Universidade Estadual de Londrina

ANA CAROLINA MIURA GIMENES

EXPRESSÃO DE CITOCINAS E QUANTIFICAÇÃO DE OOCISTOS POR qPCR EM GATOS IMUNIZADOS COM PROTEÍNA RECOMBINANTE DO *Toxoplasma gondii*

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Orientador: Prof. Dr. João Luis Garcia.

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BANCA EXAMINADORA

Orientador: Prof. Dr. João Luis Garcia
Universidade Estadual de Londrina - UEL

Prof. Dr. Alexey Leon Gomel Bogado
Universidade Norte do Paraná - UNOPAR

Prof. Dr. Andreas L. Chryssafidis
Universidade Estadual de Londrina - UEL

Prof ª Dr ª Regina Mitsuka Breganó
Universidade Estadual de Londrina - UEL

Prof. Dr. Selwyn Arlington Headley
Universidade Estadual de Londrina - UEL

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GIMENES, Ana Carolina Miura. **Expressão de citocinas e quantificação de oocistos por qPCR em gatos imunizados com proteína recombinante do Toxoplasma gondii.** 2018. 69 f. Tese (Doutorado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

Toxoplasma gondii é um protozoário intracelular obrigatório causador da toxoplasmose, uma importante zoonose. Os felídeos, dentre eles o gato doméstico, são hospedeiros definitivos do T. gondii, neles ocorre a formação de oocistos eliminados nas fezes. Visto que poucos estudos são focados em gatos, este teve como objetivo avaliar a expressão de citocinas (IL-6, IL-10, TNF-α, IFN-g) e quantificar oocistos pela técnica de qPCR em gatos imunizados com proteínas recombinantes rROP2 de T. gondii e rHSP70 de Eimeria tenella. Foram utilizados doze gatos, em 3 grupos; 4 animais cada. G1: foi imunizado com 25 µg de rROP2, 25 µg de rHSP70 mais 20 µg de Quil-A; G2: recebeu 25 µg de E. coli (vetor) mais 20 µg do Quil-A; G3: recebeu apenas solução salina; grupo controle. Todas as doses imunizantes foram administradas nos dias 0, 21 e 42 do experimento pela via nasal. O desafio foi feito no dia 0, com 600 cistos da cepa TgDoveBr8 (tipo II). Amostras de sangue foram coletadas de cada animal nos dias 0,7,14, 21 e 35 pós infecção e fezes de todos os animais foram examinadas por 9 dias após o desafio pela técnica de Sheather (centrifugo-flutuação com exame microscópico) e pela qPCR. No 7º dia pós infecção, biopsias de duodeno foram coletadas de seis animais (dois gatos/grupo) para análise histopatológica. A partir do sangue total, realizou-se a separação de leucócitos, extração de RNA, síntese de cDNA e expressão gênica relativa pela qPCR com GAPDH como controle endógeno. A análise histopatológica dos seis animais submetidos à endoscopia duodenal revelou enterite linfohistioplasmocitária moderada e difusa em todos os animais, alguns diferenciais de lesões foram encontrados entre os grupos. Quanto aos sinais clínicos foi observada diarreia leve, de forma intermitente. A expressão gênica relativa de TNF-a ($p < 0.0001$) e IL-10 ($p=0.0128$) demonstrou diferença estatística significativa em um mesmo grupo durante diferentes períodos de infecção, no entanto não foram observadas diferenças em relação às citocinas IL-6 ($p=0.3746$) e IFN-g (0.7711). No 35º dia pós desafio, os animais imunizados (G1) apresentaram a maior expressão relativa de todas as citocinas IL-6 (2.85), TNF-a (0.64), IFN-g (1.64) e IL-10 (1.42). Todos os gatos eliminaram oocistos nas fezes. Cinco gatos negativos no Sheather no (3º d.p.i) foram positivos na qPCR, com as seguintes quantificações (328; 450; 470; 509 e 1828 OOPG). A detecção de oocistos só foi possível no 4º d.p.i por Sheather. Não houve diferença estatística entre a quantificação de oocistos por qPCR ou Sheather ($p= 0.1116$). Também não houve diferença significativa quanto á quantidade média de oocistos eliminados por grupo em qualquer uma das técnicas, qPCR ($p=0.9670$) Sheather ($p=0.6534$). Houve estimulação das respostas celulares Th1 e Th2 após a fase aguda nos gatos imunizados com proteínas recombinantes associadas ao Quil-A. Os animais do G2 (Quil-A) eliminaram menor quantidade média de oocistos, porém a diferença numérica não foi estatisticamente significativa quanto aos outros grupos. qPCR pode ser usada como uma alternativa para o Sheather para detecção e quantificação de oocistos de T. gondii.

Palavras-chave: Vacina. HSP70. ROP2. Expressão gênica. Histopatologia.

GIMENES, Ana Carolina Miura. **Cytokines expression and oocysts quantification by qPCR in cats immunized with recombinant Toxoplasma gondii protein.** 2018. 69 p. Thesis (Doctor´s Degree in Animal Science) – Universidade Estadual de Londrina, Londrina, 2018.

ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan that causes toxoplasmosis, an important zoonosis. The felids, among them the domestic cat, are definitive hosts of T. gondii, in which occurs the formation of oocysts shed in the feces. Since few studies are focused on cats, this study aimed to evaluate the expression of cytokines (IL-6, IL-10, TNF-α, IFN-g) and quantify oocysts by the qPCR technique in cats immunized with recombinant proteins (rROP2 and rHSP70) from T. gondii. Twelve cats were used in three groups; four animals each. G1: was immunized with 25 µg rROP2 (T. gondii), 25 µg rHSP70 (E. tenella) plus 20 µg Quil-A; G2: received 25 µg of E. coli (vector) plus 20 µg of Quil-A; G3: received saline solution only; group control. All immunizing doses were administered on days 0, 21 and 42 of the experiment via nasal route. The challenge was done on day 0, with 600 cysts of the strain TgDoveBr8 (type II). Samples of blood were collected from each animal on days 0, 7, 14, 21, 35 of the experiment and feces from all animals were examined for 9 days after challenge by Sheather (centrifugeflotation with microscopic examination) and by qPCR. On day 79 duodenal biopsies were collected from 6 animals (two cats/group) for histopathological analysis. From the whole blood, leukocyte separation, RNA extraction, cDNA synthesis and relative gene expression were performed by qPCR with GAPDH as the endogenous control. The histopathological analysis of the six animals submitted to duodenal endoscopy revealed moderate and diffused lymphohistioplasmocitary enteritis in all animals; some lesion differentials were found between groups. As for clinical signs, mild diarrhea was observed intermittently. The relative gene expression of TNF-a ($p < 0.0001$) and IL-10 ($p = 0.0128$) demonstrated a statistically significant difference in the same group during different periods of infection, however no differences were observed in relation to IL-6 cytokines ($p = 0.3746$) and IFN-g (0.7711). On the 35th day after inoculation, the immunized animals (G1) had the highest relative expression of all cytokines IL-6 (2.85), TNF-a (0.64), IFN-g (1.64) and IL-10 (1.42). All cats shed oocysts in the feces. Five cats negative in Sheather (3rd d.p.i) were positive in qPCR, with the following quantifications (328; 450; 470; 509 and 1828 OOPG). The detection of oocysts was only possible in the 4th d.p.i by Sheather. There was no statistical difference between the quantification of oocysts by qPCR or Sheather ($p = 0.1116$). There was also no significant difference in the mean number of oocysts removed per group in any of the techniques, qPCR ($p = 0.9670$) Sheather ($p = 0.6534$). There was stimulation of Th1 and Th2 cell responses after the acute phase in cats immunized with recombinant Quil-A-associated proteins. The G2 (Quil- A) animals shed the lowest mean number of oocysts, but the numerical difference was not statistically significant for the other groups. qPCR can be used as an alternative to Sheather for detection and quantification of T. gondii oocysts.

Keywords: Vaccine. HSP70. ROP2. Gene expression. Histopathology.

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1 INTRODUÇÃO

Toxoplasma gondii é um protozoário que normalmente causa infecções subclínicas, porém, a infecção primária durante a gestação pode ser grave e até mesmo causar abortamentos em seres humanos e em algumas espécies de animais, entre as patologias mais estudadas relacionadas a este parasita estão as congênitas e oftálmicas (VERCAMMEN et al., 2000; SZABO; FINNEY, 2017). A primo-infecção durante a gestação pode levar à infecção do feto que cursa com síndrome congênita podendo ocorrer surdez, convulsões, retardo mental e danos à retina do feto (TORREY et al., 2012). Muitas vezes a criança nasce sem apresentar sinais clínicos, porém mais tarde 90% delas pode apresentar perda da visão, retardo mental, entre outros problemas neurológicos (MILLER et al., 2009; EL-TRAS, 2012).

A toxoplasmose congênita é um oneroso problema de saúde pública e além de prejuízos financeiros, causa sofrimento psicológico para a família (ELSHEIKHA, 2008). Em humanos há correlação entre as altas taxas de soropositividade para *T. gondii* e disfunções como: esquizofrenia, comportamento suicida, depressão, transtorno obsessivo compulsivo, crianças com baixo rendimento escolar, artrite reumatoide e câncer cerebral têm sido estudadas (TORREY et al., 2013).

Na área veterinária, a toxoplasmose é uma das principais causas de infecções relacionadas à problemas reprodutivos em caprinos, ovinos e suínos (DUBEY; BEATTIE, 1988). A carne desses animais pode ser uma importante via de transmissão para seres humanos, estima-se que o *T. gondii* ocupa o terceiro lugar entre patógenos de origem alimentar que mais causam mortes nos EUA (MEAD et al., 1999; HILL; DUBEY, 2002).

Estes dados demonstram a necessidade do desenvolvimento de ferramentas para diminuir a ocorrência desta doença, como as vacinas. Estas podem focar no principal hospedeiro definitivo, ou seja, o gato doméstico, com o objetivo de diminuir a eliminação de oocistos (ZULPO et al., 2012), reduzindo a contaminação ambiental. E uma vacina focada nos animais de consumo (suínos, ovinos e caprinos, principalmente) para diminuir a formação de cistos teciduais e transmissão pelo consumo de carnes malcozidas (CUNHA et al., 2012).

Apesar dos felídeos serem os hospedeiros definitivos e peças chave no ciclo do parasita, a grande maioria dos estudos realizados utilizam camundongos como modelo experimental, sendo que nos últimos anos, apenas três estudos foram realizados com o objetivo de diminuir a eliminação de oocistos nas fezes de gatos domésticos (ZULPO et al., 2012; DUBEY, 2017;

ZULPO et al., 2018). Contudo, nenhum estudo foi realizado utilizando a qPCR para dosagem de citocinas em gatos imunizados com proteínas recombinantes e desafiados com o parasita.

2 REFERENCIAL TEÓRICO

2.1 *TOXOPLASMA GONDII*

Toxoplasma gondii é um protozoário eucarionte, intracelular obrigatório, que foi isolado e descrito como *Leishmania gondii* por Nicolle e Manceaux (1908) na Tunísia. No Brasil, Splendore (1908) também isolou o parasita em um coelho de laboratório em São Paulo, classificando-o como *T. cuniculli*. No ano seguinte foi então classificado como *T. gondii* (NICOLLE e MANCEAUX, 1909). Este protozoário é formado por uma membrana externa, anéis polares, conóide, microtúbulos, grânulos densos, micronemas, roptrias, mitocôndria, retículo endoplasmático, complexo de Golgi, ribossomos, retículo endoplasmático rugoso, microporo e um núcleo com parede bem definida (METSIS, PETERSEN, 1995).

Durante a invasão celular dois tipos de proteínas se destacam: as de superfície, responsáveis pelo reconhecimento inicial da célula alvo e as excretadas/secretadas, que estão estocadas em organelas secretórias dos taquizoítos e são capazes de penetrar ativamente nas células através da formação de um vacúolo parasitóforo (VP) (METSIS, PETERSEN, 1995; CARRUTHERS, 2002). A secreção de proteínas das organelas apicais do *T. gondii* é ordenada e sequencial, primeiro são liberadas dos micronemas, das roptrias e depois dos grânulos densos (SIBLEY, 2004).

Este coccídeo infecta todos os animais de sangue quente, inclusive o homem, sendo, portanto, uma importante zoonose (DUBEY, 2009). Estima-se que 33% da população mundial possua anticorpos contra o parasita que está presente em todos os continentes, principalmente em países em desenvolvimento (DUBEY e BEATTIE, 1988; FOND et al., 2013; HIL e DUBEY, 2013).

