UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

Padronização de ELISA para detecção de anticorpos anti-*Toxoplasma gondii* e *Neospora caninum* em soro e fezes de cervídeos.

Maria Helena Mazzoni Baldini

Médica Veterinária

2021

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Maria Helena Mazzoni Baldini

Orientador: Prof. Dr. José Mauricio Barbanti Duarte

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutora em Medicina Veterinária.

2021

B177p

Baldini, Maria Helena Mazzoni

Padronização de ELISA para detecção de anticorpos anti-Toxoplasma gondii e Neospora caninum em soro e fezes de cervídeos / Maria Helena Mazzoni Baldini. -- Jaboticabal, 2022

92 p. : il.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal

Orientador: José Maurício Barbanti Duarte

1. Patologia veterinária. 2. Toxoplasmose. 3. Neospora. 4. Transmissão. 5. Animais silvestres. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

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TÍTULO DA TESE: PADRONIZAÇÃO DE ELISA PARA DETECÇÃO DE ANTICORPOS ANTI-*Toxoplasma gondii* E *Neospora caninum* EM SORO E FEZES DE CERVÍDEOS

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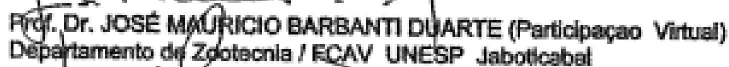

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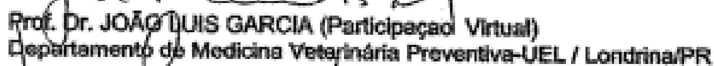

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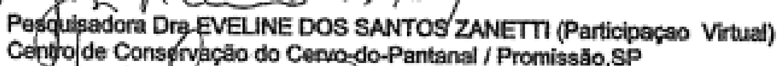

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Jaboticabal, 01 de dezembro de 2021

DADOS CURRICUALRES DO AUTOR

Maria Helena Mazzoni Baldini – Nascida em 22 de julho de 1991, na cidade de Joinville, Santa Catarina. Ingressou no curso de graduação em Medicina Veterinária na Universidade do Estado de Santa Catarina (UDESC), em março de 2009. Durante a graduação foi bolsista de extenção do Projeto Amigo do Carroceiro, sob supervisão do professor Joandes Henrique Fonteque. Cumpriu estágio extra-curricular em Clínicas veterinárias como Cia Bichos (Joinville-SC), Vida Livre (Curitiba – PR), Tukan (São Paulo – SP) e no Centro de Reabilitação de Animais Selvagens - CRAS (Campo Grande –MS). Em 2013 cumpriu estágio curricular no Zoológico de Sorocaba e Refúgio Biológico Bela vista da Itaipu Binacional. Ingressou em março de 2014 no curso de Pós-graduação em Ciencia Animal da UDESC, sob orientação do Prof. Dr. Aury Nunes de Moraes, sendo bolsista pela CAPES, durante o mestrado participou do Treinamento Técnico do NUPECCE em 2015. Em 2016 começou a trabalhar como médica veterinária do Centro de Triagem de Animais Silvestres da Associação R3 animal em Florianópolis – SC. Em março de 2018 ingressou no Curso de Pós-graduação em Medicina Veterinária na UNESP – Jaboticabal sob orientação do professor José Maurício Barbanti Duarte.

Agradecimentos

Ficam aqui registrados meus agradecimentos a todos que de alguma forma contribuíram para que eu concluísse essa tese de doutorado. Agradeço à toda minha família, em especial aos meus pais, Rosanna Mazzoni Baldini e Vagner Baldini pelo suporte e apoio para que eu continuasse meus estudos. Agradeço ao meu orientador, José Maurício Barbanti Duarte por aceitar me orientar mesmo com os desafios que o projeto proporcionava. A toda a equipe de graduandos, pós-graduandos e funcionários do Nupecce, pela amizade e auxílio durantes as atividades do setor. À Eveline Zanetti e a Tijoá por terem cedido amostras dos animais do Centro de Conservação do Cervo-do-pantanal. Às amigas e colegas Eluzai Dinai Sandoval, Yuki Tanaka e Gabrielle Queiroz pelo auxílio nas análises dos trabalhos descritos nessa tese, e à Patrícia Perin por ter contribuído na revisão dos capítulos. Agradeço também aos pesquisadores que contribuíram de forma mais efetiva para o desenvolvimento do projeto: ao Prof. Hélio Montassier, Prof. Rosângela Zacarias Machado e aos funcionários da IMUNODOT, em especial Carla Freschi. Também agradeço à Priscila Fioresi Garcia, por todos os conselhos a ajuda para organizar meus pensamentos e conseguir escrever essa tese.

Por último, agradeço a todos os animais utilizados nesses e em outros projetos, na esperança que os dados obtidos a partir deles possam um dia ser utilizados efetivamente para conservação.

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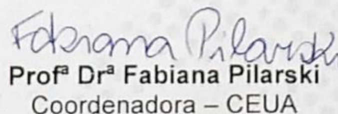
CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Uso do teste de ELISA para detecção de imunoglobulinas secretoras específicas nas fezes de cervídeos e sua correlação com imunoglobulinas séricas"**, protocolo nº 003732/18, sob a responsabilidade do Prof. Dr. José Maurício Barbanti Duarte, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 19 de abril de 2018.

Vigência do Projeto	15/04/2018 a 30/11/2021
Espécie / Linhagem	Cervídeos do gênero *Mazama sp. Odocoileus virginianus* e *Ozotocerus bezoarticus*
Nº de animais	25
Peso / Idade	13 a 50 Kg
Sexo	Ambos os sexos
Origem	Núcleo de Pesquisa e Conservação de Cervideos – NUPECCE

Jaboticabal, 19 de abril de 2018.



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Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Toxoplasmose em *Mazama gouazoubira*: Avaliação dos parâmetros clínicos, hematológicos e reprodutivos em machos experimentalmente infectados"**, protocolo nº **06892/19**, sob a responsabilidade do Prof. Dr. José Mauricio Barbanti Duarte, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 13 de junho de 2019.

Vigência do Projeto	13/07/2019 a 13/07/2020
Espécie / Linhagem	Veado – catingueiro / *Mazama gouazoubira*
Nº de animais	05 (cinco)
Peso / Idade	15 kg / 01 a 08 anos
Sexo	Machos
Origem	Núcleo de Pesquisa e Conservação de Cervídeos - NUPECCE

Jaboticabal, 13 de junho de 2019.

Fabiana Pilarski

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RESUMO

A maior parte dos dados descritos na literatura sobre patógenos de cervídeos neotropicais são provenientes de levantamentos sorológicos, informações sobre vías de transmissão e avaliações clínicas ainda são escasos. O presente trabalho teve como objetivos: a) estimar a prevalência de cervídeos positivos para *Toxoplasma gondii* e *Neospora canium* em 92 animais mantidos em cativeiro; b) Avaliar a probabilidade de diferentes vias de transmissão do *T. gondii* e *N. caninum*; c) avaliar a sintomatologia clínica e resposta imune humoral de machos de veados-catingueiros (*Mazama gouazoubira*) infectados experimentalmente com oocistos da cepa ME49 (genotipo tipo II) de *T. gondii* e d) padronizar a técnica de ELISA indireta para detecção de anticorpos fecais específicos para *T. gondii* e *N. caninum*. Inicialmente, foram colhidas amostras dos animais cervídeos pertencentes a dois criadouro conservacionistas e comparados os resultados sorológicos obtidos nos testes de imunofluorescência indireta (RIFI) e ELISA indireto pelo teste de concordância Kappa de Cohen. Em seguida, foram desenhadas árvores genealógicas dos animais de cada espécie para estimativa da taxa de transmissão congênita de cada doença. A prevalência estimada para *T. gondii* foi de 20,73% pela tecnica de RIFI e 25,60% por ELISA, apresenando uma concordância rasoável ($\varkappa$ = 0,277) entre as duas técnicas sorológicas utilizadas. Já para *N. caninum* a prevalência estimada foi de 40,24% por RIFI e 39,02% por ELISA com uma correlação quase perfeita ($\varkappa$ = 0,83) entre as técnicas. A taxa de transmissão congênita, estimada com base em números de filhos positivos de mães positivas entre o número total de filhos de mães positivas, diferiu bastante entre as doenças, sendo de 0% para toxoplasmose e 81,25% para neosporose. No capítulo 3, para a infecção experimental dos animais, quatro machos da espécie *Mazama gouazoubira* foram infectados experimentalmente com 5000 oocistos esporulados de *T. gondii* da cepa ME49, enquanto um animal soronegativo foi usado como controle. Os animais foram monitorados quanto aos parâmetros clínicos de -7 a 40 dias após a infecção (dpi). Amostras de sangue foram coletadas semanalmente até 49 dpi para detecção de anticorpos pelo teste de ELISA. A cada 15 dias os animais foram submetidos à contenção química e coleta de sêmen por eletroejaculação e o material genético das amostras de semen foi extraído para posterior análise por PCR para *T. gondii*. Apenas dois dos quatro animais infectados desenvolveram resposta de anticorpos detectável no teste de ELISA, e mesmo sem nenhum animal ter manifestado sinais clínicos da infecção, o material genético do parasita foi encontrado no sêmen dos animais soropositivos aos 35 e 49 dpi. A terceira etapa do projeto visou a padronização da técnica de ELISA para detecção de imunoglobulinas fecais contra os dois patógenos. Amostras de fezes frescas foram coletadas, extraídas e armazenadas em nitrogênio líquido ou processadas imediatamente. Um pool de amostras de animais soropositivos foi utilizado como controle positivo para *N. caninum* e um pool de amostras de animais experimentalmente infectados foi usado como controle para *T. gondii*. Mesmo com diversas alterações de protocolos não foi possível contornar o problema da baixa

