2) of *T. gondii* against oocysts shedding.

2- MATERIALS AND METHODS

2.1- Ethics Committee

This study was approved by the Institutional Ethics Committee in Animal Use (CEUA, protocol number 102/12).

2.2- *Toxoplasma gondii* strains

Two strains of *T. gondii* were used in this experiment: RH (type I) and ME-49 (type II), respectively isolated by Sabin (1941) and Lunde and Jacobs (1983). The RH strain was used for production of recombinant proteins. The ME-49 strain was used for the production of tissue cysts to challenge the cats. *T. gondii* tissue cysts of the ME-49 strain was performed as previously described (Zulpo et al., 2012), ten mice were infected with 50 sporulated oocysts of *T. gondii* by the oral route. These animals were euthanatized 60 days after being infected, and the burden of brain cysts were counted and prepared for challenge (approximated 800 cysts to each cat in a total of 2 mL of saline).

2.3- Recombinant Proteins

The recombinant proteins (rROP2) of *T. gondii* was obtained as previously described (Igarashi et at., 2010).

2.3.1- Construction of plasmids

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 ROP2 F (5'ATCGAATTCACGGATCCTGGAGAC3'-introduced *EcoRI* recognition site, underline) and ROP2R (5'TGAAAGCTTTCATGCCGGTTCTCC3' – 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

recommendations. Each PCR mixture (25µl) consisted of 10 mM Tris-HCl pH 8.3, 50 mM MgCl2, 0.5 µM of each primer, 0.2 mM nucleoside triphosphates (d-NTPs), and 1.25 Units Taq polymerase. Amplifications were carried out in a PTC-100 Programmable Thermal Controller. Thermocycling consisted of 94 °C for 2 min, followed by 35 cycles of denaturation (94 °C; 30 sec) anneling (50 °C; 30 sec) and extension (72 °C; 1 min), with a final extension at 72 °C for 5 min. After amplification, 5 µl aliquots from each reaction were analyzed by eletrophoresis in 7.5% polyacrylamide mini-gels. DNA in gels was stained with ethidium bromide, visualized by with UV light (254 nm). Each PCR product was purified by QIAquick PCR purification Kit (Qiagen) designed to purify single or double-stranded DNA fragments from PCR. A specific PCR product was obtained and after digestion with respective restriction enzyme. After it, the fragment was precipited with 100% ethanol and 3M sodium acetate and ligated into respective vector using T4 DNA Ligase (Biolabs). Sequence analysis of the DNA fragment cloned in plasmid was carried out using the DNA sequencer software.

2.3.2- Culture Conditions

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). Strain Rosetta (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 the final concentration of 1mM. The culture was incubated with shaking at 37 °C for 4 h. The cells were harvested by centrifugation and the pellets were resuspended and lysed in 20mM sodium phosphate and 500mM sodium chloride pH 7.8 followed by 3 freezing-defreezing cycles for the soluble phase.

2.3.3- Purification of rROP2

Soluble fraction was applied directly onto Ni-NTA Superflow resin (Qiagen) preequilibrated 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.

2.4- Previous monitoring of cats

The animals were previously monitored for two months prior to the beginning of the experiment. All cats were serum negative (titer <16) for *T. gondii* by the indirect immunofluorescence assay (Camargo, 1974), before being immunized. The absence of *T. gondii* oocysts was further confirmed by fecal examination (Dubey, 1995).

2.5- Immunization and challenge of cats

Twelve short hair domestic cats, of both sexes, between 3 and 6 months of age, were randomly allocated in individual cages; all cats received only commercially prepared ration and water *ad libitum*. All cats were serum negative for *T. gondii* and free from *T. gondii* oocysts shedding. The cats were divided into three groups, each group containing four animals (Table 01): 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 uL 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, these animals were anesthetized with tiletamine plus zolazepam (Zoletil®, Virbac-Brazil, 3.15 mg/kg/IM) to the challenge.

Table 01: Experimental design of cats immunized with rROP2 of *Toxoplasma gondii* (G1), G2 were adjuvant control and G3 were saline control.

Experimental groups (n=4)	Immunization route	Immunization protocols (0, 21, 42, 63 days)[1]	Challenge (Day 70)[1]
G1	Intranasally	100 µg rROP2 + QuilA (20 µg)	800 cysts ME-49
G2		100 µg BSA[2] + QuilA (20 µg)	
G3		Saline Solution	

1 Period during which procedure was realized.

2 Bovine serum albumin.

2.6- Measurements oocyst shedding

Feces from each cat were collected from 1st until 20th after being challenged and examined microscopically for oocysts as described (Garcia et al., 2007; Zulpo et al., 2012). Briefly, feces obtained over a period of 24 h were diluted in a small volume of distillated water; 2 g of this material were admixed with 10 mL of sucrose solution (specific gravity, 1.18), filtered, and centrifuged (1200xg for 10 min). One drop of solution, removed from the meniscus, was examined microscopically. When oocysts were detected the supernatant was collected (approximated 9 mL) admixed with 40 mL of water in a 50 mL tube, and centrifuged (1200xg) 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 WBC chambers of a hemocytometer. The Preventable fraction was calculated [(reference value – vaccinated group)/reference value] and multiplied by 100 to get percentage.