O parasita possui três formas infectantes para todos os hospedeiros: taquizoítos, característicos da fase aguda da infecção, bradizoítos contidos em cistos teciduais e esporozoítos provenientes de oocistos esporulados (HILL; DUBEY, 2002). A infecção dos animais e do homem pode ocorrer por ingestão de cistos teciduais em carne crua ou mal cozida, ingestão acidental de oocistos esporulados em água ou alimentos contaminados e congenitamente, via transplacentária (DUBEY; JONES, 2008).

Após a ingestão dos cistos ou oocistos, estes são rompidos e os bradizoítos ou esporozoítos, são liberados no lúmen intestinal, onde penetram rapidamente nas células do hospedeiro na forma de taquizoítos e formam um vacúolo parasitóforo (VP), que serve como proteção contra mecanismos de defesa do hospedeiro, então os taquizoítos se multiplicam várias vezes por endodiogenia, até romperem as células do hospedeiro liberando os taquizoítos (DUBEY, 2004, MAENZ et al., 2014). Estes invadem novas células e o ciclo se repete. Essa destruição celular determina os sinais clínicos da toxoplasmose.

Na fase crônica, os bradizoítos se desenvolvem e permanecem intracelularmente nos cistos teciduais, seu tamanho pode variar de cinco a 70 µm dependendo da quantidade de bradizoítos em seu interior, a diferença entre essas formas é mínima, os taquizoítos têm o núcleo mais central e são mais susceptíveis às enzimas proteolíticas (DUBEY; LINDSAY; SPEER, 1998). Os oocistos e bradizoítos desempenham um papel importante na disseminação da infecção, devido à sua maior resistência no ambiente externo e às enzimas proteolíticas no trato digestório respectivamente (FERGUSON, 2004).

A soroprevalência de anticorpos anti-*T.gondii* em humanos e felinos aumenta com a idade e não tem relação com o gênero, mas está relacionada aos hábitos higiênicos e alimentares (DUBEY et al., 1995; MONTOYA; LIESENFELD, 2004). No Brasil, França e El-Salvador, a soroprevalência em humanos pode chegar a 75- 80% (MILLER et al., 2009). E a ocorrência da toxoplasmose pode variar até mesmo dentro de um mesmo país, e entre grupos étnicos vivendo em uma mesma área (TENTER; HECKEROTH; WEISS, 2000).

2.2 FELÍDEOS E A TOXOPLASMOSE

Felídeos, dentre eles o gato doméstico, podem ser tanto hospedeiros definitivos quanto intermediários do *T. gondii* (LINDSAY; BLAGBURN; DUBEY; 1997). Nesses animais ocorre o ciclo enteroepitelial (esquizogonia e gametogonia), que culmina com a formação de oocistos não esporulados, sendo que o período pré-patente (PPP) e a quantidade de oocistos eliminados variam conforme o estágio do parasita que causa a infecção (DUBEY, 2004). O PPP após a ingestão de bradizoítos pode variar de três a 10 dias, ≥ 18 dias após a ingestão de oocistos (DUBEY, 1996), e ≥ 19 dias para taquizoítos (DUBEY, 2005). O carnivorismo é a forma mais comum e eficiente de infecção em gatos, visto que o *T. gondii* causa uma alteração no comportamento das espécies por eles predadas, favorecendo assim o ciclo do parasita (LAPPIN, 2010; VYAS et al. 2007).

A infecção em felídeos geralmente é subclínica e pós-natal. Estudos experimentais demonstram que apenas 10-20% dos gatos apresentaram diarreia branda após inoculação oral com cistos de *T. gondii* (LAPPIN, 2010), entretanto, infecções congênitas, e casos severos em animais mais jovens são descritos (DUBEY; FRENKEL, 1972). Os sinais clínicos mais comuns são febre (40-41,7ºC), dispneia, pneumonia, icterícia, uveíte, retinocoroidite, anorexia, letargia, desconforto abdominal, alterações hepáticas, pancreáticas e neurológicas (DUBEY, 2009). Na fase aguda da infecção, há invasão de tecidos extra-intestinais, sendo que fígado, linfonodos mesentéricos e pâncreas estão entre os órgãos mais afetados, e lesões severas resultam da necrose causada pela multiplicação dos taquizoítos nesses tecidos (FRENKEL, 1988; LINDSAY; BLAGBURN; DUBEY, 1997).

Dubey (2009) relata que devido ao acometimento neurológico, alterações como hipotermia, perda parcial ou total da visão, andar em círculos, torcicolo, anisocoria e convulsões podem ocorrer, porém não são frequentes. Estudos sobre infecções concomitantes por vírus da imunodeficiência felina (FIV) e vírus da leucemia felina (FeLV) apontam que estes vírus apesar de sabidamente causadores de imunossupressão, não alteram significativamente a clínica e a epidemiologia da toxoplasmose (PATTON; LEGENDRE; PELLETIER, 1991; LAPPIN et al., 1992).

Após uma infecção aguda em gatos imunocompetentes, pode ocorrer cronificação da infecção e formação de cistos teciduais, com predileção pelos músculos, diferentemente do que ocorre em camundongos (cistos no cérebro) (DUBEY, 2004; DUBEY, 2009). A toxoplasmose crônica também pode causar alterações na parede intestinal dos gatos, diminuindo a espessura da parede duodenal, bem como das células caliciformes, aumentando o diâmetro dos vilos e o número de células de Paneth, essas alterações se devem à resposta imune desencadeada pela invasão dos enterócitos (BUZONI-GATEL; WERTS, 2006; SILVA et al., 2010;), o que demonstra o papel significativo da imunidade intestinal.

O diagnóstico sorológico pode ser feito por várias técnicas: enzyme-linked immunosorbent assay (ELISA), western blotting, reação de imunofluorescência indireta e outros testes de aglutinação (LAPPIN, 1996). Geralmente, o aparecimento de anticorpos se dá uma semana após a eliminação de oocistos (DUBEY; FRENKEL, 1972). A detecção de anticorpos IgM anti-*T. gondii* não indica que o gato está eliminando oocistos, mas pode indicar toxoplasmose aguda com manifestações clínicas; 93,3% dos gatos testados sorologicamente apresentavam IgM e apenas 60% IgG (LAPPIN, 1996). Por outro lado, anticorpos IgG anti-*T. gondii* são frequentemente detectados três a quatro semanas pós infecção em gatos clinicamente saudáveis, e altos títulos podem permanecer por até seis anos (DUBEY, 1995; LAPPIN, 1996).

Isoladamente, o diagnóstico sorológico não confirma casos de toxoplasmose em felídeos, achados clínicos devem ser analisados (DUBEY, 2009), bem como exames oftalmológicos, podem ser úteis, pois em um estudo retrospectivo de 100 casos de toxoplasmose felina, iridociclocoroidite multifocal foi a lesão predominante em mais de 81% dos casos (DUBEY; CARPENTER, 1993).

A população de felinos é paralela à população humana, por exemplo, um terço dos domicílios nos Estados Unidos têm gatos (DUBEY, 2009), e a soroprevalência em gatos pode ser um indicativo da contaminação ambiental, além do mais, não devemos subestimar o risco de infecção para humanos, pois há uma correlação positiva entre a soroprevalência em gatos e humanos (GARCIA et al., 1999; DABRITZ; CONRAD, 2010).

2.3 GATOS E A ELIMINAÇÃO DE OOCISTOS DE *T. GONDII*

Os oocistos de *T. gondii* são formados apenas nos hospedeiros definitivos, felídeos domésticos ou selvagens, e podem ser eliminados após a infecção com qualquer uma das três formas (esporozoítos, taquizoítos ou bradizoítos), no entanto, a infecção por bradizoítos é mais eficaz na eliminação de oocistos, (DUBEY, 2001; FRENKEL et al., 1970). Dabritz et al. (2007) estimam que gatos naturalmente infectados eliminem um milhão de oocistos por infecção, enquanto que animais experimentalmente infectados com cistos eliminaram ≥ 20 milhões de oocistos na primo-infecção (DUBEY, 1995).

O período de eliminação de oocistos geralmente é em média, de oito dias, mas pode se estender por três semanas. Assim, o hábito dos felídeos de passar algum tempo fora da casa, enterrar suas fezes, podem favorecer a disseminação e a manutenção dos oocistos no meio ambiente (DUBEY, LINDSAY; SPEER, 1998; FIALHO, TEIXEIRA; ARAÚJO, 2009). A esporulação dos oocistos ocorre de um a cinco dias após a excreção das fezes e depende de condições ideais de aeração, temperatura e umidade, e não é um evento sincronizado, alguns oocistos podem esporular antes, outros depois (DUBEY, 2004). Oocistos esporulados tem formato sub-esférico a elíptico e medem aproximadamente 11-13 µm de diâmetro, possuem dois esporocistos com quatro esporozoítos cada, totalizando oito esporozoítos por oocisto (DUBEY, LINDSAY; SPEER, 1998).

Os oocistos esporulados são mais resistentes do que os não esporulados e possuem uma dupla parede tão forte quanto os materiais plásticos comuns, sendo resistentes à baixas e altas temperaturas ou tratamentos como: cloração, ozônio, raios ultravioleta, congelamento, e desinfetantes (JONES; DUBEY, 2010; DUMÈTRE et al., 2013). Estudos sobre oocistos de *T. gondii* demonstraram viabilidade no solo por até 18 meses (FRENKEL, RUIZ, CHINCHILLA, 1975), sob refrigeração a 4ºC por até quatro anos e meio, em água do mar por dois anos, e congelado a -10ºC por 106 dias (DUBEY, 1998; LINDSAY, DUBEY, 2009). A disseminação de oocistos no ambiente também pode ser feita por minhocas, moscas e baratas, que contaminam diretamente os alimentos (CHINCHILLA et al., 1994; HILL e DUBEY, 2002).

Além de resistentes, os oocistos são eliminados em grande quantidade, e tem alto poder infectivo para os hospedeiros intermediários, visto que um único oocisto pode causar infecção em suínos (DUBEY et al., 1997).

Diversos estudos relatam a ingestão de carne e produtos cárneos crus ou mal cozidos contendo cistos de *T. gondii* como a principal via de transmissão para seres humanos em todo o mundo (DUBEY et al., 2005; COOK et al. 2000; BONAMETTI et al., 1997; WARNEKULASURIYA; JOHNSON; HOLLIMAN, 1998). A ingestão acidental de oocistos presentes na água, nos alimentos, e no solo pode causar infecção em humanos e eventualmente surtos (BOWIE et al., 1997; MOURA et al., 2006; CARMO et al., 2010, DU et al., 2012; EKMAN et al., 2012). Um estudo apontou que uma granja de suínos que tenha a presença de gatos tem 11 vezes mais chance de ter circulação do *T. gondii* do que as que não possuem esses animais (GARCÍA-BOCANEGRA et al., 2010). Estudos epidemiológicos indicam que os gatos são essenciais na perpetuação do ciclo de vida, pois a infecção é rara ou ausente em áreas desprovidas de gatos (WALLACE, 1969; DUBEY et al., 1997).

A centrífugo-flutuação seguida de microscopia óptica é a técnica mais comumente usada para detecção de oocistos de *T. gondii* nas fezes de felinos, porém, fatores limitantes como morfologia similar entre coccídeos leva a resultados falso-positivos, bem como amostras com poucos oocistos, podem levar a resultados falso-negativos, pois o limiar de detecção se situa entre 250 a 1.000 oocistos/g de fezes (ROTHE; MCDONALD; JOHNSON, 1985; JONES; DUBEY, 2010). Uma técnica modificada de Kato-Katz com coloração de Kinyoum (KKK), demonstrou sensibilidade por maior período que a centrifugo-flutuação e pode gerar um registro permanente dos oocistos (lâminas coradas) (MEIRELES et al., 2008). O bioensaio em camundongos é a única técnica capaz de detectar oocistos viáveis, infectivos, mas é uma técnica cara e demanda recursos, tempo e infraestrutura (SALANT; SPIRA, 2010). O diagnóstico molecular, que detecta o DNA do parasita, é uma técnica altamente específica e utilizada amplamente em pesquisas científicas. Salant et al. (2007), em um estudo experimental, detectaram de um a dois oocistos em 200 μL de fezes amplificando o fragmento 529 pb na PCR. No entanto, a detecção em amostras fecais de animais naturalmente infectados e amostras ambientais (água e solo) não é tão sensível devido à dificuldade de rompimento dos oocistos e presença de inibidores da PCR (DABRITZ et al., 2007).