especificidade do teste, constatada pelos altos ruídos nas leituras das placas. Foi possível concluir que as técnicas sorológicas provaram ser muito úteis para estudos epidemiológicos em animais de cativeiro, tendo diversas aplicações além da simples estimativa da prevalência, porém sem a padronização de um método não invasivo de detecção de anticorpos, estudos epidemiológicos em populações de vida-livre ainda são um grande desafio. Nesses estudos foi possível inferir que a principal via de transmissão do *N. caninum* em cervídeos é a via congênita, de forma semelhante ao que se observa em bovinos , enquanto que *o T. gondii* parece utilizar da via horizontal para a transmissão na maioria dos casos. Além disso , a infecção experimental com 5000 oocistos esporulados de *T. gondii* não causou sinais clínicos nos animais estudados, sugerindo que a espécie apresenta certa resistência à doença, por outro lado, a detecção do DNA do parasito no sêmen dos animais sugere que a transmissão sexual é uma possibilidade nessas espécies, o que deve ser levado em consideração por criadouros conservacionistas.

ABSTRACT

Most of the data on neotropical deer pathogens come from serological surveys, little is known about transmission routes and clinical evaluations.The present study aimed to: a) estimate the prevalence of positive cervids for *Toxoplasma gondii* and *Neospora canium* in animals kept in captivity; b) Assess different routes of infection of *T. gondii* and *N.caninum* on these deer populations; c) Evaluate the clinical symptomatology and humoral immune response of male brocket deer (*Mazama gouazoubira*) experimentally infected with *T. gondii* (ME49 strain) oocysts; and d) Standardize an indirect ELISA technique for non-invasive detection of specific fecal antibodies to *T. gondii* and *N. caninum*. Initially, deer serum samples collected from animals from two conservation breeding centers were tested by both techniques making it possible to compare the results obtained in the indirect immunofluorescence tests (IFAT) and indirect ELISA by the Cohen's Kappa concordance test. Furthermore, family trees were drawn for each species to estimate the congenital transmission rate of both diseases. The *T. gondii* prevalence were 20,73% by IFAT and 25,60 by ELISA, with , with an fair agreement of ϰ = 0.277 between the two serological techniques. For *N. caninum*, the antibody prevalence was 40,24% by IFAT and 39,02% by ELISA, with a almost perfect correlation of ϰ = 0.83 between techniques. The congenital transmission rate, estimated by the number of positive offspring from positive mothers by the total of positive mother offspring, differed significantly between diseases, resulting in 0% for Toxoplasmosis and 81,25% for Neosporosis. In Chapter 3, four *Mazama gouazoubira* males were inoculated with 5000 sporulated *T. gondii* oocysts (ME49), while one seronegative animal was used as a control. Animals were monitored for clinical parameters from -7 to 40 days post-infection (dpi). Blood samples were collected weekly up to 49 dpi for antibody detection by the previously standardized ELISA test. Once every 15 days, the animals were chemical restraint for semen collection by electroejaculation. DNA of the semen samples was extracted for *T. gondii* PCR analysis. Only two from four infected animals developed an antibody response detectable in the ELISA test, and despite the animals didn’t manifest any clinical signs, the parasite's genetic material was found in the semen of the seropositive animals at 35 and 49 dpi. In the third step, the standardization of ELISA technique for detection of fecal immunoglobulins against the two pathogens was carried out, aiming to apply the methodology to epidemiological studies in free-living animals. Fresh stool samples were collected, extracted and stored in liquid nitrogen or processed immediately. A pool of samples from seropositive animals was used as a positive control for *N. caninum* and a pool of samples from experimentally infected animals was used as a control for *T. gondii*. Even with several protocol changes, it was not possible to overcome the problem of the low specificity evidenced by the high background at plate readings. Finally, it was possible to conclude that serological techniques have proved very useful for epidemiological studies in captive animals, having several applications beyond the simple estimation of prevalence, but without the standardization of a non-invasive method of detection of antibodies, epidemiological studies in free-living populations are still challenging. Whith these studies it was possible to infer tha the congenital transmission is the major route of infection os *N. caninum* in neotropical deer, in the other hand, horizontal transmission seem to be more important to *T. gondii* epidemiology in these species. Furthermore, the experimental infection with 5000 *T. gondii* oocysts did not caused clinical signs in *Mazama gouazoubira*, implying that the specie present some resistance to the disease, still, the detection of the parasite in semen samples indicate the possibility of sexual transmission of *T. gondii* in cervids,

and breeding centers should take this into account for better reproductive performance of the animals.

LISTAS DE FIGURAS

CAPÍTULO 2

Figure 1. Family tree of red brocket (*Mazama americana*) from Deer Research and Conservation Center – UNESP/ SP.

Figure 2. Family tree of brown brocket deer (*Mazama gouazoubira*), pampas deer (*Ozotoceros bezarticus*), amazonian brown brocket (*Mazama nemorivaga*) and dwarf red brocket (*Mazama nana*) and small red brocket (*Mazama bororo*) from Deer Research and Conservation Center – UNESP/ SP.

Figure 3. Family tree of marsh deer (*Blastocerus dichotomus*) from the Marsh Deer Conservation Center – Tijoá/ SP).

CAPÍTULO 3

Figure 1. Percentual of optical density (OD) values obtained in indirect ELISA readings from infected (M1, M2, M3, M4) and uninfected brown brocket deer (*Mazama gouazoubira*) during the experiment.

Figure 2. Seminal parameters. Line graphs represent the values of sperm motility index (%), membrane integrity (%), acrosome integrity (%), semen volume (ml), total defects (%), minor defects (%), major defects (%), sperm concentration (sptz/ejaculate) from male 1(●), male 2 (■), and male 3 (▲), male 4 (▼) and male 0 (♦)

CAPÍTULO 4

***Fig* 1**. Schematic representation of (A) serial dilutions of antigen in the ELISA plate wells, wells 6 received carbonate-bicarbonate buffer only and (B) serial dilutions of fecal extracts, wells F received blocking buffer (1% ovalbumin in PBS Tween 20) only.

Fig 2. Graph relating OD values for different *T. gondii* antigen concentrations against dilutions series of samples in carbonate-bicarbonate buffer. Curves show titration of

antiserum at different dilutions from wells 1-5. Wells 6 received a carbonate-bicarbonate buffer only.

Fig 3. Graph relating OD values for different *N. caninum* antigen concentrations against dilutions series of samples in carbonate-bicarbonate buffer. Curves show titration of antiserum at different dilutions from wells 1-5. Wells 6 received a carbonate-bicarbonate buffer only.

CAPÍTULO 1- Considerações gerais

1 INTRODUÇÃO E JUSTIFICATIVA

O mundo está sofrendo uma rápida mudança em seus ecossistemas, e diversos patógenos, vetores e hospedeiros acompanham essas mudanças rapidamente. Patógenos introduzidos podem causar doenças graves em animais selvagens, e em alguns casos os animais selvagens podem servir de reservatórios para agentes patogênicos sem desenvolverem sinais clínicos, exercendo um importante papel na epidemiologia de diversas doenças emergentes (Bengis et al., 2002).

Doenças infecciosas são citadas como uma das causas de declínio de populações de cervídeos neotropicais entre elas o cervo-do-pantanal (*Blastocerus dichotomus*) (Duarte et al., 2012a) e do veado-mateiro (*Mazama americana*), como consequência da perda do habitat e maior contato com animais domésticos que podem transmitir febre aftosa (Araujo Júnior et al., 2010), leptospirose (Mathias et al. 1999, Galli et al. 2014), toxoplasmose (Ferreira et al. 1997), tuberculose (Luna et al. 2003), hemoparasitos (Grazziotin et al., 2011; Sacchi et al., 2012), ectoparasitas (Szabó et al., 1999; Szabó et al., 2003) e endoparasitas diversos (Tiemann et al., 2005; Duarte et al., 2012b). As enfermidades comuns a animais domésticos também são citadas como possível causa de declínio de populações de veado-de-mão-curta (*M. nana*), porém há uma carência de estudos epidemiológicos com essas espécies (Duarte et al., 2012c). Mesmo com esses relatos, ainda pouco se sabe sobre a dinâmica das doenças infecciosas em cervídeos neotropicais. A maior parte dos estudos é proveniente de levantamentos sorológicos de populações em cativeiros e de espécies de cervídeos que ocorrem em zonas temperadas.