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

Blood and serum samples were obtained on days 0, 21, 42, 63, 70, 85, 100, 115 and 130 of the experiment to evaluate serum. The ELISA assay was performed as described previously by IgG (Garcia et al., 2007) and IgA (Zulpo et al., 2012). 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 ^{o}C. 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 ^{o}C with carbonate buffer and 8% nonfat dry milk. The control and evaluated 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 the microtitre plates in duplicate. Further, the plates were incubated for 1 h at 37 ^{o}C (IgG) and overnight at 6 ^{o}C (IgA). After rinsing, the antibodies and conjugates (Bethyl Lab, Montgomery, TX, USA) were diluted in PBS-tween 20 and 5% nonfat dry milk (for IgG, HRP anti-cat IgG antibodies were diluted 1:10,000) (for IgA, goat anti-cat IgA antibody were diluted 1:2,000 and HRP rabbit anti-goat IgG antibodies were diluted 1:1,000) after which 0.1 mL of the mixture was added to each well and incubated for 1 h at 37 ^{o}C and 2 h at

37 ^{o}C for IgG and IgA, respectively. 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. For controls, positive and negative control sera were included in every plate and a corrected OD value was calculated according to the formula in (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)].

3- RESULTS

Cats immunized with rROP2 shed oocysts in feces (Figure 01), but the burden was lower than G2, and G3. The prepatent periods (PPP), patent period (PP) and peak of oocyst excretion (POE) from G1, G2 and G3 were 4.2, 4.7 and 5th day, 7.2, 6, 8.5th day, and 5, 4 and 5th day, respectively (Table 02). The G1 animals excreted comparatively less number of oocysts per gram of feces (OOPG: 3,120) than G3 (saline control) and demonstrated a superior preventable fraction of 86.7%, when compared with G2 (Quil-A control) demonstrated a superior preventable fraction of 34.1%. G2 showed OOPG: 4,737 and cats shedding 79.8% less oocyst than G3. The G3 cats showed OOPG: 23,441.

The outcome of antibodies response can be observed in Figure 02. The cats showed levels of IgG and IgA antibodies after challenge day with ME-49. Animals from G1 showed a higher level of antibodies than G2, and G3.

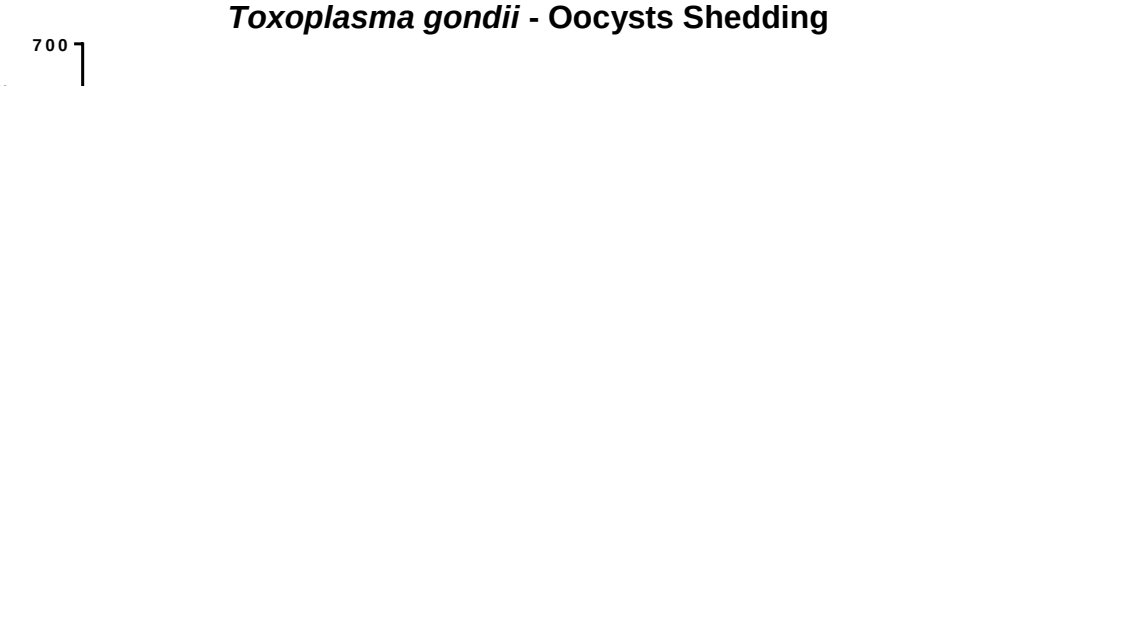

Figure 01: *Toxoplasma gondii* oocyst shedding in cats immunized with rROP2 (G1), G2 were adjuvant control and G3 were saline control. Challenged was performed at day 70 with 800 tissue cysts of ME-49 strain of *T. gondii* (**a:** Total of oocysts shedding / g of feces x 10^3; **b:** Mean of oocyst shed by four animals).