Na maioria das vezes os gatos têm acesso livre à rua, mesmo quando domiciliados, o número de animais errantes é alto, e muitos outros animais são abandonados. A junção desses fatores propicia o ciclo do *T. gondii,* visto que esses animais não têm local próprio para defecar e suas fezes são enterradas por eles no meio ambiente. A soroprevalência de anticorpos anti-*T.*

gondii é mais alta em animais mais velhos (SCHARES et al., 2008), no entanto, Zulpo et al. (2018) verificaram que mesmo gatos adultos com altos níveis de anticorpos anti-*T.gondii* podem re-eliminar oocistos em quantidade 30% menor que um gato jovem, especialmente quando re-infectados com um tipo diferente de cepa do protozoário, algo relevante em locais com alta diversidade clonal e recombinações genéticas (LEHMMAN et al., 2004; SHWAB et al., 2014; ZULPO et al., 2018).

2.4 IMUNIDADE

T. gondii possui uma estratégia de evasão sofisticada, modificando a apresentação de antígenos e as funções imunorregulatórias de células do hospedeiro por um tempo necessário para sua reprodução parasitária. Com a invasão celular, ocorre a formação do vacúolo parasitóforo (VP) que protege o parasita da fusão com endossomas e lisossomas do hospedeiro (SACKS; SHER, 2002). No entanto, quando o parasita invade um macrófago previamente ativado por IFN-γ , ocorre a ruptura do VP e eliminação do parasita (MORDUE; SIBLEY, 1997; GADDI; YAP, 2007). A imunidade humoral e o sistema complemento são importantes na destruição de parasitas extracelulares, no sangue, fluídos corporais e teciduais (GARCIA, 2009).

Apesar de ser uma única espécie, com base em estudos sobre a estrutura genética populacional global do parasita, pelo menos seis grupos são descritos e variações quanto à transmissão, virulência, antigenicidade, podem ocorrer entre cepas de grupos clonais. (SU et al., 2012) Por exemplo, no Brasil a maioria das infecções são brandas, no entanto, algumas cepas atípicas são altamente virulentas, causando severos casos de toxoplasmose ocular (BELFORT-NETO et al., 2007).

Os mecanismos envolvidos na proteção do hospedeiro contra a toxoplasmose são complexos e envolvem a resposta imune celular e humoral mediadas respectivamente por Th1 e Th2 (GARCIA, 2009). Macrófagos, células dendríticas e linfócitos intraepiteliais no intestino são a primeira barreira contra o parasita, que invade primeiramente células epiteliais intestinais (BUZONI-GATEL et al., 2006). A resposta imune inata utiliza receptores pré-existentes tipo *Toll* (TLR), que são expressos em células sentinela (macrófagos, mastócitos, células dendríticas, eosinófilos e células epiteliais que revestem trato respiratório e intestinal), esses receptores podem ser intra ou extracelulares e reconhecem padrões moleculares associados aos patógenos (PAMPs) (MILLER et al., 2009a). A ativação desses receptores leva à expressão de citocinas, que são mediadores na comunicação celular, especialmente envolvidas na defesa do organismo e orquestram as atividades celulares estimulando a imunidade adaptativa (MONTOYA; LIESENFELD, 2004; GAZZINELLI; DENKERS, 2007; HUNTER; SIBLEY, 2012).

IFN-γ é uma citocina pró-inflamatória e é produzida por resposta Th1, sendo o maior fator mediador de resistência adquirida ao *T. gondii,* além de ativar várias atividades antimicrobianas celulares alterando metabolismos que limitam a replicação do parasita (SUZUKI et al., 1988). No entanto, IFN-γ demonstra uma dependência de TNF-α e outros fatores, como IL-12 para exercer essa atividade antimicrobiana em macrófagos, pois ambas (IFN-γ e IL-12) atuam sinergicamente (SIBLEY et al., 1991; DENKERS; GAZZINELLI, 1998). Tanto os linfócitos Th1 CD4+ auxiliares e CD8+ citotóxicos são essenciais para uma imunidade protetora e sobrevivência do hospedeiro durante a infecção crônica, pois são fonte importante de IFN-γ (DENKERS et al., 2004).

IFN-γ exerce um papel importante tanto na fase aguda quanto crônica da infecção, induzindo cronificação da infecção aguda, bem como prevenindo a conversão de bradizoítos em taquizoítos e evitando uma re-agudização (JONES; BIENZ; ERB, 1986; BOHNE et al., 1993). Outros mecanismos dependentes de IFN-γ são a geração de intermediários reativos de oxigênio (ROI), privação de oxigênio, degradação do triptofano (aminoácido essencial ao protozoário), indução da síntese de GTPases relacionadas à imunidade (IRG) (MILLER et al., 2009).

TNF-α é secretado por leucócitos e faz a ativação de macrófagos, além de ser predominante na fase aguda, essa citocina multifuncional é importante no controle de encefalite em infecções crônicas (SCHLÜTER et al., 2003). A IL-6 é uma citocina pró-inflamatória importante para resposta imune adaptativa promovendo a diferenciação de células B e T, também pode induzir febre, atua junto à IL-1 e IL-5 para estimular a síntese de IgM e IgA, respectivamente (SIRAGE; LEAL, 2014; KANG; TANAKA; KISHIMOTO, 2015). Níveis elevados das citocinas (IL-12, TNF-α, IL-6, IL-10 e IFN-γ) são observados na fase aguda da infecção, com efeito microbicida devido à produção de óxido nítrico (NO) e intermediários reativos de oxigênio (ROI) (DENKERS; GAZZINELLI, 1998; HUNTER; SIBLEY, 2012).

A IL-10 é uma citocina regulatória marcadora de Th2, produzida por células dendríticas, macrófagos, células B e certos tipos de células T, e têm um papel inibitório sob IFN-γ, IL-12, modulando a resposta imune e prevenindo patologias relacionadas à hipersensibilidade, por seu efeito anti-inflamatório (GADDI; YAP, 2007; DA SILVA; LANGONI, 2009). Visto que o hospedeiro necessita de uma resposta imune para potencializar a eliminação do patógeno, evitando ao mesmo tempo os danos a si próprio, a IL-10 é vital na obtenção desse equilíbrio (SHAW et al., 2006). Portanto, a imunidade celular é vital para determinar o curso da infecção, até mesmo sendo avaliada para detecção precoce da infecção (YIN et al., 2015; ZULPO et al., 2018).

Anticorpos específicos contra o parasita são detectados 10 a 12 dias após a infecção inicial e o pico de produção de anticorpos coincide com o desaparecimento dos taquizoítos viáveis (FRENKEL, 1988; INNES; VERMEULEN, 2006). A resposta humoral é de longa duração, porém não protegeu gatos experimentalmente infectados contra a re-eliminação de oocistos (ZULPO et al., 2012; 2017; 2018). Em suínos e camundongos a presença de anticorpos induziu uma proteção parcial contra formação de cistos de *T. gondii* (GARCIA et al., 2005; IGARASHI et al., 2008). Portanto, isoladamente a resposta imune humoral não é suficiente para promover imunidade no hospedeiro, devido a localização intracelular do parasita (BUXTON, 1993; MILLER et al., 2009)

2.5 Vacinas em gatos

O fato dos gatos serem hospedeiros definitivos desse parasita e estarem muito próximos aos seres humanos, torna-se necessário controlar a contaminação ambiental para reduzir ou controlar a eliminação de oocistos (DUBEY, 2017). No entanto, poucos trabalhos foram desenvolvidos com o objetivo de controlar a eliminação de oocistos em gatos, devido ao fato de ser um modelo experimental mais caro, trabalhoso, mais propenso à efeitos vacinais adversos como sarcomas (ELSTON et al., 1998). Além de serem *pets* e ter um apelo emocional.

Os primeiros a imunizarem felinos com este objetivo foram Frenkel et al. (1991) e Freyre et al. (1993). Estes autores, juntamente com Mateus-Pinilla et al. (1999), utilizaram a cepa T-263, uma cepa mutante com ciclo enteroepitelial incompleto em gatos, conferindo uma imunidade estéril de até 100% de proteção contra a eliminação de oocistos (FREYRE et al.,1993). Apesar da proteção induzida por esta vacina, a produção comercial foi descartada devido a dificuldades na conservação, alto custo de produção, curta vida de prateleira (precisa ser mantida congelada em nitrogênio líquido e administrada em no máximo 15 minutos após descongelada para manter sua eficiência), além do risco de infecção acidental para manipuladores e outros animais (DUBEY, 1995; MATEUS-PINILLA et al., 1999; DUBEY, 2017).

Outras formas de imunização, utilizando desde taquizoítos irradiados com cobalto 60 até o uso de vacinas de DNA, foram testadas para avaliar a proteção contra a eliminação de oocistos do *T. gondii* pelas fezes de gatos, porém, os autores não observaram redução significativa (OMATA et al., 1996; MISHIMA et al., 2002). As vacinas de subunidade (proteínas purificadas ou recombinantes) são seguras, no entanto, muitas vezes não são tão imunogênicas quanto as vacinas vivas atenuadas e para promover um estímulo imunológico satisfatório a associação com diversos adjuvantes tem sido relatada (BOWERSOCK; MARTIN, 1999).

As roptrias são organelas secretoras localizadas no complexo apical, com função de penetração celular e formação do VP, e estão presentes em igual número em taquizoítos, bradizoítos e esporozoítos (BECKERS et al., 1994; DUBEY; LINDSAY; SPEER, 1998). A ROP2 tem sido apontada como um importante imunógeno, indutor de altos níveis de IFN- γ em humanos (SAAVEDRA et al., 1991). Tem sido utilizada experimentalmente em vacinas contra *T. gondii* em algumas espécies animais, tais como: suínos, camundongos e gatos (VERCAMMEN et al., 2000; GARCIA et al., 2005; GARCIA et al., 2007; IGARASHI et al., 2008; CUNHA et al, 2012;). Garcia et al. (2007) e Zulpo et al. (2012) utilizaram a ROP2 em gatos e obtiveram respectivamente uma redução de 89% e 98% na eliminação de oocistos.

As proteínas de choque térmico ou “proteínas de estresse” (HSPs) (RITOSSA, 1962), apesar do nome, são expressas sob condições normais tanto em células procariontes e eucariontes, dentre elas a família mais conservada é a HSP70 (BOOTHROYD, 2009; HUNT; MORIMOTO, 1985). Zhang et al. (2012), imunizando frangos de corte com HSP70 obtiveram um efeito protetor contra infecção por *Eimeira tenella*, com indução significativa da resposta imune celular, assim é uma candidata à vacina contra parasitas intracelulares.

Adjuvantes podem atuar de diversas maneiras visando melhorar a imunogenicidade das vacinas por manter alta concentração do imunógeno e por direcionar a resposta imune (BOWERSOCK; MARTIN, 1999). A maioria dos adjuvantes são moléculas derivadas de fatores imunes inatos do próprio parasita, tais como TgHSP70, profilina, lipídio monofosforil A e são propostos como potentes adjuvantes endógenos contra *T. gondii* (SCHAAP et al., 2007).

A capacidade de imunomodulação dos adjuvantes é relacionada à estimulação de algumas citocinas e inibição de outras (COX; COULTER, 1997), portanto, a escolha do adjuvante levando em conta o efeito desejado é importante.

As saponinas são complexos derivados vegetais utilizadas como adjuvantes em vacinas na medicina veterinária, o Quil-A se destaca por ser barato, seguro, de fácil formulação e fortes indutores de resposta Th1 e Th2 (COX; COULTER, 1997). Quil-A foi utilizado incorporado ao complexo imunoestimulante (ISCOM) e também isoladamente como adjuvante em vacinas utilizando proteínas de roptrias contra a formação de cistos teciduais de *T. gondii* em suínos, respectivamente, por Garcia et al. (2005) e Cunha et al. (2012) e ambos obtiveram bons resultados. Utilizando Quil-A como adjuvante em uma vacina intranasal em gatos, Zulpo et al. (2012) obtiveram estimulação da resposta imune celular, com proliferação de linfócitos e uma redução significativa na eliminação de oocistos de *T. gondii*. Utilizando o mesmo adjuvante em suínos, houve uma potente estimulação na expressão de genes regulados por IFN, demonstrando assim que o Quil-A auxilia a resposta imune inata (CHARERNTANTANAKUL; FABROS, 2018).

2.6 Considerações Finais

Visto que os felinos são de extrema importância na cadeia epidemiológica da toxoplasmose, estudos sobre a modulação da resposta imune nesses animais são vitais para o desenvolvimento de uma vacina que bloqueie ou reduza a eliminação de oocistos. Ainda mais, são necessárias técnicas de alta sensibilidade e especificidade para detecção e quantificação de oocistos nas fezes desses animais, estimando assim a potencial contaminação ambiental. Estudar a expressão de citocinas em diferentes momentos da infecção pode ajudar a elucidar pontos importantes, até mesmo possibilitando a identificação de animais infectados antes que possam eliminar oocistos.