A forma como uma doença se dissemina em um ecossistema depende de vários fatores, incluindo espécies envolvidas, o nicho ecológico e a estrutura social dos animais. Para a realização de um estudo epidemiológico é necessária colheita de dados e amostras de vários indivíduos. Em um estudo envolvendo animais selvagens de vida-livre, a colheita de amostras geralmente requer que os animais sejam capturados, o que frequentemente é um manejo problemático. Isso faz com que experimentos de larga escala sejam prejudicados devido à maior parte dos estudos serem realizados com número de amostras insuficientes, levando a uma

dificuldade de extrapolação desses dados, o que pode conduzir a erros de interpretação (Caley et al. 2009). Dentre os animais selvagens, os cervídeos são animais reconhecidamente sensíveis aos efeitos do estresse, sendo comum advirem acidentes traumáticos, sérios problemas cardiorrespiratórios e distúrbios metabólicos graves como a acidose e a miopatia de captura (Nunes et al., 2007). Portanto, procedimentos de captura de cervídeos requerem muita cautela e habilidade da equipe e podem representar um grande risco à vida desses animais (Duarte et al., 2010). Por esse motivo, técnicas não invasivas de diagnóstico de enfermidades representariam um grande avanço no estudo de patologia e epidemiologia em cervídeos neotropicais.

Parasitas que causam problemas reprodutivos em ruminantes domésticos também representam um fator de risco em potencial para o sucesso na reprodução de cervídeos em cativeiro. O conhecimento das manifestações clínicas e como a doença é introduzida e se mantém em populações de cervídeos é muito importante para programas de conservação de espécies silvestres. Tendo isso em vista, o presente trabalho teve como objetivo utilizar técnicas sorológicas, especialmente o teste de ELISA para avaliar a prevalência, vias de transmissão e susceptibilidade clínica à toxoplasmose e neosporose de duas populações de cervídeos mantidos em cativeiro. Além disso, realizar a padronização do teste de ELISA indireto para procura de imunoglobulinas fecais específicas para *Toxoplasma gondii* e *Neospora caninum*, buscando assim uma forma de diagnóstico não invasivo que pudesse ser utilizada em animais de vida livre.

2 REVISÃO DE LITERATURA

2.1 Os cervídeos brasileiros

Animais da família Cervidae pertencem à ordem Cetartiodactyla, caracterizados pela presença de cascos que recobrem totalmente os dedos. São ruminantes verdadeiros e como tal regurgitam e mastigam seu alimento várias vezes (Duarte e Merino, 1997). Atualmente a família Cervidae engloba 55 espécies que estão distribuídas amplamente na América, Europa, Ásia, norte da África e ,mais recentemente, na Oceania, onde diferentes espécies foram introduzidas entre o final do século XVII e começo do século XIX (Jesser, 2005).

Até o momento, foram oficialmente descritas oito espécies de cervídeos no Brasil, são elas: o cervo-do-pantanal (*Blastocerus dichotomus*), veado-da-cauda-branca (*Odocoileus virginianus*), veado-campeiro (*Ozotocerus bezoarticus*), veado-catingueiro (*Mazama gouazoubira*), veado-roxo (*Mazama nemorivaga*) veado-mateiro (*Mazama americana*), veado-mateiro-pequeno (*Mazama bororo*) e veado-de-mão-curta (*Mazama nana*). Porém, atualmente, a taxonomia das espécies passa por uma revisão aliando critérios morfológicos, citotaxonomia e genética molecular (Duarte e Merino, 1997).

O cervo-do-pantanal é a maior espécie de cervídeo da América Latina, alcançando até 130 Kg. Habita várzeas das planícies de inundação dos grandes rios e seus tributários (Duarte et al., 2012a) e está classificado como vulnerável pelos critérios da IUCN (Duarte et al., 2016). As principais ameaças para a espécies são a perda de habitat, a caça e enfermidades introduzidas por animais domésticos (Duarte et al., 2012). Já o veado-da-cauda-branca é uma espécie classificada como pouco preocupante (Gallina et al., 2016) e possui 38 subspécies, entre elas o cariacou (*Odocoileus virginianus cariacou*) que ocorre no extremo norte do Brasil (Gallina *et al*., 2010). O cariacou é bem menor fisicamente do que as subspécies mais conhecidas na américa do norte, e existem apenas dois exemplares desse animal em cativeiro. O veado-campeiro, é uma espécie que habita áreas abertas incluindo regiões dos pampas, cerrado e pantanal e é mais facilmente avistado em comparação com outros cervídeos brasileiros. Essa espécie é classificada como quase ameaçada pela IUCN (González et al., 2016). Já os cervídeos do gênero *Mazama* são animais elusivos, sendo solitários e florestais, e dificilmente são avistados. Uma exceção pode ser feita ao veado-catingueiro (*Mazama gouazoubira*) que possui bastante plasticidade ecológica, podendo ser encontrado em áreas mais abertas e cultivadas, em proximidade a animais domésticos (Duarte et al., 2012c).

O manejo em cativeiro da maior parte das espécies de cervídeos neotropicais é bastante desafiador devido ao comportamento muito reativo e à susceptibilidade a doenças, levando a perdas frequentes e baixa eficiência reprodutiva. Por esse motivo, a criação de cervídeos no Brasil tem sido pouco desenvolvida, mesmo sendo um passo fundamental no esforço conservacionista (Gasparini et al., 1997).

2.2 Toxoplasma gondii

Toxoplasma gondii é um protozoário pertencente ao Filo Apicomplexa, Classe Sporozoea, Ordem Eucoccidiida, Família Sarcocystiidae e ao Gênero *Toxoplasma*. O Filo Apicomplexa é caracterizado pela presença de um complexo apical composto de organelas secretoras especializadas, abrigando patógenos de importância médica e veterinária como o *Plasmodium* spp, *Cryptosporidium* spp. e *Babesia spp* (Dubey et al.,1998b).

Toxoplasma foi descrito inicialmente por Splendore (1908) causando infecções em coelhos, e quase simultaneamente por Nicolle & Manceaux (1908) quando identificado na Tunísia em um roedor da espécie *Ctenodactylus gondi*. Foram esses últimos autores os responsáveis pela determinação do nome da espécie. O nome *Toxoplasma* é derivado de sua forma em lua crescente do (toxon = arco, plasma = forma, grego), (Dubey, 2008). Mesmo com a descrição do protozoário acontecendo no início do século XX, foi apenas em 1970 que o seu ciclo de vida foi completamente elucidado, com a descoberta da fase de reprodução sexual do parasita no intestino delgado do gato (Frenkel et al., 1970). Até então, apesar da transmissão congênita e por carnivorismo já ser aceita, não se podia explicar a infecção adquirida em indivíduos vegetarianos e animais herbívoros (Dubey, 2009).

T. gondii é um parasita intracelular obrigatório e invade diversos tipos de células nucleadas. A interação do parasita com a célula hospedeira acontece mediante dois processos principais: a adesão e a invasão. A baixa restrição por células hospedeiras indica que os ligantes e receptores que mediam a adesão do parasita às células são altamente conservados e igualmente distribuídos (Tomavo,1996). *T. gondii* apresenta três estágios infecciosos: os taquizoítos (em grupos ou clones), bradizoítos (em cistos teciduais) e esporozoítos, presentes no interior dos oocistos (Dubey, 1988). Os oocistos são o produto final da reprodução sexuada que ocorre no trato digestivo dos felídeos, que são seus hospedeiros definitivos. Estes se desenvolvem na presença de oxigênio e em temperaturas mais baixas que a corpórea, permanecendo viáveis por período de tempo variável dependendo das condições ambientais (Dubey, 2020). Os oocistos esporulam no ambiente formando dois esporocistos com quatro esporozoítos cada, e tornando-se uma das formas infectantes. Após a ingestão pelos hospedeiros intermediários os

esporozoítos são liberados no sistema gastrointestinal e invadem a mucosa intestinal tornando-se taquizoítos e migrando para a corrente sanguínea (Gennari et al. 2010). O termo "taquizoíto" foi atribuído por Frenkel (1973) para descrever a forma de proliferação rápida em células do hospedeiro intermediário e em células não intestinais do hospedeiro definitivo. Os taquizoítos possuem forma de meia lua e medem 2-6 µm e se multiplicam assexuadamente na célula do hospedeiro por endodiogenia, uma forma de reprodução em que duas células-filhas se formam do parasita original consumindo-o (Dubey,1998). Após replicações sucessivas em diversos tecidos, os taquizoitos adentram o tecido muscular, encistando na forma de bradizoítos. Estes são a forma de reprodução lenta, e apesar dos cistos teciduais poderem se formar em diversos órgãos incluindo pulmão, fígado e rins, são mais prevalentes em tecidos neurais e musculares como cérebro, olhos e músculo esquelético e cardíaco.