Table 02: Parameters of *Toxoplasma gondii* oocyst shedding after challenge with ME-49 strain. Cats were immunized with rROP2 + Quil-A (G1), G2 received BSA + Quil-A and the G3 received only saline.

Groups[1]	Pre patent period[2]	Patent Period[2]	Peak of oocysts shedding[2]	Oocysts shedding[3]	Preventable fraction (%)	Preventable fraction (%)
G1	4.2	7.2	5	3,121	86.7	34.1
G2	4.7	6	4	4,737	79.8	Reference value
G3	5	8.5	5	23,481	Reference value	

1: Mean of four animals.
2: Period in days.
3: Average totals of oocyst per garm of feces (OOPG) during 20 days.

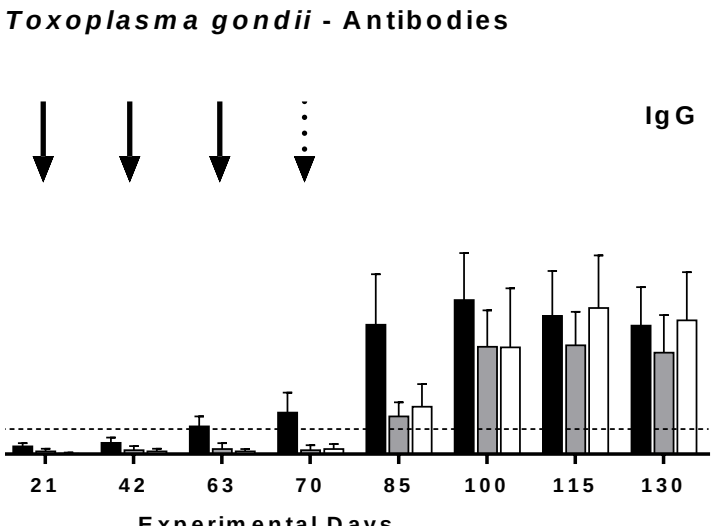

Figure 02: *Toxoplasma gondii* serum IgG and IgA antibody responses of cats evaluated by the indirect enzyme-linked immunosorbent assay (MEAN ± SD). G1, vaccinated with rROP2 plus Quil-A, G2 received bovine serum albumin (BSA) plus Quil-A and G3 received only saline, by nasal route. Treatments were made at days 0, 21, 42, and 63 (solid arrow). All groups received a challenge of 800 cysts from the ME-49 strain on day 70 (dashed arrow). Dashed line indicates positive cut-off.

4- DISCUSSION

In the present study we observed that cats immunized with rROP2 of *T. gondii* by nasal route showed partial protection against oocysts shedding. Differently from our study that used the DNA vaccine expressing ROP2 did not reduce oocysts shedding in cats (Mishima et al., 2002), this could be related to the fact that DNA vaccines are usually used by systemic route, and we used the intranasal route.

The results from oocyst shedding showed that animals that received rROP2 plus Quil-A (G1) excreted 34.1% less oocysts than animal that received only adjuvant (G2). This result is very interesting since ROP2 is just one protein among over 20 thousands of proteins from this protozoa (Dubey, 2010), and there was a specific humoral immune response based on IgG and IgA. Thus, the use of others recombinants proteins in formulation of a recombinant vaccine could improve its efficacy.

The CD8 cells are responsible for immunity against *T. gondii* (Frenkel, 1990; Sher and Coffman, 1992; Buxton; 1993). An efficient vaccine should stimulate these cells populations. Additionally, a previous study has supported the role of mucosal CD8+ cell population in mucosal protection of cats (Finerty et al., 2000).

The G2 group, that received BSA plus Quil-A, also had a partial protection, when compared with the G3 that received only saline solution. The adjuvant could have contributed to the immunity in cats, because the last vaccine dose was very next to the challenge day. The Quil-A adjuvant induce strong Th1 and Th2 responses and moderate CTL responses to some proteins probably as a result of forming mixed protein-saponin micelles (Cox and Coulter, 1997). Therefore, the G2 showed an efficacy against the challenge of *T. gondii*, this fact reveals the importance of the adjuvant in the vaccine in producing nonspecific immune response. The Quil-A adjuvant is a saponin, it is widely used in animals due to its low cost, simple design and it's generally safe (Cox and Coulter, 1997; Garcia et al., 2005, 2007; Igarashi et al, 2008, 2010; Cunha et al, 2012;. Zulpo et al, 2012).