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4 OBJETIVOS

4.1 Objetivo Geral

Avaliar se as proteínas recombinantes ROP2 (*T. gondii*) e HSP70 (*E. tenella*), utilizados como imunógeno em gatos desafiados com *T. gondii*, interfere na expressão de citocinas e eliminação de oocistos.

4.2 Objetivos Específicos

Analisar a expressão de citocinas IL-6, IL-10, IFN-γ, e TNF-α nos gatos imunizados durante diferentes períodos pós-infecção.

Avaliar a eliminação de oocistos e comparar pelas técnicas de Sheather (centrifugo-flutuação e microscopia óptica) e qPCR.

5. ARTIGO A

Cytokines gene expression in cats immunized with recombinant *Toxoplasma gondii* proteins

Expressão gênica de citocinas em gatos imunizados com proteína recombinante de *Toxoplasma gondii*

ABSTRACT

The immune mechanisms of the host against *T. gondii* are complex and the cellular response Th1 and Th2 are important in inducing the expression of proinflammatory and regulatory cytokines, which orchestrate the cellular activities involved in the defense of the organism stimulating the adaptive immunity. This study evaluated the expression of cytokines (IL-6, IL-10, TNF-α, IFN-γ) by the qPCR technique in cats immunized with recombinant proteins. Twelve cats with 4 animals in each group were used. G1: was immunized with rROP2 plus rHSP70 and Quil-A; G2: Quil-A (adjuvant); G3 control group. All immunizing doses were administered on days 0, 21 and 42 of the experiment via the nasal route. The challenge was done on day 72, with 600 cysts of the strain TgDoveBr8 (type II), via stomach catheter. On the 7th day pos-infection two cats from each group were submitted to duodenum endoscopy and biopsy for histopathological evaluation. Blood samples were collected from all cats on days 0, 7, 14, 21, 35 after infection. Relative gene expression was performed by qPCR using GAPDH as endogenous control. The histopathological analysis of the duodenal biopsies revealed moderate, diffused lymphohistioplasmocitary enteritis in all the animals, some lesion differentials were found between the groups. As for clinical signs, mild diarrhea was observed intermittently. The relative gene expression of TNF-α ($p < 0.0001$) and IL-10 ($p = 0.0128$) demonstrated a statistically significant difference in the same group during different periods of infection, however no differences were observed in relation to IL-6 cytokines ($p = 0.3746$) and IFN- (0.7711). On the 35th day after the challenge, the immunized animals (G1) had the highest relative expression of all cytokines IL-6 (2.85), TNF-α (0.64), IFN-γ (1.64) and IL-10 (1.42). We conclude that there was stimulation of Th1 and Th2 cell responses after the acute phase in cats immunized with Quil-A-associated recombinant proteins.

Key words: cytokine profile, histopathology, real-time PCR, toxoplasmosis.

RESUMO

Os mecanismos de imunidade do hospedeiro contra *T. gondii* são complexos e a resposta celular Th1 e Th2 são importantes para induzir a expressão de citocinas pró-inflamatórias e regulatórias, que orquestram as atividades celulares envolvidas na defesa do organismo estimulando a imunidade adaptativa. Este estudo teve como objetivo avaliar a expressão de citocinas (IL-6, IL-10, TNF-α, IFN-γ) pela técnica qPCR em gatos imunizados com proteínas recombinantes (rROP2 de *T. gondii* e rHSP70 de *Eimeria tenella*). Foram utilizados 12 gatos, com quatro animais em cada grupo. G1: foi imunizado com rROP2, rHSP70 mais Quil-A; G2: adjuvante Quil-A; G3 grupo controle. O desafio foi feito no dia zero, com 600 cistos da cepa TgDoveBr8 (tipo II), via sonda estomacal. No 7º dia pós desafio, seis animais, dois gatos de cada grupo foram submetidos à endoscopia e biopsia de duodeno para exame histopatológico. Amostras de sangue foram coletadas de cada animal nos dias 7, 14, 21, 35 do experimento. A expressão gênica relativa foi realizada por qPCR utilizando GAPDH como controle endógeno. A análise histopatológica das biopsias duodenais revelou enterite linfohistioplasmocitária moderada e difusa em todos os animais, alguns diferenciais de lesões foram encontrados entre os grupos. Quanto aos sinais clínicos foi observada diarreia leve, de forma intermitente. A expressão gênica relativa de TNF-α ($p < 0.0001$) e IL-10 ($p = 0.0128$) demonstrou diferença estatística significativa em um mesmo grupo durante diferentes períodos de infecção, no entanto não foram observadas diferenças em relação às citocinas IL-6 ($p = 0.3746$) e IFN-γ (0.7711). No 35º dia pós desafio, os animais imunizados (G1) apresentaram a maior expressão relativa de todas as citocinas IL-6 (2.85), TNF-α (0.64), IFN-γ (1.64) e IL-10 (1.42). Conclui-se que houve estimulação das respostas das células Th1 e Th2 após a fase aguda nos gatos imunizados com proteínas recombinantes associadas ao Quil-A.

Palavras-chave: perfil de citocinas, histopatologia, *real-time* PCR, toxoplasmose.

Introduction

Toxoplasma gondii is an important zoonotic agent and cats are definitive hosts, playing an important role in the toxoplasmosis' spread (FRENKEL et al., 1991). Although in healthy individuals infection is generally subclinical, in immunocompromised or during a primary infection in pregnancy, it can be severe and lead to miscarriage in humans and animals, among other pathologies related to toxoplasmosis, congenital and ocular are the most researched (JONES; PARISE; FIORE, 2014; SZABO; FINNEY, 2017). Pathogenesis of nfection depends on many factors including *T. gondii* genotype, infectious dose, parasite stage, and immunity status host, age, nutritional condition, pregnancy and concomitant infections (SILVA et al., 2017).

Host's immunity mechanisms against *T. gondii* are complex and involve both, cellular and humoral immune responses (GARCIA, 2009). The innate immune response utilizes pre-existing *Toll* receptors (TLRs) that are expressed in sentinel cells (macrophages, mast cells, dendritic cells, eosinophils, and epithelial cells lining the respiratory and intestinal tract). These receptors may be intra or extracellular and are able to recognize molecular patterns associated with pathogens (PAMPs)(MILLER et al., 2009). Activation of these receptors leads to the expression of pro-inflammatory (IL-6, IFN-γ, TNF-α) and regulatory (IL-10) cytokines, which orchestrate the cellular activities involved in the defense of the organism and stimulate adaptive immunity (MONTOYA; LIESENFELD, 2004; HUNTER; SIBLEY, 2012; GAZZINELLI; DENKERS, 2017).

Protozoan proteins ROP2 and HSP70 have been used in vaccines due to their potential for increasing the immunogenicity against intracellular pathogens in several animal species, such as pigs, mice, chickens and cats (CUNHA et al., 2012; GARCIA et al., 2007; IGARASHI et al., 2008; ZHANG et al., 2012; ZULPO et al., 2012). Zhang et al. (2012) obtained a strong stimulation of cellular immune response by poultry immunization with *Eimeria tenella* HSP70.

The association between protozoan antigens and adjuvants such as, Quil-A could result in enhanced immunogens being widely applied in veterinary area (BOWERSOCK; MARTIN, 1999). Saponin Quil-A stands out as a cheap, safe, easy to formulate adjuvant and strong Th1 and Th2 response inducers (COX; COULTER, 1997).

Cytokine detection is difficult due to low concentration in blood and plasma, however, a gene expression assay using probes require less than 1 ng of the total RNA per cytokine determination (LEUTENEGGER et al., 1999). The role of cytokine expression is well established in immunosuppressive retroviral diseases of humans and cats (MAHER et al., 2014), and it can help to elucidate some aspects of toxoplasmosis in cats, contributing to develop a feasible and efficient vaccine. IL-6, IL-10, IFN-γ, TNF-α, were chosen by us for acting as important inflammatory mediators against intracellular parasites as described above by the fact that they are Th1 and Th2 markers.

The object of this study was to evaluate gene expression of proinflammatory cytokines IL-6, IFN-γ, TNF-α and IL-10 anti-inflammatory cytokine in cats immunized with recombinant proteins rROP2 (*T. gondii*) and rHSP70 (*E. tenella*).

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Material and methods

Ethics Committee

Animal housing and experimentation were performed according to guidelines established by the Institutional Ethics Committee in Animal use of Londrina State University, Brazil. The protocol of this study (CEUA, number 102/12).

***Toxoplasma gondii* strains**

Two strains of *T. gondii* were used in this experiment: RH (type I) isolated by Sabin (1941) was used for DNA extraction and *rop*2 gene amplification and TgDoveBr8 (type II) isolated by Barros et al., (2014) was used for tissue cysts production. 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 brain cyst burden was counted and prepared for challenge (approximated 600 cysts to each cat in a total of 2 mL of saline) as previously described (Zulpo et al., 2012).

Recombinat proteins

rROP2– Expression and purification

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

E. coli 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 (DO600) 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.

The soluble fraction was applied directly onto Ni-NTA Superflow resin (Qiagen, QIAGEN Biotecnologia Brasil Ltda., São Paulo, Brazil) 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. The protein concentration was determined using Pierce® BCA Protein Assay Kit (Thermo Scientific®, San Jose, CA, USA).

rHSP70– Expression and purification

Eimeira tenella HSP70 gene cloning was perfomed previously by Bogado et al., (2017) and the rHSP70 protein was obtained as described by the same authors.

Clinical evaluation of cats

In this experiment, we used twelve (8 male and 4 female) short hair domestic cats (*Felis catus*), between 6 and 9 months of age, examined, vaccinated against feline viral diseases (Rhinotracheitis, Calici and Panleukopenia viruses) (Feligen®, Virbac, Brazil). The cats were also submitted to vermifugation (Vetmax plus, Vetnil, Brazil). All cats were clinically healthy and had no detectable specific IgG anti-*T. gondii* titres by IFAT (Camargo, 1974) and absence of *T. gondii* oocysts was confirmed by fecal examination (Dubey, 1995).

Cats were randomly allocated in individual cages and all cats received only commercial dry food and water *ad libitum*. Clinically evaluated continued to other diseases for two months prior to the beginning of the experiment. The clinical observations were conducted daily, as well as cleaning, feeding, welfare and rectal temperature.

Cats Immunization

The cats were divided into three groups, each group containing four animals (Table 1). G1 animals received 25 µg of rROP2 plus 25 µg of rHSP70 and 20 µg of Quil-A. G2 received 25 µg of *E. coli* and 20 µg of Quil-A; G3 (control) received only saline solution. The animals were immunized by administrating (140 µl of final solution for each cat 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 and 42 of the experiment.

Experimental inoculation

The animals were anesthetized with tiletamine plus zolazepam (Zoletil®, Virbac – Brazil, 3.15 mg/kg/IM) before the challenge. All cats from G1, G2 and G3 were challenged on day 72 with 600 cysts of the TgDoveBr8 strain (contained in a volume of 2 mL) administered via stomach tube, after which they were injected with 5 mL of saline, to avoid remaining residuals.

Table 1. Experimental design of cats immunized with rROP2 plus rHSP70 (G1), adjuvant (G2) and negative control (G3).

Experimental groups	Immunization route	Immunization protocols (0, 21, 42 days)[1]	Challenge[2] (Day 72)
G1	Intranasal	25 µg rROP2 + 25 µg rHSP70 + QuilA (20 µg)	600 cysts
G2	Intranasal	QuilA adjuvant (20 µg)	600 cysts
G3	Intranasal	Saline Solution	600 cysts

[1] Period during which procedure was performed. [2]TgDoveBr8 (BARROS et al., 2014).

Duodenal endoscopy and histopathology

Duodenal biopsies were collected from six cats (two animals/group) on day 7 (pos-infection), general anesthesia was performed with 5mg/kg ketamine plus 4 mg/kg diazepam and maintenance with 5mg/kg Propofol. During anesthetic procedures, the animals were observed for the physiological parameters (rectal temperature, respiration and cardiac rate) and duodenal endoscopy was performed to take samples from the duodenum using flexible tubes 1m and 11 mm diameter gastroscope and a 1.50 m and 13 mm diameter colonoscope specific for cats (Video Processor EPK-100, Pentax Medical).

A duplicate set of biopsies was kept in 10% neutral buffered formalin for 24h, transferred subsequently to a 70% ethyl alcohol solution and embedded in paraffin. Paraffin-embedded tissues were serially sectioned at 4 µm thick and stained with hematoxylin-eosin (HE) for histopathological evaluation.