Infecções causadas por *T. gondii* são amplamente difundidas pelo mundo e afetam diversas espécies de animais, incluindo humanos (Dubey, 1998; Tenter et al., 2000; Pappas et al., 2009). A prevalência global da toxoplasmose varia de 10 a 30%, sendo que no Brasil, a proporção de humanos positivos fica em torno de 60% (Pappas et al., 2009). Essa ampla distribuição se deve à característica latente da infecção crônica e à capacidade de *T. gondii* de coexistir benignamente com seu hospedeiro, tornando-o um parasita extremamente bem-sucedido (Dubey, 2009).

As principais formas de transmissão de *T. gondii* são por via transplacentária, por carnivorismo e via fecal-oral. A infecção congênita foi inicialmente descrita em 1939 (Wolf et al., 1939, Dubey 2020) em seres humanos, e mais tarde, foi comprovada em diversas outras espécies, em especial ovinos, caprinos e roedores. A infecção por carnivorismo ocorre por ingestão de carne crua, no caso de seres humanos, e a predação de outras espécies de animais parece ter um papel epidemiológico muito significativo para a toxoplasmose. Os bradizoítos presentes nos cistos são resistentes às enzimas proteolíticas, e apesar da parede do cisto ser logo dissolvida no estômago do hospedeiro, os bradizoítos liberados parecem sobreviver por tempo suficiente para que a infecção ocorra. A descoberta da via fecal-oral foi tardia, e foi comprovada apenas após o esclarecimento do ciclo de vida do protozoário. Essa via é a predominante de infecção adquirida em herbívoros e pessoas vegetarianas. A excreção dos oocistos ocorre nas fezes de felinos após o parasito realizar a fase de reprodução sexuada nas células intestinais do hospedeiro

definitivo (Dubey, 2020). Um único gato pode liberar 20 milhões de oocistos por dia em aproximadamente 20 gramas de fezes (Fayer, 1981) e, após decomposição das fezes, a contaminação do solo pode ser de 100.000 oocistos/grama (Frenkel et al., 1995). Todos os gatos domésticos são susceptíveis à infecção por *T. gondii,* embora animais mais jovens quando infectados pela primeira vez, eliminem um número maior de oocistos nas fezes em comparação com gatos adultos que eliminam oocistos em menor quantidade e por um período mais curto (Lindsay et al.,1997).

Infecções causadas por *T. gondii* em seres humanos possuem alta prevalência em várias regiões do mundo, porém na sua maior parte, são assintomáticas. Entretanto, o parasita pode causar doença severa principalmente no caso de crianças infectadas por via congênita e pessoas com imunossupressão como nos casos de portadores de HIV, e que passaram por transplantes de tecidos (Robert-Gangneux e Dardè, 2012, Torgerson e Mastroiacovo, 2013, Kolören e Dubey, 2020).

A toxoplasmose já foi relatada em diversas espécies de animais domésticos e silvestres com diferentes graus de susceptibilidade entre grupos. Em relação aos ruminantes domésticos, os bovinos raramente apresentam manifestação clínica da doença e casos de aborto são raros (Lindsay e Dubey, 2020). Muitos dos relatos de abortos causados por *T. gondii* na espécie bovina foram na realidade causados por *Neospora caninum* (Dubey e Lindsay, 1996).

Já em ovinos e caprinos, *T. gondii* é considerado como a causa mais comum de abortos, sendo um problema importante na ovinocultura. Abortos consecutivos ocorrendo em um rebanho, geralmente indicam uma fonte comum de oocistos (Dubey e Lindsay, 2020).

Em cervídeos, a doença clínica não foi descrita em animais naturalmente infectados na América do Norte, porém a infecção tem alta prevalência entre os veados-da-cauda-branca (*Odocouileus virginianus*), e o consumo da carne desses animais já foi relacionado com a toxoplasmose em humanos (Sacks et al., 1983; Ross et al., 2001, Lindsay e Dubey, 2020). Formas viáveis de *T. gondii* já foram isoladas de fetos de veados-da-cauda-branca e veado-da-cauda-preta (*Odocoileus heminionus*) em início e meio de gestação (Dubey et al., 2008). Infecção congênita foi observada em renas (*Rangifer tarandus*) de uma propriedade nos Estados Unidos e animais jovens dessa mesma espécie desenvolveram enterite hemorrágica e foram a óbito após infecção experimental (Dubey et al.,2002; Oksanen et al., 1996).

O diagnóstico da toxoplasmose pode ser feito por testes sorológicos, amplificação de sequências específicas de ácidos nucleicos (PCR), demonstração do parasita e antígenos em fragmentos histológicos e pelo isolamento do organismo (Remington et al 2001; Montoya, 2002). Testes sorológicos para pesquisa de anticorpos específicos para *T. gondii* são o método primário de diagnóstico e diferentes testes detectam classes diferentes de anticorpos em estágios distintos da infecção. A detecção de anticorpos IgG é muito utilizada para diagnóstico da doença e estudos epidemiológicos. Em humanos, os anticorpos dessa classe aparecem em torno de uma a duas semanas após a infecção, atingem o pico entre um e dois meses, e declinam em taxas variadas geralmente persistindo por toda a vida do indivíduo.

2.3 *Neospora caninum*

Neospora caninum é um reconhecido protozoário parasita de animais que até 1988 era identificado erroneamente como *T. gondii* (Dubey e Lindsay, 1996). O primeiro relato desse parasita foi feito em 1984 na Noruega, quando foi identificado em cães. Quatro anos depois, Dubey et al. (1988) descreveram o novo gênero e espécie, e esse protozoário emergiu como causador de doenças importantes em gado e cães. O parasita é um coccídeo muito semelhante em estrutura e em ciclo de vida do *T. gondii,* porém com duas principais diferenças: a neosporose é primariamente uma doença de bovinos e o seu hospedeiro definitivo é o cão e outros canídeos, enquanto que a toxoplasmose é uma doença que afeta principalmente ovelhas, cabras e humanos, e os hospedeiros definitivos são os felídeos (Dubey et al. 2007).

O ciclo de vida envolve as três formas do protozoário, o taquizoíto, cistos teciduais e oocistos. Os taquizoítos medem 2 a 6 µm e são encontrados intracelularmente, assim como os cistos teciduais que possuem formato oval e podem atingir 107µm de comprimento. Os cistos são formados por bradizoítos e acometem primariamente o sistema nervoso central e com menos frequência, tecidos extraneuronais como músculos (Dubey et al., 2004, Dubey et al., 2007). A forma resistente ao ambiente são os oocistos eliminados por cães e coiotes (Gondim et al., 2004, McAllister et al.,1998) que esporulam no ambiente. Pouco se sabe sobre quanto tempo os oocistos permanecem viáveis no ambiente, mas pela sua

semelhança com o *T. gondii* é assumido que o tempo de sobrevivência seja parecido (Dubey et al., 2007).

A maior parte dos estudos envolvendo vias de transmissão no *N. caninum* foram realizados com bovinos. As vias a transmissão natural ainda não são completamente elucidadas, porém é sabido que o parasita pode ser transmitido por via transplacentária em vários hospedeiros, sendo a rota vertical é a principal via pela qual o parasita se mantém em bovinos. *N. caninum* é um dos parasitas mais eficientes em transmissão placentária em bovinos, e em certos rebanhos, praticamente todos os bezerros nascidos infectados são assintomáticos (Dubey et al., 2007). Em contraste, existem poucos relatos de transmissão horizontal (evidenciado por soroconversão) nessa espécie (Hietala e Thurmond 1999; Davison et al., 1999). Entretanto, estudos por modelos matemáticos indicam que mesmo taxas baixas de transmissão horizontal em um rebanho de vacas de leite podem ser suficientes para a manutenção da infecção entre rebanhos (French et al., 1999; Davinson et al., 2001). Os cães, hospedeiros definitivos do parasita, se infectam por meio do consumo de tecidos contendo cistos teciduais (Dubey et al., 1998a). A ingestão de oocistos do ambiente é a única via de infecção natural demonstrada em bovinos após o nascimento. Até o momento, não foi comprovada a transmissão por excreções ou secreções de animais adultos assintomáticos (Dubey et al., 2007). Infecções experimentais demonstraram que bezerros podem se infectar após ingerir leite contendo taquizoítos, porém ainda não foi comprovada que a transmissão lactogênica ocorra naturalmente. A transmissão venérea é uma possibilidade, porém apesar do material genético do parasita ter sido encontrado no sêmen de touros naturalmente infectados (Silva et al., 2004; Ferre et al., 2005), estudos sugerem que formas viáveis do parasita no sêmen seriam poucas, se presentes (Canada et al., 2006).