A previous study (Garcia et al., 2007) did not report the shedding of oocyst in two out of three cats immunized with crude rhoptry of *T. gondii*, which was different from the present study that used recombinant protein (rROP2). Another study using crude rhoptry of *T. gondii* obtained an effectiveness of 98.6% in oocysts shedding (Zulpo et al., 2012). The challenge from those studies were performed with different strains VEG and ME-49, respectively. The ME-49 strain was the same as used in our study, this strain is considered with a higher pathogenicity and able to produce larger amount of oocysts (Dubey, 1995). This strain is a genotype II, and it is the most frequently used to challenge cats (Dubey and Thulliez, 1989; Lappin et al., 1989, 1994; Burney et al., 1995; Dubey, 1995; Dubey et al., 1995; Zulpo et al., 2012). The peaks of oocysts shedding was approximately five days herein described (from all

groups) were similar to previously described studies, where the PPP varied between 3 and 7 days, with PP ranging from 5 and 19 days.

Few additional studies have investigated the immunization of *T. gondii* in cats: some have utilized the T-263 live strain to evaluate oocyst shedding protection in cats (Frenkel et al., 1991; Freyre et al., 1993; Mateus-Pinilla et al., 1999); these studies have described reduced of toxoplasmosis. However, one of the disadvantages of the T-263 vaccine is that it contains live bradyzoites of *T. gondii* and the risk of human contamination is high (Dubey, 1995). Omata et al. (1996) evaluated a ^{60}Co-irradiated tachyzoites vaccine in cats but only 43% (3/7) of infected cats did not shed oocysts, but the challenge was realized with cysts of same strain. Therefore, the results of this study are important due to the demonstration of partial protection in cats that received rROP2 against oocyst shedding of *T. gondii*.

The contact with the ground was described as a major risk of acquiring *T. gondii* during pregnancy (Decavalas et al. 1990). Thus, a vaccine with the goal of decreasing oocyst shedding, would decrease environmental contamination and consequently the risk of acquiring the parasite. Thus, the potential of rROP2 plus Quil-A (G1) used here in reduce environmental contamination could be 86%, when compared with G3. Dabritz et al. (2007) 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 4671 oocysts/m^2. Additionally, other environmental survey has suggested that 22.58% (7/31) of soil samples obtained from the playgrounds of elementary public schools in the state of São Paulo were positive for *T. gondii*, and it was concluded that *T. gondii* oocysts are widely distributed within areas of public schools located in that region (Santos et al., 2010). Further, an outbreak of human toxoplasmosis was described in Santa Isabel do Ivai city, Paraná state, Brazil, where approximately 426 people were infected, and the source of contamination was the water reservoir (De Moura et al., 2006).

Studies about *T. gondii* in Brazil show an occurrence of antibodies in cats quite variable ranged from 14,3% to 87,3% (Garcia et al., 1999; Silva et al., 2001; Silva et al., 2002; Meireles et al., 2004; Cavalcante et al., 2006; Pena et al., 2006; Dalla Rosa et al., 2010 and Cruz et al., 2011). The animals with antibodies against *T. gondii* show the risk of environmental contamination by oocyst shedding and reveal the importance of controlling the infection in cats that can infect humans and animals.

This fact has been described in susceptible human populations (Jones et al., 2006; Santos et al., 2010).

Humoral immune response from IgG and IgA could have contributed to the control of *T. gondii*, because cats that were vaccinated intranasally (G1) also demonstrated more elevated levels of IgG and IgA and eliminated less oocysts relatively to those not immunized (G2 and G3). This fact was reported by Frenkel and Smith (1982) who observed that 25% of cats that developed antibodies did not shed oocysts when challenged with *T. gondii*.

Frenkel et al. (1991) suggested that the primary focus of immunity against oocyst shedding might be the intestinal epithelium of kittens Therefore, the elevated intestinal stimulation observed in vaccinated cats intranasally with the rROP2 of *T. gondii* during this study could have contributed.

This shows that we should study further the mucosal immunoglobulin immunity, for there is possibly a relation between IgA production and lower oocysts shedding by cats since the parasite multiplies in cells of the intestinal mucosa (Bonefant et al., 2001; Dimier-Poisson et al., 2006; Mack and McLeod, 1992). The IgG immunoglobulin could also have contributed, because this is responsible for opsonizating to *T. gondii* outside the host cell.

Consequently, the immunization with rROP2 of *T. gondii* in cats, using Quil-A as an adjuvant would be important to control toxoplasmosis in either human or animal populations. The results of this study are desirable since environmental contamination by oocysts can be significantly reduced, but other proteins must be combined to increase the efficacy of the vaccine in future studies.