Sample collection, leukocyte's separation and RNA preservation

Blood samples (2–4ml) were collected from cat's jugular vein using syringe and needle on days 0, 7, 14, 21 and 35 of the experiment to evaluate cytokines expression. It was placed immediately into sodium heparin vials (Vacutainer; Becton Dickinson and Co, NJ, USA) and then transported at room temperature for processing until 3 h after collection. Leukocytes were obtained as described by Boyum (1964) and kept at -20°C in RNA later® stabilization solution (Ambion, Thermo Fisher, USA) until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA from leukocytes was extracted using a commercial kit (PureLink™ RNA Mini kit, Thermo Fisher®, USA) according to manufacturer's protocol. RNA elution was performed in 50µL of ultra-pure water and samples were immediately submitted to cDNA transcription or kept in -80ºC until transcription. cDNA was synthesized using the commercial High-capacity cDNA Reverse Transcription Kit (Life Technologies®, USA) following all manufacturer's recommendations. The reverse transcription reaction consisted of 10 min at 25ºC, 120 min at 37ºC, 5 min at 85ºC and the sample was held at 4ºC. The cDNA quality and quantity were checked by UV spectrophotometer (Evolution 60S, Thermo Fischer Scientific, USA) at 260/280 nm and maintained -20ºC until use.

Cytokines Expression by qPCR

Gene expression assays were performed using the Taqman® Applied Biosystems system according to the manufacturer's instructions. All qPCR assays were performed in duplicate using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as endogenous control, as well as a negative control and probe concentrations of 100 nM. The specific sequences for feline cytokines were based in a prior study (VAN et al., 2006) and Genbank accession numbers were:

IL-6: D13227; IL-10: AF060520; IL-12 p40: U83184, IFN-γ: X86972; TNF-α: M92061; GAPDH: AF054608. The primer, probe sequences, and product lengths are shown in Table 2.

The reaction mixture contained 1 µL of cDNA, 5 µL of TaqMan® Universal Master Mix II with UNG (Applied Biosystems, CA, USA) 0.5 µL primer / probe, plus 1.5 µL GAPDH mix (FW - 0.7 µL, RV - 0.7 µL; 0.1 µl probe), 0.4 µL of BSA; H2O MilliQ 1.10 µL resulting in a final volume of 10 µL per reaction. Cycling conditions (ABI 7500 StepOnePlus™ Real-Time PCR system Applied Biosystems, Foster City, CA, USA) were 95ºC for 10 min (activation) followed by 45 cycles of 95ºC for 15s (denaturation), 58ºC (60ºC for TNF-α) for 60s (annealing), and 72ºC for 60s (extension). All reactions were submitted to the same analysis conditions and normalized by ROX dye passive reference.

Cytokines relative quantitation (RQ) was calculated by $2^{-\Delta\Delta Ct}$ method as described (SCHMITTGEN; LIVAK, 2008) using StepOne™ Software v.2.2. 2 (Applied Biosystems, USA). This method expresses the fold change that a given gene is more or less expressed in the infected group relative to the control group.

Table 2. Sequence of qPCR Primers and TaqMan Probes used in this study.

Cytokine	Primer 5'-3'	Probe 5'-3'	Fluorophore/Quencher	Amplicon size (pb)
IL-6	Fw-CTCCACAAGCG CCTTC Rv-TGCAGAGGTGA GTGGTAGTC	CCCTGGGAGGAG ATGCCACCTCAA	FAM/ QSY	127
IL-10	Fw- ACTTTAAGGGTTA CCTGGGTTG Rv- CGTGCTGTTTG ATGTCTGG	TTGGAGGAGGTG ATGCCCCA	FAM/ QSY	108
IFN-ɣ	Fw-TGCAAGTAATCCA GATGTAGCAG Rv-GTTTTATCACTC TCCTCTTTCCAG	CAAAATGTCTACG AAAAGCGACCCACC *	FAM/ QSY	81
TNF-α	Fw-CACATGGCCTGCT GCAACTAATC Rv-AGCTTCGGGGTT TGCTACTAC	TCTCGAACTCCGAG TGACAAGCCA	FAM/ QSY	104
GAPDH	Fw-GCTGCCCAGAAC ATCATCC Rv-GTCAGATCCACG ACGGACAC	TCACTGGCATGGC CTTCCGT	VIC/QSY	134

* Antisense probe

Statistical analysis

Data were log-2 transformed and comparison of cytokines IL-6, and IFN-γ gene expression between the three groups (G1, G2, G3) was performed by Kruskal-Wallis test, except for IL-10 and TNF-α for these cytokines was used ANOVAs to compare the effect of factors and interactions. All analyses were made in GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA), and were considered significant if the p value ≤ 0.05.

Results

After the infection, animals from all groups presented mild diarrhea, intermittently from the 4th to 12th day after infection, and no clinical signs were observed.

The relative gene expression results of the analyzed cytokines are shown in figure 1. All animals were challenged on day 72, and on day 79 (7 dpi) the only cytokine that showed positive expression was TNF-α in the G1 (vaccinated) and G3 (control), for IL-6 was not detected, all groups had negative expression of IFN-γ and IL-10 on the same date. At day 86, the G1 animals had negative regulation of IFN-γ (-1.99) and positive regulation of IL-10 (0.74). On day 93 (21 dpi), G1 animals presented 3.2-fold higher expression of IL-10 and G2 cats presented lower expression of TNF-α (-0.73) and IFN-γ (-1.04) while G3 animals presented regulation negative IL-6 (-0.57). On the 35th day after the challenge, the immunized animals (G1) had the highest relative expression of all cytokines IL-6 (2.85), TNF-α (0.64), IFN-γ (1.64) and IL-10 (1.42). The relative gene expression of TNF-α ($p < 0.0001$) and IL-10 ($p = 0.0128$) demonstrated a statistically significant difference in the same group during different periods of infection, however, no differences were observed in relation to IL-6 cytokines ($p = 0.3746$) and IFN-γ (0.7711).

Histopathological analysis of the biopsies of the six cats submitted to duodenal endoscopy revealed moderate, diffused lympho-histio-plasmocitary enteritis in all animals. Some lesion differentials are shown in figure 2. In a G1 animal, a focus of villous necrosis was observed. In G2, one animal had mild necrosis, and the other presented mild edema and vascular congestion. In G3 one animal also presented focal necrosis of crypt and in another cat it was observed a marked vascular congestion.

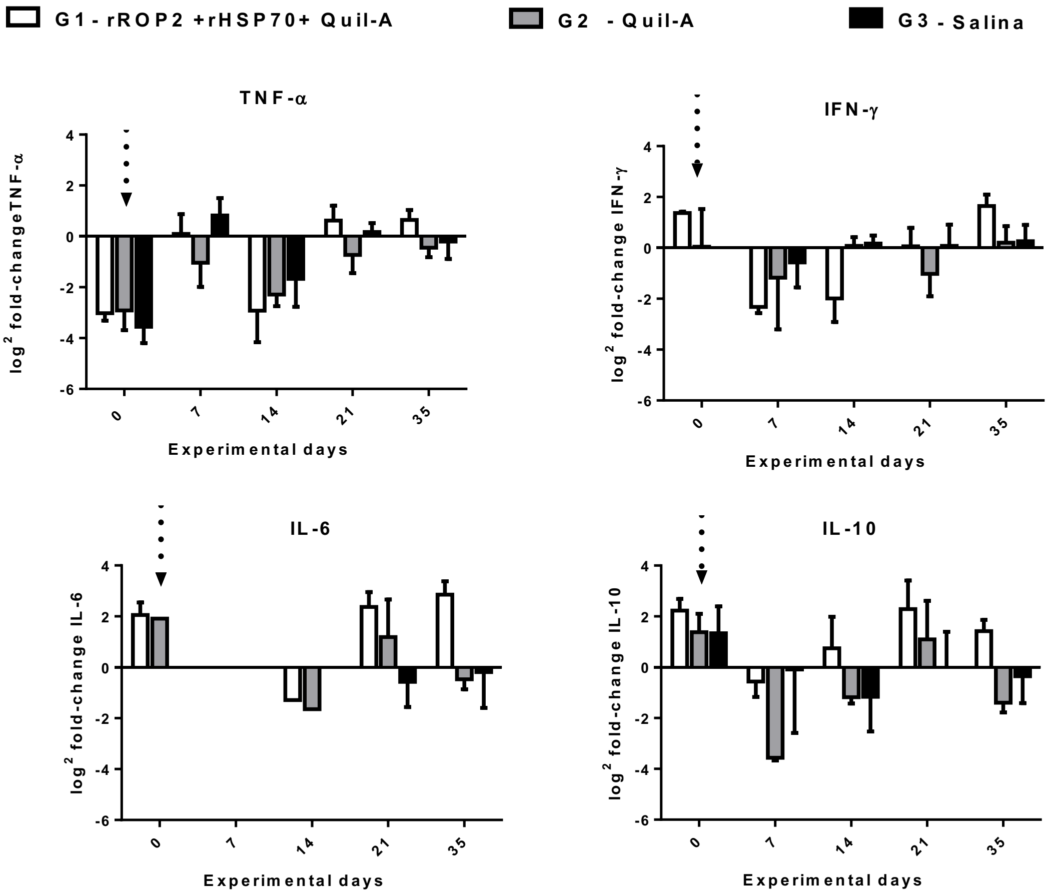

Figure 1. Cytokines profile in cats immunized with recombinant proteins rROP2 (*T. gondii*) and rHSP70 (*E. tenella*) during different periods after infection. The animals were challenged (arrow) on day 72 with 600 tissue cysts of the TgDoveBr8 strain of *T. gondii.* The values are on the log 2 base scale indicate how many times (fold change) the gene for each cytokine is more expressed (positive values) or less expressed (negative values) in relation to the animals in the control group. The data normalization factor used was the signal of the GAPDH gene endogenous control, according to $2^{-\Delta\Delta Ct}$ method.

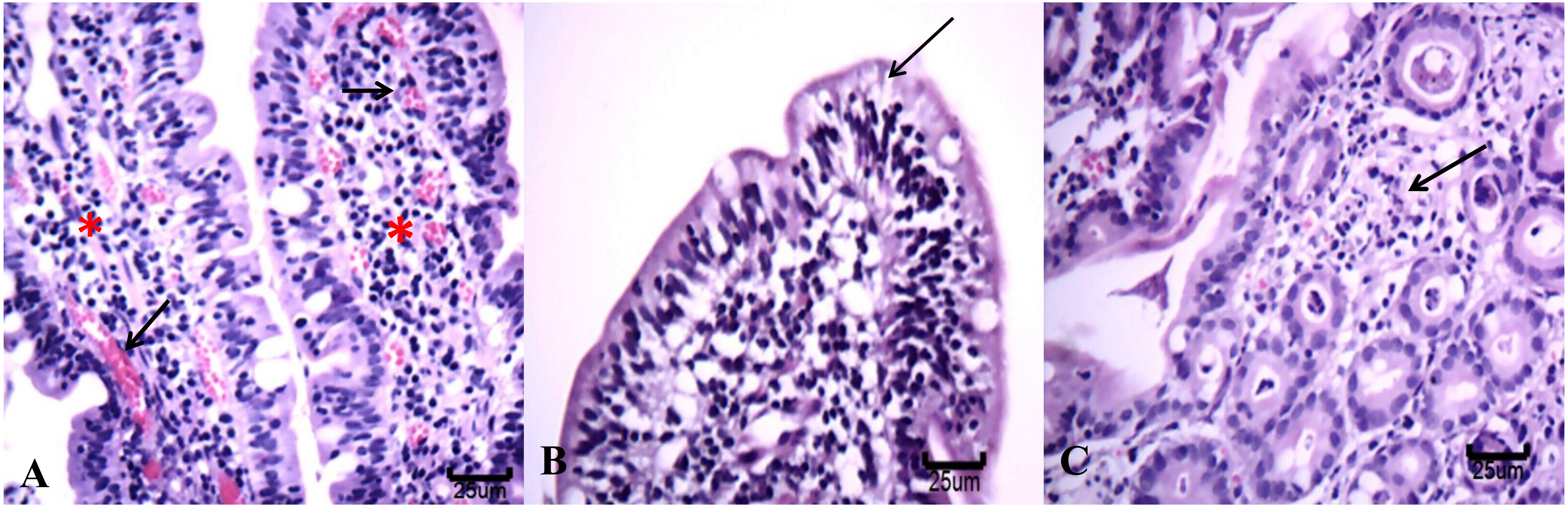

Figure 2. Experimentally infected cats' duodenum with *Toxoplasma gondii*. Biopsies were collected on day 79 (7 d.p.i) from two animals from each group**.**

A- Cat from G1 with moderate diffuse lymphoplasmacytic inflammatory process by lamina propria (*) and vascular congestion (arrows), 25µm bar, HE. **B-** Cat from G2 with cytoplasmic vacuolar degeneration in apical enterocytes (arrow). 25µm bar, HE. **C-** Cat from G3 with focal necrosis of crypt (arrow), 25µm bar. HE.