A neosporose causa abortos em bovinos, tanto de leite quanto de corte. Vacas infectadas podem sofrer aborto a partir dos três meses de gestação, sendo que na maior parte dos casos, abortos ocorrem entre o quinto e sexto mês. Os fetos podem ser reabsorvidos, mumificados, natimortos ou até mesmo nascer com sintomatologias clínicas. Vacas soropositivas para *N. caninum* têm mais chances de sofrer abortos do que as soronegativas, porém até 95% dos bezerros infectados por via congênita permanecem assintomáticos (Dubey, 2003). Os abortos podem se manifestar de forma epidêmica ou endêmica. A ocorrência é considerada epidêmica

quando 10% ou mais das gestações acabam em aborto dentro de 6 a 8 semanas (Anderson et al., 1995). Em vacas soropositivas para *N. caninum* os títulos de anticorpos aumentam entre 4 e 5 meses antes do parto, isso sugere uma reativação de uma infecção latente. A característica da infecção intracelular do parasita promove uma resposta inflamatória mediada por células reguladas por linfócitos Th1. Esta resposta é crítica para proteção contra a doença, e é caracterizada pela produção de interferon-gama (IFN-γ) e outras citocinas. Durante a gestação ocorre uma diminuição da resposta imune mediada por células para evitar rejeição do feto. Essa situação causa uma variação no equilíbrio Th1/ Th2 o que pode permitir a reativação dos bradizoítos de *N. caninum* que se diferenciam em taquizoítos permitindo a disseminação do parasita pela placenta e para o feto. Esse desbalanceamento também torna as vacas gestantes mais susceptíveis a primoinfecção do que vacas não gestantes (Marugan-Hernandez, 2017). Infecções experimentais em bovinos mostraram que o feto pode ser infectado quatro semanas após a infecção da mãe, e mesmo em estágios iniciais são observadas lesões em placenta e sistema nervoso central como encefalite (Buxon et al 2002). Porto et al. (2017) observou que cabras infectadas com *N. caninum* que abortavam durante estágios anteriores da gestação apresentavam níveis mais altos de IFN-γ periférico do que as que sofriam abortos em estágios mais avançados, sugerindo que o aborto causado por *N.caninum* pode ter um mecanismo imunomediado no qual uma resposta imune do tipo Th1 desencadeada pela infecção tenha efeitos deletérios para a gestação.

Sinais clínicos da neosporose foram apenas relatados em animais de até dois meses de idade. Os bezerros infectados podem apresentar sinais neurológicos, baixo ganho de peso, dificuldade de se levantar ou nascer sem sinais clínicos da doença. Os membros pélvicos e torácicos podem estar flexionados ou hiperestendidos. Os animais ainda podem apresentar ataxia, reflexos patelares diminuídos, perda de consciência e propriocepção. Exoftalmia e aparência assimétrica dos olhos, além de hidrocefalia também já foram relatados (Dubey, 2003; Marugan-Hernandez, 2017).

O primeiro diagnóstico de neosporose em cervídeos ocorreu em um exemplar de veado-da-cauda-preta (*Odocoileus heminionus columbianus*) de vida-livre encontrado morto na Califórnia (Woods et al.,1994). Pouco depois, Dubey et al. (1996) relataram a infecção em um cervo-de-eld (*Cervus eldi siamensis*) natimorto

em um zoológico da França. Mortalidade perinatal também foi observada em cervos-axis (*Axis axis*) em um zoológico da Argentina, que confirmou o diagnóstico de infecção em um filhote e quatro neonatos que foram a óbito(Walter et al., 2014). Estudos soroepidemiológicos em veados-da-cauda-branca (*Odocoileus virginianus*) mostrou que 40 a 50% das amostras testadas eram positivas para anticorpos para *N. caninum*. Alguns animais apresentavam alta titulação de anticorpos, sugerindo que essa espécie possa participar do ciclo silvático do parasita na América do Norte. A titulação desses animais testados não aumentava com a idade, o que poderia indicar infecção congênita (Dubey et al., 1999). A transmissão congênita foi comprovada depois do parasita ter sido isolado do cérebro de dois fetos de veados-da-cauda-branca, cujas mães eram soropositivas (Dubey et al. 2013). Outros estudos epidemiológicos demonstraram que *N. caninum* isolados a partir de amostras de veados-da-cauda-branca naturalmente infectados, apresentavam sequências de espaçador interno transcrito 1 (ITS1) idênticos àqueles encontrados em amostras obtidas de animais domésticos (Godim et al., 2004; Vianna et al., 2005) sugerindo a ocorrência de transmissão entre animais domésticos e silvestres (Godim et al., 2004).

O diagnóstico da neosporose em casos de aborto depende da identificação de anticorpos específicos no sangue da mãe e identificação do agente em tecidos do feto e placenta. A técnica de imuno-histoquímica é necessária, pois frequentemente há pouco *N. caninum* no tecido autolisado e são perceptíveis de forma esporádica pela histologia. O teste de PCR pode ser utilizado, porém sua eficiência vai variar conforme o procedimento de colheita de amostra e grau de autólise dos tecidos. Técnicas sorológicas são muito úteis no diagnóstico da infecção e incluem vários tipos de ELISAs, Imunofluorescência indireta, e Teste de aglutinação de *Neospora* (TAN). O nível de anticorpos definitivo para ser considerado diagnóstico de neosporose ainda não foi estabelecido para bovinos. Em testes sorológicos, os títulos e valores de absorbância dependem da composição dos antígenos, anticorpos secundários e outros reagentes (Dubey, 2003).

2.4 Imunoglobulinas específicas fecais

Embora os mamíferos possuam uma extensa variedade de mecanismos de defesa de imunidade inata e adaptativa nos tecidos, é nas superfícies que os

microrganismos invasores são primeiramente encontrados e amplamente repelidos ou destruídos. A parede intestinal contém linfócitos B (ou células B) que se dividem repetitivamente em resposta ao antígeno. Alguns desses linfócitos B migram para os linfonodos regionais e para os vasos linfáticos intestinais, de onde alcançam o ducto torácico e entram na circulação sanguínea. Esses linfócitos B IgA-positivos circulantes possuem afinidade por todas as superfícies corpóreas, portanto, as superfícies do aparelho gastrointestinal e respiratório são preenchidas por células plasmáticas produtoras de IgA em associação com a cadeia J, um polipeptídio de 15 Da. A cadeia J está ligada pelo terminal carboxila de 18 aminoácidos com IgA e IgM levando a formação da imunoglobulina polimérica. As imunoglobulinas poliméricas (pIg) são transportadas ativamente através do epitélio pelo receptor de imunoglobulinas poliméricas (pIgR). Este se encontra em altos níveis no duodeno, jejuno, e colón e em baixos níveis no pulmão, rim, pâncreas e endométrio. (Strugnell; Wijburg, 2010). A IgA produzida em mucosas é transportada pelas células epiteliais para secreções externas. A maior parte de IgA produzida na parede intestinal, por exemplo, é carreada para o fluido intestinal. (Tizard, 2014). A IgA predomina nas secreções de superfície. Ela é encontrada em quantidades significativas em saliva, fluido intestinal, secreções nasal e traqueal, lágrimas, leite, colostro, urina e secreções do trato urogenital (Macpherson et al., 2008).

Apesar da detecção por teste de ELISA específico para IgA fecal não ser um método diagnóstico usado rotineiramente, alguns estudos mostram resultados positivos de detecção de anticorpos contra antígenos específicos nas fezes e secreções intestinais. Anticorpos IgA anti-Norwalk vírus foram detectados nas fezes de seres humanos antes e depois de uma infeção experimental (Okhuysen et al.,1995). Esse tipo de teste também foi usado para avaliar a eficácia resposta imune protetora contra agentes patogênicos como no estudo de Lee et al. (1995), no qual avaliou-se a resposta imune secretória e sistêmica contra *Helicobacter sp.* em ratos usando ELISA para detecção de anticorpos IgA anti-urease presente em secreções intestinais. Em outro estudo, objetivou-se avaliar a resposta imune secretória contra *Cryptospiridium* em pacientes humanos positivos para HIV, no qual foram analisados os níveis de IgA e IgM anti-*Cryptosporidium* nas fezes dos pacientes (Benhamou et al.,1995).

Anticorpos específicos IgA anti-trofozoitos de *Giardia lamblia* foram encontrados e mensurados nas fezes de ratos infectados experimentalmente

(Heyworth et al., 1988), e no estudo de Chardes et al. (1990) foram verificados os níveis de anticorpos IgA, IgM, e IgG fecais específicos contra *Toxoplasma gondii* em ratos infectados por via oral. Os autores foram capazes de detectar títulos altos IgA e IgG contra toxoplasma nas secreções intestinais dos camundongos, sendo que os títulos de IgA apareceram no 21º dia de infecção, atingiram níveis máximos no 49° dia e persistiram em títulos mais baixos até o final do estudo de 17 semanas.

A IgA pode não ser a única imunoglobulina secretória com importância diagnóstica. O estudo de Besser et al. (1988) demostraram que 68% da depuração de IgG1 sanguínea em bezerros ocorre pelo trato gastrointestinal. Tendo isso em vista, Carvalho et al. (2012) avaliaram a técnica dosagem fecal de IgG e propuseram a mesma como forma de monitoramento não invasivo da resposta imune humoral em camundongos.