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CAPÍTULO 03

(Este capítulo está nas normas da revista Veterinary Parasitology)

Toxoplasma gondii and domestic cats: re-shedding of oocysts after heterologous infection

ABSTRACT

The cats are definitive hosts of *Toxoplasma gondii*, and they are considered the key in the life cycle of this parasite because they are able to shed millions of oocysts by feces. Usually, this occurs when cats are infected for the first time while kittens. However, the potential of a cat re-shed oocyst further after re-infections is not totally know. Thus, the aim of the present study was to evaluate the re-shedding of *T. gondii* oocysts in cats fed with tissue cysts from heterologous strains. Ten cats were used in the present study. All animals were infected with ME-49 (type II) strain of *T. gondii* on zero day. After infection these cats were divided in three groups; G1 (n=2), G2 (n=5), and G3 (n=3). Animals from G1 and G2 were re-infected after twelve months with cysts of VEG (type III) and PB-1 (type II), respectively. G2 and G3 were re-infected thirty-six months later with cysts of VEG strain. The infections were performed by the use of ≅800 tissue cysts. Animals had their feces evaluated by fecal flotation and serology by ELISA. All cats sheded oocysts at the infection with ME-49 strain. Cats from G1 when re-infected after twelve months with VEG strain, one cat (1/2) shed oocysts. G2 when re-infected after twelve months with PB-1 strain did not shed oocysts, and when re-infected after thirty-six months with VEG strain, three out of four cats sheded oocysts. The G3 when re-infected after thirty-six months with VEG strain, two out of three cats sheded oocysts. All animals showed IgM, IgG and IgA after infection with ME-49 and remained positive until the last analysis of experiment (39 months). In conclusion, the excretion of *T. gondii* oocysts had kept high in experimentally re-infected cats during the years, mainly when different stains are used, what is more probably to happen in natural conditions.

Key words: definitive host, oocyst shedding, re-infection and toxoplasmosis.

1- INTRODUCTION

Toxoplasma gondii is an obligatory intracellular protozoan that can infect over 380 species of animals, including humans. Felids are the only definitive host of *T. gondii* (Frenkel et al., 1970; Hutchison et al., 1971), these animals at the primary infection shed milions of oocysts by feces contaminating the environment (Dubey and Beattie, 1988, Garcia, 2009) and generally develop immunity to re-excretion of oocysts after challenge with homologous or heterologous strains of *T. gondii* (Dubey and Frenkel, 1972, 1974; Sheffield and Melton, 1976; Ruiz and Frenkel, 1980; Frenkel and Smith, 1982), but Dubey (1995), in a unique study that evaluated the immunity for long term, showed that four out of nine re-infected cats orally with tissue cysts after 77 months after the first infection with *T. gondii*, re-sheded oocysts. The number of oocysts eliminated during the second infection was lower, but these animals were adults and the amount of fecal matter is higher. However, the potential of naturally infected cats in re-eliminate oocysts is unknown (Dubey and Frenkel, 1974).

Toxoplasmosis in cats is usually subclinical, although clinical disease has been well documented in both kittens and adults (Dubey and Beattie, 1988). Importance is given to the zoonotic potential of domestic cats, chiefly focusing on the transmission of *T. gondii* to humans by oocysts shedding (Dabritz and Conrad, 2010). Thus, the aim of the present study was to evaluate re-shedding of *T. gondii* oocysts in cats fed with tissue cysts from different strains.

2- MATERIALS AND METHODS

2.1- Ethics Committee

This study was approved by the Institutional Ethics Committee in Animal Use (CEUA, protocol number 51/07 and 102/12).

2.2- *Toxoplasma. gondii* strains

Three strains of *T. gondii* were used in this experiment: ME-49 (type II strain, Lunde and Jacobs (1983)), PB-1 (type II strain isolated from Brazilian pigeons by our group, Barros et al. (2011)) and VEG (type III strain, Dubey (1996)). For tissue cysts

production ten mice were infected with 50 sporulated oocysts of specific strain by the oral route. The mouse were euthanatized 60 days after being infected, and the burden of brain cysts were counted and prepared for challenge (approximated 800 cysts to each cat in a total of 2 mL of saline).

2.3- Previous monitoring of cats

The animals were previously monitored for 2 months prior to the beginning of the experiment. All cats were serum negative (titer <16) for *T. gondii* by the indirect immunofluorescence assay (Camargo, 1974), before being immunized. The absence of *T. gondii* oocysts was further confirmed by fecal examination.

2.4- Infection of cats

Ten short hair domestic cats, of both sexes, between 3 and 6 months of age, were randomly allocated in individual cages; all cats received only commercially prepared ration and water *ad libitum*. All animals were infected on zero day with cysts of ME-49 strain, type II. After infection the animals was divided in three groups, G1 (n: 2), G2 (n: 5), and G3 (n: 3). Animals from G1 was infected 12 months later with cysts of VEG strain (type III). G2 were infected with PB-1 (Brazilian type II strain) with 12 months and infected with VEG with 36 months and cats from G3 were infected 36 months later with cysts of VEG strain. The infections were performed by the use of ≅800 tissue cysts (contained in a volume of 2 mL) administered via stomach tube, after which they were injected with 5 mL of saline, these animals were anesthetized with tiletamine plus zolazepam (Zoletil®, Virbac-Brazil, 3.15 mg/kg/IM) to the infection. One cat from G2 died until of infection with 36 months from a noninfectious cause. The G1 group was accompanied until 15 months and the G2 and G3 were accompanied until 39 months.