Discussion

The intranasal route was chosen because it induces a high number of CD8 (+) T cells, high levels of proinflammatory cytokines such as IL-2 and IFN-γ, promotes local and systemic immunity and requires fewer antigenic particles than oral route, in addition, this route increase the survival rate and a lower rate of brain cyst formation in mice (VELGE-ROUSSEL et al. 2000; WANG et al., 2017). The endogenous GAPDH gene was chosen as normalizer because it has constant expression in blood and feline tissues (KESSLER et al., 2009).

T. gondii is an intracellular protozoan highly adapted to different host species and has the ability to interfere with the macrophage activation pathway making them unable to produce IL-12 and consequently other key proinflammatory cytokines in the development of acquired pathogen resistance (SACKS; SHER, 2002). In the acute period of infection (7 d.p.i), this negative regulation was observed in G2 animals for TNF-α, and there was also negative regulation of IFN-γ, and IL-10 in all groups.

IL-10 expression was measured at four different periods after *T. gondii* infection, in three of these periods, immunized (G1) animals showed greater IL-10 expression than the other animals. IL-10 is a regulatory cytokine produced by dendritic cells, macrophages, B cells and some types of T cells and is Th2 response marker that is critical to avoid damage due to uncontrolled inflammatory response (GADDI; YAP, 2007; DA SILVA; LANGONI, 2009; MILLER et al., 2009). The increase in IL-10 expression during the course of infection is due to the accumulation of IL-10 produced by regulatory B cells and leads to the formation of tissue cysts and is in agreement with another recent study (JEONG et al., 2016).

The vaccine was formulated based on rROP2, rHSP70 and Quil-A, as they are capable of enhancing Th1 and Th2 cellular immune response. In addition, rHSP70 is a trigger for IL-6 and IFN-γ, which may stimulate differentiation of bradyzoites (WEISS et al., 1995). Accordingly, IL-6 and IFN-γ showed increased expression at the end of experimental days (93), which may be related to chronic infection.

Further studies should be performed to evaluate the low regulation of the proinflammatory cytokines (IL-6, IFN-γ, TNF-α) observed at the acute of the infection, however, the stimulation of these cytokines in acute infection is important to control the (NO) and reactive oxygen species (ROS) (MILLER et al., 2009). IFN-γ is the main mediating factor of acquired resistance to *T. gondii* (SUZUKI et al., 1988) and demonstrates a dependence of TNF-α, both act in synergy (DENKERS; GAZZINELLI, 1998; SIBLEY et al., 1991). A recent vaccine study in mice showed an efficient Th1, Th2 and Th17 response against *T. gondii* reduced the number of tissue cysts and promoted enhanced protection, attesting to the importance of a vaccine capable of stimulating this type of response (WANG et al., 2017). Our study was the first to use qPCR to evaluate cytokines in cats immunized and challenged against *T. gondii.*

The small intestine is an important organ in the life cycle of *T. gondii* as well as being the host's first barrier against the parasite (DUBEY; FRENKEL, 1972). Despite the importance of intestinal wall integrity in mucosal immunity only one study analyzed the effect of *T. gondii* infection on the intestinal wall of living cats by biopsy, however in association with inflammatory intestinal disease (PETERSON et al., 1991), histopathological analysis is usually performed *post mortem* (DUBEY; FRENKEL, 1972; FERGUSON et al., 1974; FERGUSON, et al., 1991; SILVA et al., 2010). The histopathological lesions in the duodenum of experimentally infected cats were classified as characteristics of moderate to severe enteritis with mononuclear infiltrate, without wall and villous atrophy, probably because it is a recent infection, as Silva et al. (2010) in a challenge with 200 cysts of a type II strain observed severe lesions, with atrophy of the intestinal wall of the duodenum, increase of intraepithelial lymphocytes and increase in the number of Paneth cells.

The lesions observed in the present study were similar in all animals, with small variations, the non-immunized G3 presented focal necrosis of crypt, to verify if there was intestinal protection in the immunized animals it would be interesting to repeat the analysis after the chronification of the infection, since in the present study a larger number of cysts were used in the challenge (600 cysts).

Conclusion

In conclusion, stimulation of the Th1 and Th2 response by the upregulation of cytokines (IL-6, TNF-α, IFN- and IL-10) after the acute phase of infection was observed in cats immunized with Quil-A-associated recombinant proteins and experimentally infected with *T. gondii.*

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6. ARTIGO B

Oocysts quantification by qPCR in cats immunized with *Toxoplasma gondii* recombinant protein.

Quantificação de oocistos por qPCR em gatos imunizados com proteína recombinante de *T. gondii.*

ABSTRACT

Felines, including the domestic cat, are definitive hosts of *Toxoplasma gondii* and are able to shed oocysts in their feces, contaminating the environment. Oocysts are largely shed and sporulation become them more resistant, besides that, it has high infective power for intermediate hosts, a single oocyst can cause infection in pigs, even outbreaks were caused by oocysts contaminated water and food. However, few studies have been developed aiminig to control oocysts shedding by cats, due to the fact that it is a more expensive and laborious experimental model. This study aimed to quantify oocysts by the qPCR technique in cats immunized with recombinant proteins (rROP2 from *T. gondii* and rHSP70 from *E. tenella*). Twelve cats were used divided into three groups with four animals each. G1: was immunized with 25 µg rROP2 (*T. gondii*), 25 µg rHSP70 (*E. tenella*) plus 20 µg Quil-A; G2: received 25 µg *E. coli* plus 20 µg Quil-A; G3: only received saline solution; group control. All immunizing doses were administered on days zero, 21 and 42 of the experiment via the nasal route and the faeces of all the animals were examined for nine days after challenge by Sheather (centrifugal-flotation technique with microscopic examination) and by qPCR. After feces purification, DNA extraction was performed by phenol-chloroform method. All cats shed oocysts in the feces. Five negative cats in the Sheather technique at (3 rd d.p.i) were qPCR positive (328; 450; 470; 509 and 1828 OOPG). Detection of oocysts was only possible in 4th d.p.i by the Sheather technique, however there was no statistical difference between qPCR and Sheather ($p = 0.1116$). There was a statistically significant difference compared to the other groups in both tests Sheather ($p = 0.6534$) and qPCR ($p = 9670$). qPCR can be used as an alternative to Sheather for the detection and quantification of *T. gondii* oocysts.

Key words: Vaccine, Real-time PCR, toxoplasmosis, Sheather, feline.

RESUMO

Felinos, incluindo o gato doméstico, são hospedeiros definitivos de *Toxoplasma gondii* e são capazes de eliminar oocistos nas fezes, contaminando o meio ambiente. Os oocistos são eliminados em alta quantidade e a esporulação os torna mais resistentes, além disso, possuem alto poder infectivo para hospedeiros intermediários, um único oocisto pode causar infecção em suínos, até mesmo surtos foram causados por água e alimentos contaminados com oocistos. No entanto, poucos estudos foram desenvolvidos para controlar a eliminação de oocistos de *T. gondii* por gatos, devido ao fato de ser um modelo experimental mais caro, laborioso, e por se tratar de uma espécie "*pet*". Este estudo teve como objetivo quantificar os oocistos pela ténica de qPCR em gatos imunizados com proteínas recombinantes (rROP2 de *T. gondii.* e rHSP70de *E. tenella*). Doze gatos foram utilizados divididos em três grupos com quatro animais cada. G1: foi imunizado com 25 µg de rROP2 (*T. gondii*), 25 µg de RHSP70 (*E. tenella*) mais 20 µg de Quil-A; G2: recebeu 25 µg de *E. coli* mais 20 µg de Quil-A; G3: apenas recebeu solução salina; controle de grupo. Todas as doses imunizantes foram administradas nos dias zero, 21 e 42 do experimento através da via nasal e as fezes de todos os animais foram examinadas durante nove dias após o desafio por Sheather (técnica de centrífugo-flutuação com exame microscópico) e por qPCR. Após purificação das fezes, a extração de DNA foi feita pelo método do fenol clorofórmio. Todos os gatos eliminaram oocistos nas fezes. Cinco gatos negativos na técnica de Sheather no (3º d.p.i) foram positivos para qPCR (328; 450; 470; 509 e 1828 OOPG). A detecção de oocistos só foi possível no 4º d.p.i pela Técnica de Sheather, contudo não houve diferença estatística entre qPCR e Sheather (p = 0,1116). Houve diferença numérica quanto à média de oocistos eliminados por cada grupo, G2 (adjuvante) eliminou em média menos oocistos, mas não houve diferença estatística comparado aos outros grupos em ambos os testes Sheather (p=0.6534) e qPCR (p=9670). qPCR pode ser usada como uma alternativa para o Sheather para a detecção e quantificação de oocistos de *T. gondii.*

Palavras-chave:. Vacina, *Real-time* PCR, toxoplasmose, Sheather, Felinos.

Introduction

Felines, including the domestic cat, are definitive hosts of *Toxoplasma gondii* and are able to shed oocysts in their feces, contaminating the environment (FRENKEL; DUBEY; MILLER, 1970; DABRITZ et al., 2007). A single bradyzote can lead a cat shed millions of oocysts, which in ideal conditions (temperature, humidity, aeration, pression), become infectious in one to five days, in a process called sporulation, that is not a synchronized event and some oocysts can sporulate before while others can take more time (DUBEY, 2001, DUBEY et al., 2004). Sporulated oocysts are more resistant than non-sporulated oocysts, and have a double wall as strong as ordinary plastics, resistant to low and high temperatures or treatments such as chlorination, ozone, ultraviolet rays, freezing, disinfectants (JONES; DUBEY, 2010; DUMÈTRE et al., 2013).

Studies on *T. gondii* oocysts have demonstrated viability in the soil for up to 18 months (FRENKEL; RUIZ; CHINCHILLA, 1975), under refrigeration at 4ºC for up to 4.5 years, in sea water for 2 years, and frozen at -10ºC for 106 days (DUBEY, 1998; LINDSAY; DUBEY, 2009). The presence of oocysts in the environment can also be spread by worms, flies and cockroaches, which directly contaminate food (CHINCHILLA et al., 1994; HILL; DUBEY, 2002). In addition, to being resistant, oocysts are largely eliminated and have high infective power for intermediate hosts, where a single oocyst can cause infection in pigs (DUBEY et al., 1996).

The accidental intake of oocysts is an important route of transmission that directly causes infection in humans, even outbreaks by contaminated water, food, and soil (BOWIE et al., 1997; MOURA et al., 2006; CARMO et al., 2010; DU et al., 2012; EKMAN et al., 2012). Livestock animals has also become infected by oocysts and many studies have shown that the presence of cats is a risk factor to *T. gondii* infection*;* moreover in one of these studies, a pig farm that has the presence of cats have 11 times more chances to been exposed to the parasite than a farm without cats (GARCÍA-BOCANEGRA et al., 2010; VIEIRA et al., 2018). Ferreira et al., (2014) found that presence of cats poses a 2.2 more risk to ocular toxoplasmosis in Brazil.

Centrifugal-flotation followed by optical microscopy examination (Sheather) is the most common technique used *T. gondii* oocysts for detection in feline feces. However, limiting factors such as similar morphology with other coccidian oocysts lead to false-positive results, as well as samples with few oocysts can lead to false-negative results, since the detection threshold is between 250 to 1,000 oocysts/g of feces (JONES; DUBEY, 2010). A modified

Kato-Katz technique with Kinyoum's staining (KKK) demonstrated sensitivity for a longer period than the centrifugal-flotation and can generate a permanent record of oocysts (stained slides) but it is a semi quantitative technique such as centrifugal-flotation (MEIRELES et al., 2008). The mice bioassay is the only technique capable of detecting viable and infectious oocysts, but it is an expensive technique and demands resources, time and infrastructure (SALANT; SPIRA, 2010).

A molecular approach, which detects the DNA of the parasite, is a high specific technique widely used in scientific research (DABRITZ et al., 2007). Salant et al. (2007) in an experimental study, detected 1-2 oocysts in 200 µl of feces amplifying the 529 bp fragment in the PCR. However, the detection in the fecal samples of naturally infected animals and environmental samples (water, soil) is not such sensitive due to the difficulty of breaking the oocysts, and the presence of PCR inhibitors (Dabritz et al., 2007).