O uso de amostras fecais para pesquisa de anticorpos como método não invasivo para estudo epidemiológico de animais de vida-livre já foi proposto no estudo de Nieto-Pelegrın et al. (2015). Nesse experimento, suínos foram infectados com o vírus da peste suína africana (ASFV), e os níveis de anticorpos fecais foram comparados com o do sangue. O estudo obteve uma correlação satisfatória,e tendo em vista a importância desse vírus em populações selvagens, os autores sugerem a pesquisa de anticorpos fecais contra o ASFV para monitoramento dessa doença infecciosa em javalis selvagens.

A obtenção de material fecal em substituição a sangue e tecidos apresenta diversas vantagens, tanto do ponto de vista econômico, quanto de facilidade de acesso ao material e bem-estar dos animais envolvidos. Diversos estudos têm sido realizados com técnicas genéticas não invasivas utilizando-se as fezes de cervídeos brasileiros (González et al., 2009; González e Duarte, 2007; Oliveira e Duarte, 2013). Além de material genético também é possível mensurar hormônios nas fezes desses animais (Krepschi et al. 2013; Pereira et al., 2005). As técnicas para a obtenção de fezes variam conforme a espécie e o habitat, podendo ser usados colheita após a observação da defecação pelo animal, identificação de latrinas e trilhas deixadas por alguns cervídeos, atração do animal com alimento palatável (González e García, 2010) e rastreamento do material fecal com o uso de cães treinados (González; García, 2010; Duarte et al., 2016; Oliveira et al., 2012).

3 OBJETIVOS

CAPÍTULO 2:

- Estimar a prevalência de animais positivos para *T. gondii* e *N. caninum* nos plantéis de cervídeos do Núcleo de pesquisa e conservação de cervídeos (NUPECCE) e Centro de Conservação do cervo do pantanal (CCCP).

- Avaliar a probabilidade das vias de transmissão vertical e horizontal do *T. gondii* e *N. caninum* em cervídeos.

CAPÍTULO 3:

- Descrever a sintomatologia clínica e resposta imune humoral de machos de veados-catingueiros (*Mazama gouazoubira*) infectados experimentalmente com oocistos da cepa ME49 de *T. gondii*.

- Avaliar a possibilidade de transmissão sexual do *T. gondii* em cervídeos buscando o DNA do parasito no sêmem dos animais experimentalmente infectados.

CAPÍTULO 4:

- Padronizar a técnica de ELISA indireta para detecção de anticorpos fecais específicos para *T. gondii* e *N. caninum*.

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CAPÍTULO 2 - Assessment of transplacental transmission of *Neospora caninum* and *Toxoplasma gondii* in Neotropical deer: an estimative based on serology[1]

Highlights

- Congenital transmission rate of *Neospora caninum* appears to be high in South-American deer species.

- There is no evidence of congenital transmission of *Toxoplasma gondii* in the present study.

- Indirect fluorescent antibody test and ELISA developed "in house" had a better agreement for *N. caninum* than for *T. gondii*.

Abstract - Transplacental transmission of *Neospora caninum* and *Toxoplasma gondii* is well known in some domestic species and despite both parasites having been proved to infect deer fetuses during gestation, the congenital transmission rate in South-American deer species is still unknown. This study aimed to estimate the congenital transmission rate of neosporosis and toxoplasmosis in captive deer populations from Brazil, through serological techniques (IFAT and ELISA). Serum samples from 82 deer were tested by both techniques 20,73% (IFAT) to 25,60% (ELISA) were seropositive for *T. gondii*; the kappa test showed an index of 0,277 of agreement between both techniques. For *N. caninum*, 40,24% (IFAT) to 39,02% (ELISA) were seropositive, with an index of 0,833 of agreement between techniques. Family trees for each species were drawn and we estimated the congenital transmission rate of the diseases. We found a rate of 81,25% congenital transmission of *N. caninum* and no evidence of congenital transmission of *T. gondii*. Vertical transmission appears to be the main route of introduction and maintenance of *N. caninum* in these captive deer populations, while *T.gondii* seems to be favored by the horizontal route.

Keywords: Congenital transmission; Coccidiosis; serologic surveys; cervids.

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[1] Este capítulo corresponde ao artigo científico submetido à revista Veterinary Parasitology

1 INTRODUCTION

Neospora caninum and *Toxoplasma gondii* are closely related species of cyst-forming coccidian parasites and are known to infect a variety of warm-blooded animals (Dubey and Lindsay, 1996; Lindsay and Dubey, 2020). Both Neosporosis and Toxoplasmosis are diseases of great importance in domestic ruminants, mainly causing reproductive impairment (Bártová et al., 2009).

Neosporosis is primarily a disease of cattle; dogs and related canids are the definitive hosts of the parasite. *N. caninum* is one of the most efficiently transplacentally transmitted parasites among all known microbes in cattle and in some herds, virtually all calves are born infected but asymptomatic (Dubey et al., 2007). Cows infected by this parasite before pregnancy may suffer abortion, suggesting that immunity acquired in the first exposure to *N. caninum* is not enough to protect them from disease (Stenlund et al., 1999).

The first report of Neosporosis in deer occurred in California, where two black-tailed deer (*Odocoileus heminionus columbianus*) found dead in the wild were diagnosed through necropsy (Woods et al 1994). *N. caninum* tissue cysts were also found in the brain of a full-term stillborn Eld's deer (*Cervus eldi siamensis*) in a zoo in Paris (Dubey et al., 1996). However, congenital transmission of *N. caninum* in white-tailed deer has only been verified when viable *N. caninum* was isolated from the brains of two fetuses (Dubey et al., 2013). The parasite was also diagnosed at axis (*Axis axis*) fawn and neonates in Argentina suggesting its association with perinatal mortality in captive axis deer (Basso et al., 2014).

Similar to *N. caninum*, *T. gondii* also causes reproductive impairment and abortions on domestic ruminants, but this protozoan is primarily a pathogen of humans, goats and sheep. Both parasites have a similar structure and life cycles but felids are the only definitive hosts of *T. gondii* (Dubey et al., 2007). In sheep, infection with this parasite may cause early embryonic death and reabsorption, fetal death and mummification, abortion, stillbirth, and neonatal death. The severity of infection is associated with the pregnancy stage at which the ewe becomes infected (Dubey, 2009).

Congenital transmission rates in sheep vary by study. Research conducted using PCR revealed a congenital transmission rate of 65% of pregnancies with a higher level (91%) in unsuccessful pregnancies (Ducanson et al., 2001; Williams et

al., 2005; Hide, 2016). In deer, congenital transmission was also identified when viable *T. gondii* was isolated from white-tailed deer (*Odocoileus virginianus*) fetuses (Dubey et al., 2008b).

Despite reports of congenital neosporosis and toxoplasmosis in cervids, there is no data about vertical transmission rate in neotropical deer species. The present study aimed to estimate the probability of congenital transmission of both parasites in captivity deer in Brazil. Usually, transplacental transmission can be estimated from the number of seropositive offspring born from seropositive mothers, soon after their birth and before ingestion of colostrum (Paré et al., 1996). However due to the difficulty in handling these animals we used a different approach, drawing family trees for each species in order to identify patterns and assessing the probability for vertical and horizontal transmission of *T. gondii* and *N. caninum* on these deer populations.

2 MATERIALS AND METHODS

2.1 Ethical approval

The study was approved by the Animal Ethics and Welfare Committee (Comitê de Ética e Bem-Estar Animal, CEUA, Protocol number: 003732/18) of the Faculty of Agrarian and Veterinary Sciences (Faculdade de Ciências Agrárias e Veterinárias, FCAV), UNESP, Jaboticabal, SP, Brazil.

2.2 Experimental design

This study was carried out at São Paulo State University (UNESP)/Jaboticabal, Brazil. Blood samples from 82 captivity deer from two breeding centers (Deer Research and Conservation Center – UNESP/ SP and Marsh Deer Conservation Center – Tijoá/ SP) were used in this study. In total, 16 brown brocket deer (*Mazama gouazoubira*), 5 amazonian brown brocket (*Mazama nemorivaga*), 18 red brocket (*Mazama americana*), 5 small red brocket (Mazama bororo), 4 Brazilian dwarf brocket (*Mazama nana*), 4 pampas deer (*Ozotoceros bezoarticus*), 2 white-tailed deer (*Odocoileus virginianus cariacou*) from Deer Research and Conservation Center in Jaboticabal – SP; and 28 marsh deer (*Blastocerus dichotomus)* from Marsh Deer Conservation Center in Promissão- SP were used in the experiment.

Animals were physically or chemically restrained with an association of xylazine hydrochloride 2% [Rompum® 1 mg/kg i.m.] and ketamine 10% [Dopalen® 7 mg/kg i.m.] during blood collection. Also, females Marsh Deer allowed the blood

collection during estrus reflex . Blood samples were collected by venipuncture of the external jugular, using the Vacutainer® system (B.D. Indústria Cirúrgica, Juiz de Fora, MG), in silicon-treated tubes without anticoagulants. Samples were then centrifuged at 3.500 rpm for 10 minutes for serum obtainment, which were aliquoted in polypropylene microtubes and then kept frozen at -20°C until the time of use.