Table 01: Experimental design of cats after first infection tissue cysts of ME-49 strains of *T. gondii*[1].

Groups	Cats*	Month 12	Month 36
G1	2	VEG	NC
G2	5	PB-1	VEG
G3	3	NC	VEG

[1]: The G1 was evaluated for 15 months and the G2 and G3 for 39 months.
NC: Not challenge.
*: All cats were infected on zero day with ME-49 strain.

2.5- Oocyst shedding

Feces from each cat were collected from the 1st to the 20th day after challenged and examined microscopically for oocysts as described previously by Garcia et al. (2007). Briefly, feces obtained over a period of 24 h were diluted in a small volume of distillated water; 2 g of this material were admixed with 10 mL of sucrose solution (specific gravity, 1.18), filtered, and centrifuged (1200xg for 10 min). One drop of solution, removed from the meniscus, was examined microscopically. When oocysts were detected the supernatant was collected (approximated 9 mL) admixed with 40 mL of water in a 50 mL tube, and centrifuged (1200xg 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 WBC chambers of a hemocytometer.

2.6- Enzyme-linked immunosorbent Assay (ELISA) - IgM, IgG and IgA

Animals were bleeding and serum samples were obtained on the day of challenge, and further samples obtained on months 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 of the experiment to detect antibodies in sera. The ELISA assay was performed as described previously by IgG and IgM (Garcia et al., 2007) and IgA (Zulpo et al., 2012). Optimal dilutions were established by using checkerboard titrations with dilutions of sera and conjugates. Proteins from membrane tachyzoites of *T. gondii* were used as antigens (Garcia et al., 2004) 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 ^{o}C. 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 ^{o}C with carbonate buffer and 8% nonfat dry milk. The control and evaluated sera were diluted (1:100 for IgM and 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 the microtitre plates in duplicate. Further, the plates were incubated for 1 h at 37 ^{o}C (IgM and IgG) and overnight at 6 ^{o}C (IgA). After rinsing, the antibodies and conjugates (Bethyl Lab, Montgomery, TX, USA) were diluted in PBS-tween 20 and 5% nonfat dry milk (for IgG, HRP anti-cat IgG antibodies were diluted 1:20,000 and for IgM, HRP anti-cat IgM antibodies were diluted 1:5,000) (for IgA, goat anti-cat IgA antibody were diluted 1:2,000 and HRP rabbit anti-goat IgG antibodies were diluted 1:1,000) after which 0.1 mL of the mixture was added to each

well and incubated for 1 h at 37 ^{o}C for IgM an d IgG and 2 h at 37 ^{o}C for IgA. 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. For controls, positive and negative control sera were included in every plate and a corrected OD value was calculated according to the formula in (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)].

3- RESULTS

The overall result of oocyst shedding is shown in Table 02. All animals shedded oocysts after infection with ME-49 strain, and the average of oocysts shedding was 23,087 oocyst per gram of feces (OOPG) considering average of 20 days of excretion. When animals from G1 were re-infected with VEG strain 50% (1/2) re-shed oocysts. Animals from G2 did not shed oocysts after PB-1 infection, however, 75% (3/4) shedded oocysts after a third infection with VEG strain. At the G3 67% (2/3) of cats re-shed oocysts after infection with VEG strain.

The prepatent period (PPP) at first infection with ME-49 strain was 6.9 days, and the patent period (PP) was 5.7 days and peak of oocyst excretion (POE) was at 7th day. The PPP, PP, and POE from G1, G2 (after third infection) and G3 (after second infection) were 5, 4 and 6th day, 6.3, 5, and 7.6th day, and 6, 3.5 and 6.5th day, respectively.

The outcome of antibodies response can be observed in Figure 01. The cats showed levels of IgG, IgM and IgA antibodies just after oocysts shedding of ME-49 strain and remained positives until the last analysis.

Table 02: Oocyst shedding of cats evaluated by the fecal examination. All cats were infected with ME-49 on zero day. G1 were re-infected twelve months later with VEG strain. G2 were re-infected after twelve months with PB-1 strain and thirty-six months later were re-infected with VEG strain. G3 were re-infected thirty-six months later with VEG strain.

	Primary Infection (Day zero)		12 months after primary infection		36 months after primary infection
	ME-49		VEG	PB-1	VEG
Cats	OOPG (mean)	Groups	OOPG	OOPG	OOPG
1	23,087	G1*	Neg	NC	NC
2			5,937	NC	NC
3		G2[#]	NC	(-)	(-)
4			NC	(-)	187
5			NC	(-)	30,875
6			NC	(-)	Died
7			NC	(-)	10,750
8		G3[#]	NC	NC	(-)
9			NC	NC	2,750
10			NC	NC	187

OOPG: oocyst per gram of feces.
(-): Negative.
NC: Not Challenged.
*: This group was evaluated for 15 months.
[3]: This groups were evaluated for 39 months.