Most cats have free access to the street even when domiciled, the number of wandering animals is high, and many other animals are abandoned. The junction of these factors propitiates the cycle of *T. gondii* since these animals do not have proper place to defecate and their feces are buried by them in the environment. Seroprevalence of antibodies anti-*T. gondii* is higher in older animals (SCHARES et al., 2008), however it is not a protection factor, Zulpo et al. (2018) found that even adult cats with high levels of *T. gondii* antibodies can re-eliminate oocysts in large numbers (only 30% less than a young cat), especially when re-infected with a different type of protozoan strain, which may be relevant in sites with high clonal diversity and genetic recombination (LEHMAN et al., 2004, SHWAB et al., 2014, ZULPO et al., 2018).

Although the oocysts shedding by cats is epidemiologically important in the *T. gondii* spread, few studies were developed with the objective of controlling oocysts shedding by cats, due to the fact that it is a more expensive, laborious experimental model and a pet specie. Moreover, high sensitivity and specificity techniques are required for the detection and quantification of oocysts in the feces of these animals, thus estimating environmental contamination. The aim of this study was to quantify oocysts by the qPCR in feces of cats immunized with recombinant proteins (rROP2 from *T. gondii* and rHSP70 from *E. tenella*) and challenged with *T. gondii* cysts.

Material and methods

Ethics Committee

Animal housing and experimentation were performed according to guidelines set forth by the Institutional Ethics Committee in Animal Use from Londrina State University, Brazil. The protocol of this study (CEUA, number 102/12).

2.2 *Toxoplasma gondii* strains

Two strains of *T. gondii* were used in this experiment: RH (type I) isolated by Sabin (1941) was used for DNA extraction and *rop*2 gene amplification and TgDoveBr8 (type II) isolated by Barros et al., (2014) was used for tissue cysts production. 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 brain cyst burden was counted and prepared for challenge (approximated 600 cysts to each cat in a total of 2 mL of saline) according as previously described (Zulpo et al., 2012).

Recombinant proteins

rROP2– Expression and purification

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

E. coli 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.

The soluble fraction was applied directly onto Ni-NTA Superflow resin (Qiagen, QIAGEN Biotecnologia Brasil Ltda., São Paulo, Brazil) 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. The protein concentration was determined using Pierce® BCA Protein Assay Kit (Thermo Scientific®, San Jose, CA, USA).

rHSP70– Expression and purification

The recombinant rHSP70 protein was obtained from *Eimeria tenella* as described by Bogado et al., (2017).

The rHSP70 was expressed on *E. coli* One Shot® TOP10 (Invitrogen, Carlsbad, CA, USA). The cells were grown in LB with 100 µg/ml ampicillin, at 37°C to an optical density (DO600) of 0,5-0,7. The expression of the protein was induced with isopropyl-D-thiogalactopyranoside (IPTG) at the final concentration of 1mM, at 37°C for 4 hours.

The purification was realized in denaturing conditions with guanidine in Ni-NTA Superflow resin (Qiagen, QIAGEN Biotecnologia Brasil Ltda., São Paulo, Brazil). The recombinant antigen was eluted from the resin by gravity flow with urea 8M.

Clinical evaluation of cats

In this experiment, we used twelve (8 male and 4 female) short hair domestic cats (*Felis catus*), between 6 and 9 months of age, examined, vaccinated against feline viral diseases (Rhinotracheitis, Calici and Panleukopenia viruses) (Feligen®, Virbac, Brazil). The cats were also submitted to vermifugation (Vetmax plus, Vetnil, Brazil). All cats were clinically healthy and had no detectable specific IgG anti-*T. gondii* titres by IFAT (Camargo, 1974) and absence of *T. gondii* oocysts was confirmed by fecal examination (Dubey, 1995).

Cats were randomly allocated in individual cages and all cats received only commercial dry food and water *ad libitum*. Clinically evaluated continued to other diseases for two months prior to the beginning of the experiment. The clinical observations were conducted daily, as well as cleaning, feeding, welfare and rectal temperature.

Cats Immunization

The cats were divided into three groups, each group containing four animals (Table 1). G1 animals received 25 µg of rROP2 plus 25 µg of rHSP70 and 20 µg of Quil-A. G2 received 25 µg of *E. coli* and 20 µg of Quil-A; G3 (control) received only saline solution. The animals were immunized by administrating (140 µl of final solution for each cat 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 and 42 of the experiment.

Experimental inoculation

The animals were anesthetized with tiletamine plus zolazepam (Zoletil®, Virbac – Brazil, 3.15 mg/kg/IM) before the challenge. All cats from G1, G2 and G3 were challenged on day 72 with 600 cysts of the TgDoveBr8 strain (contained in a volume of 2 mL) administered via stomach tube, after which they were injected with 5 mL of saline, to avoid remaining residuals.

Table 3. Experimental design of cats immunized with rROP2 plus rHSP70 (G1), adjuvant (G2) and negative control (G3).

Experimental groups	Immunization route	Immunization protocols (0, 21, 42 days)[1]	Challenge[2] (Day 72)
G1	Intranasal	25 µg rROP2 + 25 µg rHSP70 + QuilA (20 µg)	600 cysts
G2	Intranasal	QuilA adjuvant (20 µg)	600 cysts
G3	Intranasal	Saline Solution	600 cysts

[1] Period during which procedure was performed. [2]TgDoveBr8 (BARROS et al., 2014).

Fecal examination

Fecal samples from each cat were examined daily for oocysts search day 3rd to 11th after inoculation as previously described (Garcia et al., 2007). The total volume of feces from each cat, in each day, were pooled, homogenized and weighed. Two grams from this homogenate were admixed with 10 mL of sucrose solution (specific gravity, 1.18), filtered with gauze, 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, the pellet ressuspended in water (1 mL) and storage in labeled microtubes at -20º C. Oocysts quantification was then determined in four chambers of Neubauer's chamber.

DNA extraction of oocysts

The yield solution from centrifugal-flotation in sucrose solution were placed into new microtubes (1.5 mL), 100 uL of the oocysts sample were homogenized by vortexing with 900 uL TE buffer (100mM Tris e HCl,10mM EDTA, pH8), and centrifuged at 10000x g for 5 minutes at room temperature The supernatant was discarded and the pellet was resuspended by vigorous vortexing in 300 µl of lysis buffer (T1 buffer, Macherey-Nagel, NZ740952250), following 5 freeze-thaw cycles in -80ºC for 10 minutes and a thawing for 5 minutes at 65ºC. Lysis were performed with proteinase K (2 mg/ml) and overnight incubation at 65ºC.

Total DNA was extracted by adding ultrapure buffer satured phenol (v:v:) to the samples, which were homogenized and centrifuged (10,000x g) for 5 minutes at room temperature. The supernatant was then transferred to a new clean tube, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the samples were again mixed and centrifuged as above. 250 µL of the translucid aqueous phase was transferred to a clean tube to proceed the DNA precipitation, with cold absolute ethanol (3:1 ratio) and ammonium acetate (10M). The mixture was homogenized and stored at -20°C for 1 hour. The sample was centrifuged (10,000x g) for 15 minutes at 4°C to pellet precipitated DNA. The supernatant was discarded and DNA purification was done by washing with 1mL of 70% ethanol for 15 min at 10,000x g. After pellet drying at 37ºC, the DNA was eluted with ultrapure water (25 µl) and stored at -20ºC until PCR reactions.

qPCR assay quantitation

Quantitative Real-time PCR (qPCR) was performed with a standard reaction mixture: 5 uL of Sybr™ Select Master Mix (Applied Biosystems, Foster City, CA, USA, 4472908), 0.2 µl (10 µM) of each the primer, 0.4 µl of BSA3.2ul ultrapure water and 1,0 µl of DNA template, resulting in a final volume of 10 µL per reaction. BSA (10µg/µl) was included in reaction mix as this concentration since it reduced the effect of PCR inhibitors without affecting the PCR (Data not shown). The forward primer (Tox-9) and reverse primer (Tox-11) targeted a 529-bp repeat element as described previously (GenBank AF146527) (HOMAN et al., 2000; REISCHL et al., 2003).

Cycling conditions (ABI 7500 StepOnePlus™ Real-Time PCR system Applied Biosystems, Foster City, CA, USA) were 95ºC for 10 min (activation) followed by 40 cycles of 94ºC for 15s (denaturation), 59ºC for 30s (annealing), and 72ºC for 30s (extension). The fluorescence was measured at the end of each cycle. An additional step from 95ºC for 15s, 60ºC to 95ºC for 1 min (0.3ºC/sec) was added to obtain a melting curve to verify the specificity of the amplified products by their specific melting temperatures (Tm). Post amplification melting curve with SYBR Green dye of *T. gondii* positive DNA samples revealed one peak. Data were analyzed in StepOne™ Software v.2.2. 2 (Applied Biosystems, USA). Each sample was tested in duplicate, and each qPCR plate contained a negative control.

qPCR standards

T. gondii oocysts genomic DNA standards (five points at 1:10 dilution) obtained from 1,422500 oocysts) were included on each run to enable calculation of a standard curve for determination of oocysts per gram of feces in samples. The slope of standard curve was used to calculate amplification efficiencies by the formula: E=10(−1/slope) −1, where E is the efficiency of amplification and s is the slope.

Statistical analysis

To compare difference between Sheather (centrifugal-flotation) and qPCR, the *T* test was performed, and to compare difference between groups (G1, G2, G3) Kruskal-Wallis test was used, in GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, and were considered significant if the p value < 0.05.

Results

After immunization (three doses) and challenge on day 72, were collected feces daily for 9 days post-infection, and a total of 62 samples were examined by qPCR. It was possible to quantify oocysts in all samples analyzed by qPCR (Figure 1 and 2).

All cats shed *T. gondii* oocysts in feces, but no animals showed significant clinical signs except mild diarrhea. Five negative animals in the centrifugal-flotation technique (Sheather) on the 3rd dpi were positives in the qPCR with the following quantifications (328; 450; 470; 509; 1828 OOPG), while the detection and quantification of oocysts was only possible on 4th d.p.i by Sheather. Although qPCR detects and quantifies oocysts earlier than Sheather, there was no statistical difference between the two techniques ($p = 0.1116$).

Oocysts shedding per group in each of the techniques is shown in Figure 3. G2 animals shed less oocysts per gram of feces in comparison to the other groups, with an average of OOPG in Sheather= 90,888 and in qPCR = 144,976; while G1 cats shed an average of OOPG in

Sheather=114,120 and in qPCR= 160,760; G3 (control) excreted an average of OOPG in Sheather: 134,263 and in qPCR: 162,907, a bigger quantity than another groups. However, there was no statistically significant difference between the groups in both Sheather and qPCR (p value = 0.6534 and 0.9670, respectively).

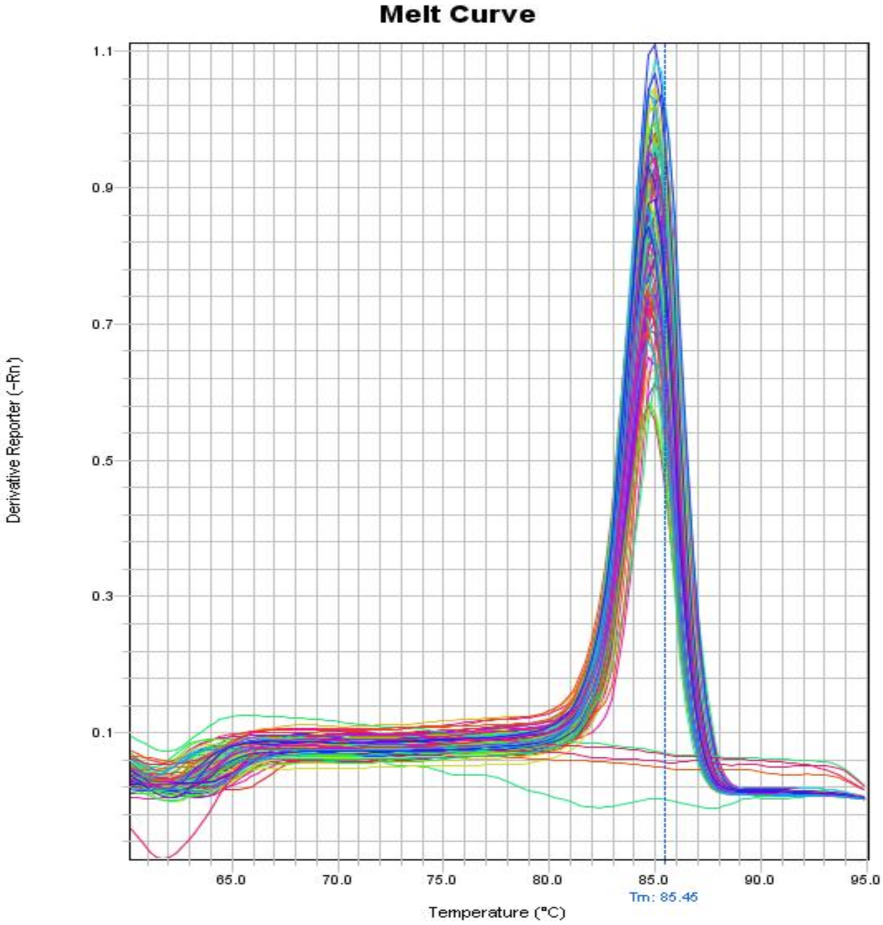

Figure 1. Post amplification melting curve with SYBR Green dye of *Toxoplasma gondii* positive DNA samples revealed one peak.