2.3 Indirect fluorescent antibody test (IFAT) for *N. caninum* and *T. gondii*

For IFAT, slides containing tachyzoites of *N. caninum/T. gondii* (IMUNODOT®, Jaboticabal - São Paulo, Brazil) in marked wells were used. Ten serum samples were tested on each slide and were compared with negative and positive controls. Test serum samples and positive e negative controls were diluted at 1:40 for *T. gondii* and 1:25 for *N. caninum* (Dubey et al., 2008a). After dilution in phosphate-buffered saline solution (PBS) (pH 7.2; 1.3 M NaCl, 27 M KCl, 56 mM Na2HPO4, 10 mM KH2PO4 and 9.2 mM NaH2PO4), 10 µL of diluted serum samples were deposited in each slide well. The slides were then incubated at 37 °C for 30 minutes in a humid chamber. After incubation, slides were washed three times with PBS for five minutes, consecutively, and then dried at room temperature. Following this, 10 µL of conjugate (anti-deer-IgG FITC-labeled SeraCare®, Milford, MA, United States) diluted in PBS containing Evans Blue 10%, at 1:20 was added to each slide well. Then, slides were incubated in a humid chamber again, at 37 °C for 30 minutes, with subsequent washing, as described previously.

After the slides had been dried at room temperature, buffered glycerin was added (glycerin and 0.5 M carbonate-bicarbonate buffer at pH 9.6), and then covered with coverslips, for observation at a magnification of 400X, under a microscope equipped with fluorescent light (Olympus BX-FLA). Test positivity was determined through observation of total peripheral fluorescence of the tachyzoites (Paré et al., 1995).

2.4 Enzyme-Linked Immunosorbent Assay (ELISA) for *N. caninum* and *T. gondii*

Indirect ELISA tests to *N. caninum* and *T. gondii* were developed in house adapted from protocols described by Silva et al. (2007). To sensitize the ELISA plate (NUNC MaxiSport®), 5 µg/mL and 2,5µg/mL of crude soluble antigen of *N. caninum and T. gondii* respectively were diluted in carbonate-bicarbonate buffer (pH 9.6;

0.5M). One hundred microliters of the diluted antigen was added to each plate well and then were incubated for 12-14h in a humid chamber at 4 °C. After this period, the excess antigen was discarded and the plates were washed with PBS Tween-20 three times and the plate was blocked using 1% bovine serum albumin (BSA) in PBS Tween-20. For every plate well 200 µL of the blocker was added followed by incubation in the humid chamber at 37 °C for 1:30 hour. After removal of the blocking solution, the plate was washed three times with PBS Tween-20. Positive and negative controls were selected from samples previously tested by IFAT. One hundred microliters of the serum diluted at 1:200 (*N. caninum*) and 1:100 (*T.gondii*) in PBS Tween-20 were added to each well and, once again, the plate was incubated in the humid chamber at 37 °C for one hour with subsequent washing as previously described.

One hundred microliters of anti-deer-IgG peroxidase-labeled (KPL, Gaithersburg, MD, USA), diluted at 1:2000 (*N. caninum*) and 1:1000 (*T. gondii*) in PBS Tween-20, was added to each plate well. Plates were then incubated and washed in the same manner as described previously. One hundred microliters of the enzyme substrate 3,3′, 5,5′-Tetramethylbenzidine (SureBlue™ TMB 1-Component Microwell Peroxidase Substrate) was added to each well.

Plates were incubated at room temperature protected from the light for 15 minutes. The reaction was stopped by adding 50µl of 3 M H2SO4 per well. Absorbance at 450 nm was determined using a TP-Reader-Basic (Thermo Plate®, China).

2.5 Animals genealogy and statistical analysis.

We designed family trees for each species evaluating the compared serology results of mothers and offspring. Congenital transmission rate for each disease was estimated by the number of seropositive offspring from seropositive mothers, compared to the total offspring from positive mothers. The data was expressed as measurements of relative frequency.

The cut-off point for ELISA was calculated based on the arithmetic mean of the percentage of optical density (%OD) from 10 serum samples negative by IFAT technique plus three standard deviation with the formula: cut-off point = (m%OD) + 3s (Paredes et al., 1999).The coefficient of concordance between IFAT and ELISA

was estimated using the kappa test (Landis and Koch, 1977). The results were analyzed on the R Core Team (2020).

3 RESULTS

3.1 IFAT and ELISA

Of the total number of evaluated animals, 20,73% (17/82) and 25,60% (21/82) were seropositive for *T. gondii*, according to IFAT and ELISA respectively. Kappa test was performed showing an index of 0,277 (fair correlation) with an agreement of 74%. For *N. caninum*, 40,24% (33/82) of animals tested positive by IFAT, compared to 39,02% (32/82) on ELISA test. Kappa test revealed an index of 0,833 (almost perfect) with an agreement of 92,1%. Interpretation of the results was carried out as suggested by Landis and Koch (1977).

3.2 Congenital transmission rate

Seven of the 20 female breeders tested were positive for *N. caninum,* and they gave birth to 16 offspring in total. The estimated congenital transmission rate for *N. caninum* was at least 81,25% (12/16). Only two breeding females were positive for *T. gondii*, and congenital transmission did not seem to have occurred (0%).

The family trees are represented in Figures 1 to 3.

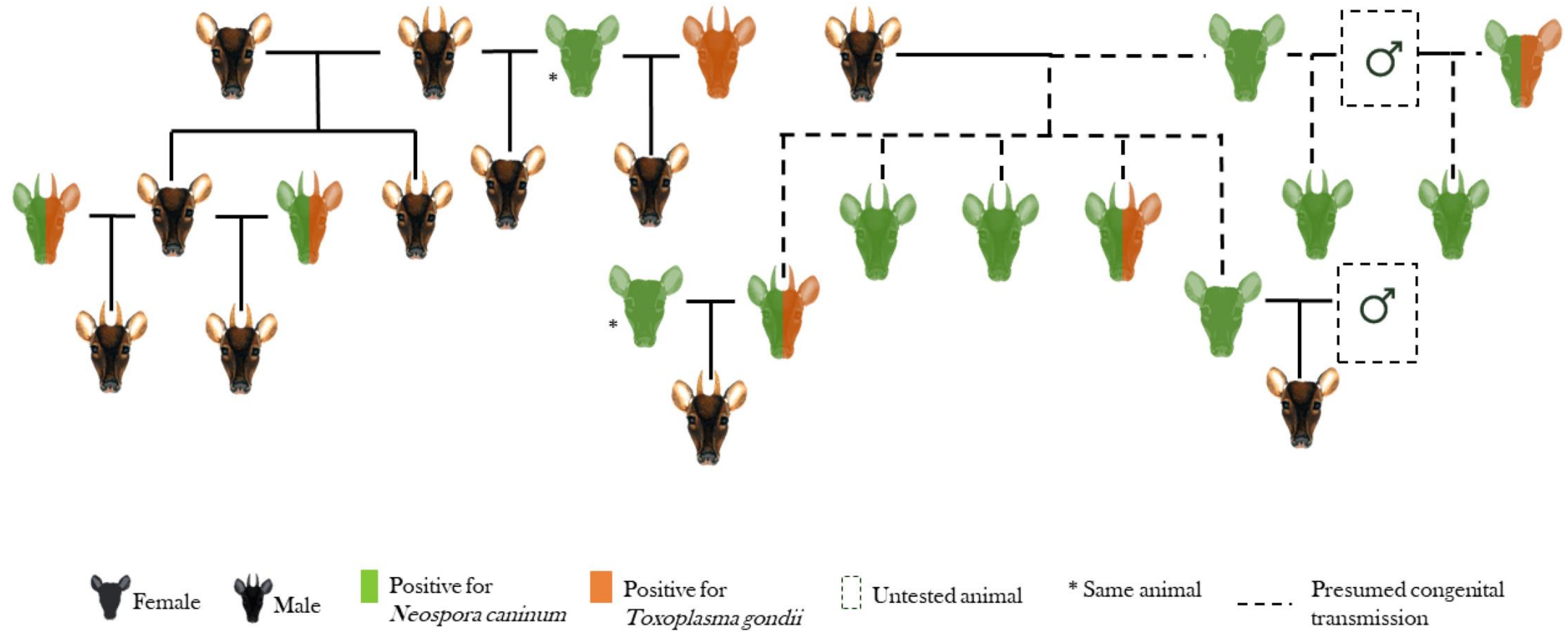

Figure 1. Family tree of red brocket (*Mazama americana*) from Deer Research and Conservation Center – UNESP/ SP.