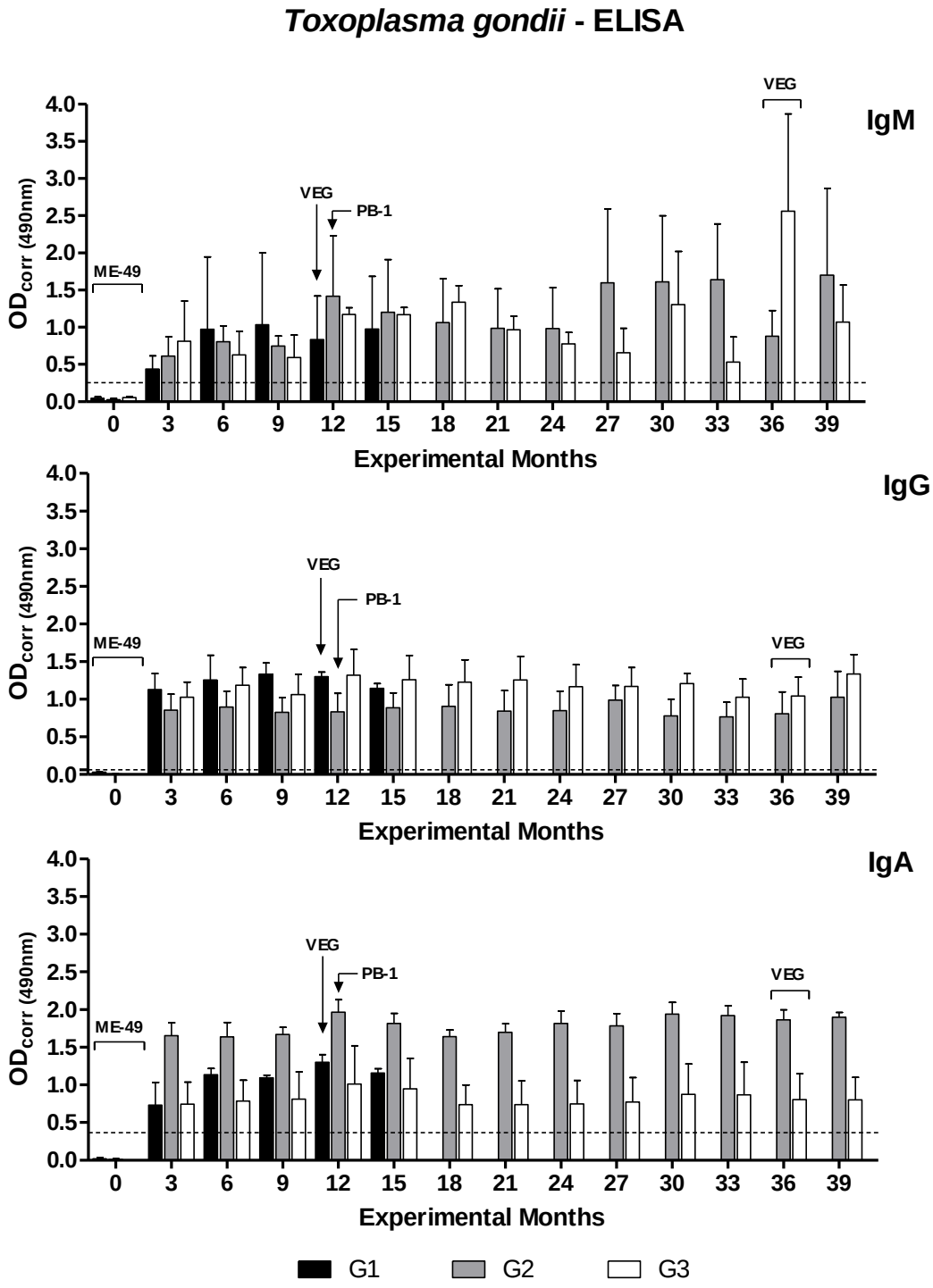

Figure 01: Serum IgM, IgG and IgA antibody responses of cats evaluated by the indirect enzyme-linked immunosorbent assay (ELISA) (MEAN ± SD). All cats were infected whit ME-49 on zero day. G1 were re-infected twelve months later with VEG strain. G2 were re-infected with twelve months with PB-1 strain and thirty-six months later were re-infected with VEG strain. G3 were re-infected thirty-six months later with VEG strain. All groups received an infection of ≅800 cysts of strain. Dashed line indicates positive cut-off. The G1 was evaluated for 15 months and G2 and G3 were evaluated for 39 months.