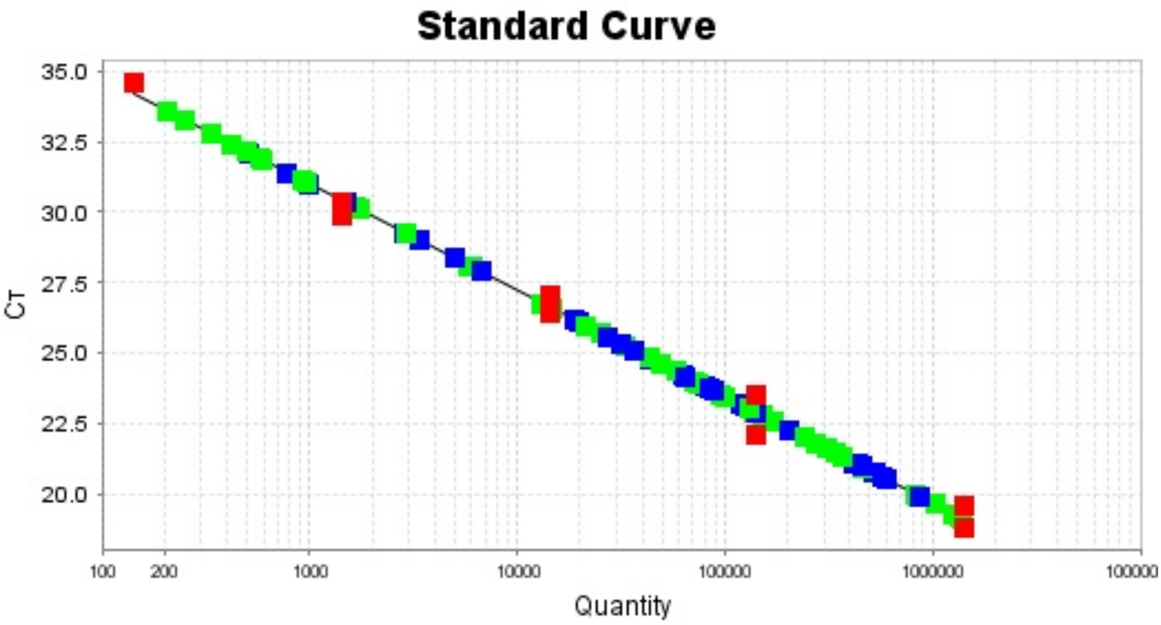

Figure 2. Standard curve for a serial 10-fold dilution of *T. gondii* oocysts in cat faeces. Slope:-3.772; Y-interception: -42.3089; correlation (r^2)= 0,991.

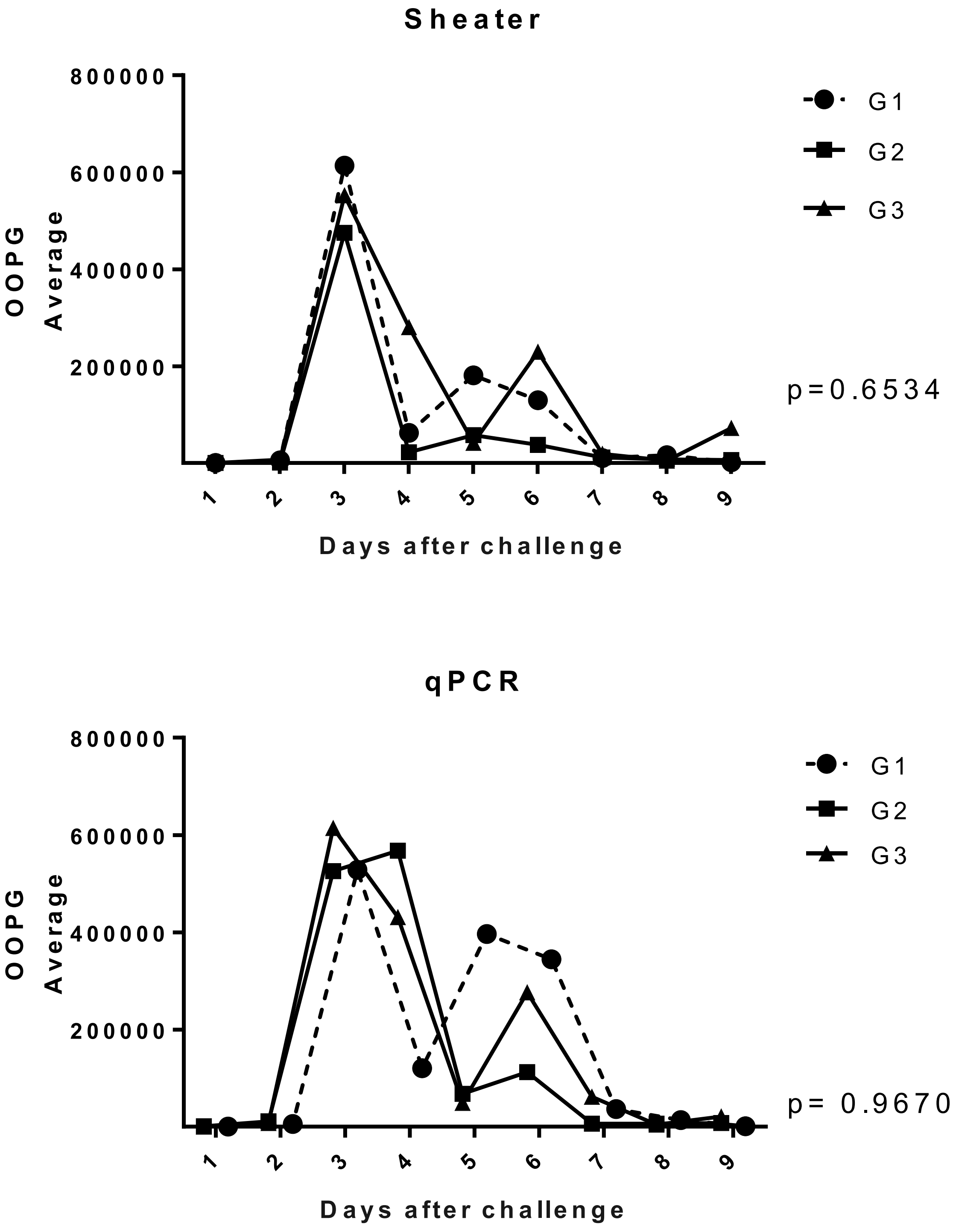

Figure 3. *Toxoplasma gondii* oocysts shedding in cats shed by a group of cats immunized with rROP2 and rHSP70 (G1), G2 were adjuvant and vector control and G3 were saline control. Challenge was performed on day 72 with 600 tissue cysts of the TgDoveBr8 strain of *T. gondii.*

Discussion

We choose a *T. gondii* type II strain for the cats' challenge because it is an intermediate virulence genotype**,** being able to produce tissue cysts to mice, is the most common genotype associated with human toxoplasmosis in patients with AIDS, as well as in congenital infections in animals and humans, in Brazil this strain was isolated in eared doves, guinea-fowl and cattle (HOWE; SIBLEY, 1995; BARROS et al., 2014 ; DUBEY et al., 2011; MACEDO et al., 2012).

In this study, we observed that DNA extraction of unsporulated oocysts was not successful when we used 0.2 g of total feces. Total feces contain a lot of organic material and PCR inhibitors, moreover low levels of oocysts shedding hamper *T. gondii* detection and DNA extraction. It was only possible to extract DNA after the centrifugal flotation technique because the final solution of this technique contains less impurities and a higher concentration of oocysts allowing the extraction of *T. gondii* oocysts DNA thereby reduce the amount of potential PCR inhibitors found in feces. Adaptations in extraction protocols to disrupt highly resistant oocysts wall (freezing and thawing 5x, lysis buffer, and proteinase K incubation overnight) had to be done, in another study they reported similar procedures to disrupt *T. gondii* oocyst wall (CORNELISSEN et al., 2014).

The centrifugal-flotation technique is widely used and is an inexpensive technique, which does not require many equipments, but it has some disadvantages such as the high detection threshold (1000 OOPG) (JONES; DUBEY, 2010), requires training and experience for oocysts identification and quantitation in Neubauer' chamber. In this study, a sample with a low number of oocysts was detected and quantified by qPCR: 328 oocysts/gram of feces. Another negative sample in Sheather was quantified with 1828 OOPG by qPCR thus demonstrating that the Sheather's detection threshold may be greater than 1828 OOPG. However, the centrifugal-flotation was an indispensable step for the successful accomplishment of qPCR, considering the need for purification and concentration of the samples.

Among molecular methods of *T. gondii* oocysts detection regular and nested PCR assays are sensitive, but not quantitative. qPCR allows an accurate quantification because the concentration of amplicons can be monitored continuously during the reaction, and samples with differing amounts of starting material can be discriminated from another one (HUNT, 2011).

The target sequence of 529 bp repeats 200-300 times in the genome of the parasite and is the most used for qPCR because it demonstrates high sensitivity (HOMAN et al., 2000), being able to detect 6-7 times more DNA than n- PCR (CONTINI et al., 2005). In this study qPCR detected *T. gondii* oocysts earlier and in greater quantity than Sheather, but Cornelissen et al. (2014) and Poulle et al. (2016) reported that the high sensitivity of the qPCR technique can detect *T. gondii* DNA fragments without the presence of oocysts, and therefore qPCR should be carefully analyzed, especially in studies to verify the efficacy of vaccines and different doses of challenge, we must take into account the type of strain, infectious dose, standard curve, reaction efficiency. qPCR is a highly sensitive quantitative technique that uses minimal sample quantities, however, requires improved laboratory structure with high-cost reagents and equipment as well as personnel technically capable of performing and interpreting the results.

As for oocyst elimination pattern, our study showed the expected pattern for *T. gondii* cysts infection, with qPCR positivity at the 3rd dpi, and an increase in the number of oocysts on subsequent days up to the 11th dpi, as well as other studies using a type II strain (DUBEY, 1996; DUBEY; FRENKEL, 1972; CORNELISSEN et al., 2014).

There was a numerical difference in the number of oocysts eliminated between groups, but this difference was not statistically significant. The results obtained in these two techniques were similar, the G3 (control) that shed on average more oocysts in qPCR was also what most shed oocysts in the Sheather. G1 had an intermediate shedding mean and G2 (adjuvant) was what eliminated on average less number of oocysts, Zulpo et al. (2012) obtained similar results using Quil-A and reported the immunomodulation potential of this adjuvant, in this study Quil-A effect could be observed in the two groups that eliminated less oocysts than G3.

In experimental studies oocysts quantification is commonly performed, however, few studies have been carried out to quantify the oocysts' shedding in feces of naturally infected cats (DUBEY, 2010), qPCR and Sheather can be used concomitantly to estimate potential environmental contamination by *T. gondii* oocysts in naturally infected feline stool samples.

Conclusion

Results obtained with the qPCR method were comparable to centrifugal-flotation (Sheather) results for experimentally infected cats. The qPCR method can be used as an alternative to Sheather for detection and quantification of *T. gondii*.

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7 CONCLUSÃO

- As proteínas recombinantes rROP2 (*T. gondii)* e rHSP70 (*E. tenella*) promoveram estimulação das respostas celulares Th1 e Th2 nos gatos imunizados, em relação aos do grupos controle após a fase aguda da infecção.

- Não houve efeito protetor utilizando proteínas recombinantes rROP2 de *T. gondii*, rHSP70 de *E. Tenella* associadas ao Quil-A em animais experimentalmente infectados com cistos de *T. gondii,* porém houve a modulação da resposta imune com maior indução de IL-10 nos animais imunizados.

- O grupo imunizado com adjuvante Quil-A eliminou menor quantidade de oocistos em relação aos outros grupos, porém a diferença numérica não foi estatisticamente significativa.

- Os resultados obtidos com o método qPCR foram comparáveis aos resultados obtidos pela técnica de centrifugo-flutuação seguida de exame microscópico (Sheather) para gatos infectados experimentalmente, a qPCR pode ser usada como uma alternativa para o Sheather para detecção e quantificação de oocistos de *T. gondii*