4- DISCUSSION

Little is known if naturally infected cats can shed oocysts more than once (Dubey, 2010). The present study showed that all cats shedded oocysts after primary infection with ME-49 (type II) and when the G1, G2 and G3 were re-infected with VEG strain (type III) 67% (6/9) re-shedded oocysts However, when animals from G2 were re-infected with the PB-1 strain (same genotype), no animal shedded oocysts;

Similar results were showed by Dubey (1995) when 12 cats infected with the ME-49 and TS-2 strain shedded a high amount of oocysts in the primary infection. After 39 days, five cats initially infected were re-infected with an homologous strain and no animal shedded oocysts. At 77 months after the primary infection, nine cats were re-infected with the P-89 strain and 44% (4/9) re-sheded oocysts. Dubey and Davis (1995) infected cats with the ME-49 strain and three months later re-infected them with the same strain and no animal shedded oocysts. Other study reported that eleven out of 18 wild cats with natural *T. gondii* infection, verified by seropositivity, shedded oocysts after being fed with tissue cysts (Dubey et al., 1970;. Piekarski and Witte, 1971). This shows that the animals are protected by the homologous strain, which is not the reality of a natural infection and when they were re-infected with a heterologous strain, they re-shed oocysts, so cats should not be considered immune after oocysts shedding.

Cats when re-infected with the PB-1 strain (Brazilian type II) had 100% immunity. This fact should be further investigated because the immunity of *T. gondii* may be related to the genotype of the strain. Brazil has a large genetic diversity of isolates of *T. gondii* (Ajzenberg et al., 2004; Dubey et al., 2004; Pena et al., 2006).

Most cats are infected for the first time as kittens (Dubey, 2010), kittens eliminate a smaller amount of feces than adults. An adult cat in a second or third infection can shed oocyst and lead to an environmental contamination similar to a kitty's. Even though they shed less oocysts per gram of feces, the amount of fecal matter is higher, since an adult cat has the potential to eliminate approximately 40 grams of fecal matter per day (Dabritz et al., 2006) against kittens that eliminate approximately 5 grams of fecal matter per day. Our concern must be increased and we must have in mind that the cat can transmit *T. gondii* during its lifetime.

The ME-49 and VEG strains are considered as large producers of oocysts in cats and infected cats can eliminate millions of oocysts on the first infection (Dubey,

1995; Garcia et. al., 2007, Zulpo et al., 2012). This fact was confirmed by infection in cats in our study, were the $OOPG_{average}$ of ME-49 strain was 23,087. The number of oocysts shed during the second infection is usually lower than the first infection (Dubey and Frenkel, 1974). Only one cat (1/9) when re-infected with VEG strain eliminated more oocysts in third infection than in first infection. The environmental contamination by oocysts reveals the importance of studying the immunity against oocyst shedding in cats.

Several factors other than the strain of *T. gondii* can affect the shedding of oocysts, including the age of the cat, the nutritional status, the number of tissue cysts fed during the infection, the stage and the taken route, primary and secondary infections and concurrent infections (Dubey, 1995).

Cats develop antibodies against *T. gondii* within 10 days of ingesting tissue cysts, the animals that have antibodies are more likely to had shed oocysts before (Dubey, 1995b). All cats seroconverted for IgG, IgM and IgA after infection with the ME-49 strain. However, in studies of naturally infected cats, IgM has been detected for many months after primary infection, showing that it does not always correlate with recent infection (Lappin et al., 1993). IgM, IgG and IgA remained positive until the last analysis of experiment.

This study revealed that cats remain with high titers of antibodies against *T. gondii* for several years. A relevant fact is that these antibodies do not protect the animals against re-infections. Thus, antibodies should be useful in epidemiological studies to determine environmental contamination by oocysts. As cats shed oocysts for a short period (1 week or less), and the oocysts are dispersed over a wide area, the detection of oocysts in cat feces or in the environment is more complicated and less sensitive than determining the seropositivity (Dubey, 1995b).

Zulpo et. al (2012) reported that antibodies formation in vaccinated cats was not sufficient to induce total immunity against infection. In other animals species the presence of antibodies from immunization against *T. gondii* was also not sufficient for animals to become immune to infection (Garcia et al, 2005; Igarashi et al, 2008; Cunha et al, 2012.). The information shows that antibodies do not create an effective immune response against the protozoa, but assist on the immunity, because they operate on the opsonization and on the complementary system against extracellular parasites (Frenkel, 1990; Wastung et al., 1995).

We could assume that the quantity of oocyst shedded by adult cats was lower than when they were young, however, the amount of feces that an older cat produce is much higher than a younger one. In conclusion, the excretion of *T. gondii* oocysts may stay high in experimentally re-infected cats during the years, mainly when different strains are used, what is more probably to happen in natural conditions. The potential of environmental contamination from a cat may be much higher than is thought, because 67% of the cats shed oocyst in secondary or third infections.

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CONCLUSÕES

Os gatos que receberam a rROP2 pela via nasal eliminaram menos oocistos que os grupos controles.

A rROP2 administrada pela via nasal foi capaz de estimular a resposta imune humoral específica para IgG e IgA.

A eliminação de oocistos do *T. gondii* manteve-se elevada em gatos re-infectados com diferentes cepas mesmo após 36 meses da primeira infecção.

Gatos infectados pelo *T. gondii* permaneceram com IgM, IgG e IgA em níveis elevados até a última análise do experimento.