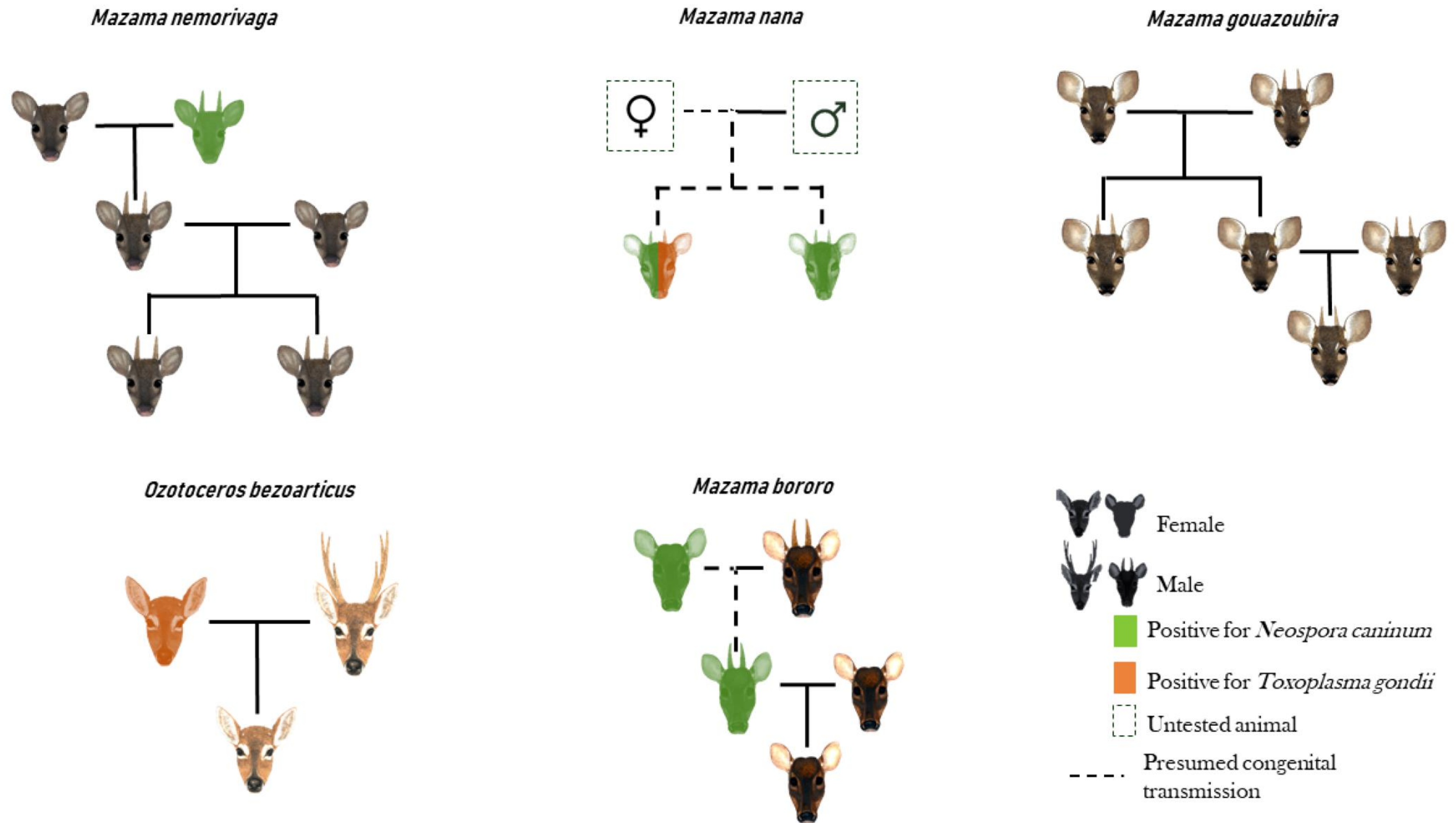

Figure 2. Family tree of brown brocket deer (Mazama gouazoubira), pampas deer (Ozotoceros bezarticus), amazonian brown brocket (Mazama nemorivaga) and dwarf red brocket (Mazama nana) and small red brocket (Mazama bororo) from Deer Research and Conservation Center – UNESP/ SP.

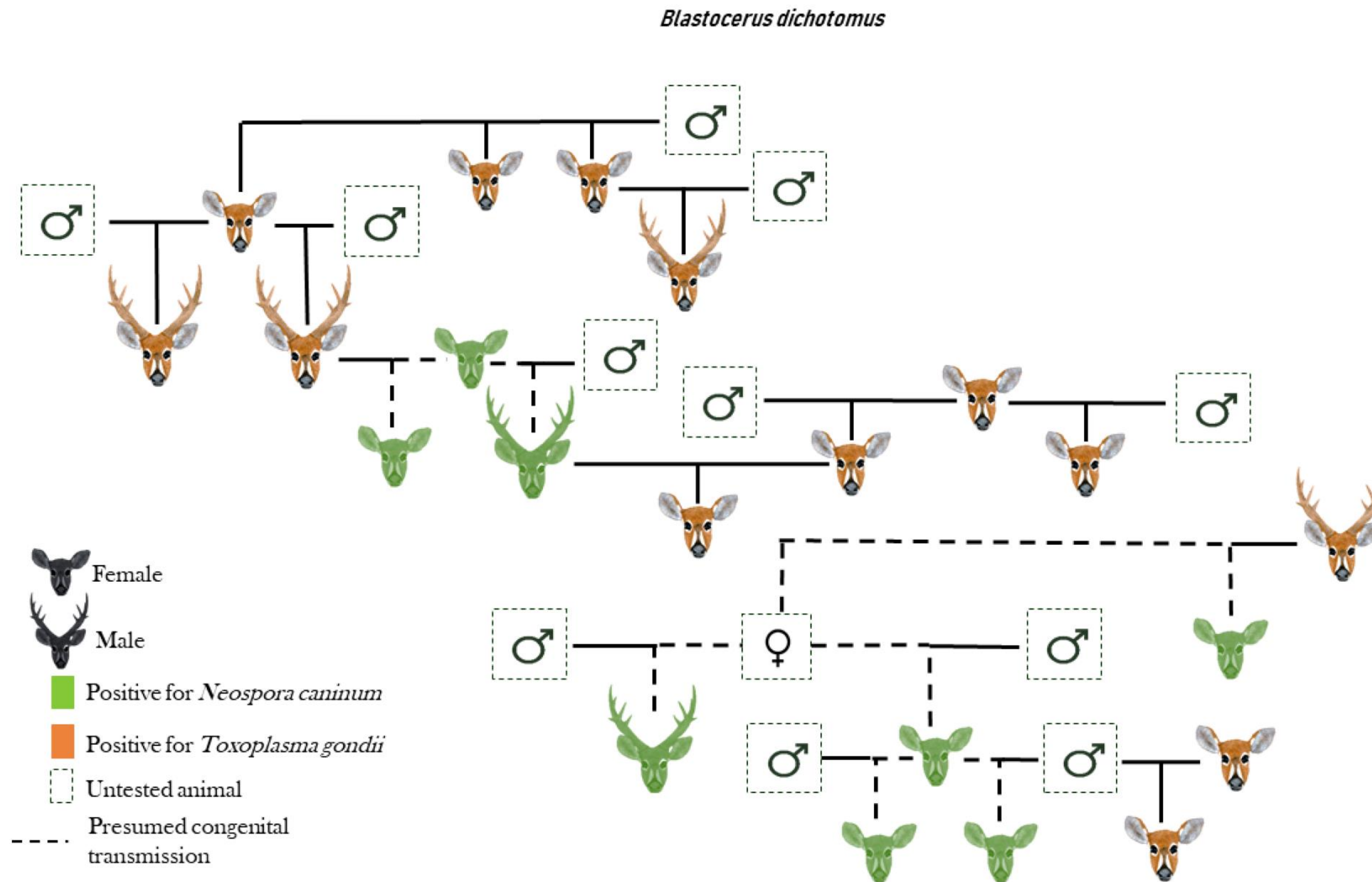

Figure 3. Family tree of marsh deer (Blastocerus dichotomus) from the Marsh Deer Conservation Center – Tijoá/ SP)

4 DISCUSSION

The seroprevalence found in the present study varied according to the diagnostic technique, for *T. gondii* 20,73% to 25,69% of the animals were positive, while for *N. caninum* frequencies were between 39,02% to 40,24%. IFAT has been the choice test for the diagnosis of toxoplasmosis (OIE 2018) and was the first serologic technique developed for the diagnosis of neosporosis (Dubey et al., 1988). Therefore, we used IFAT as the gold standard to select the positive and negative controls and to calculate the cut-off point for ELISA.

Cohen's kappa coefficient (ϰ) showed a better agreement between IFAT and ELISA for *N. caninum* tests (ϰ= 0,83) than *T. gondii* (ϰ = 0,277). The agreement between the two techniques vary by study, for *T. gondii* high values (ϰ=0,85 and ϰ=1) were found for IFAT and ELISAs developed “in house” (Sroka et al., 2008; Pereira-Bueno et al. 2004). For *N. caninum,* low ϰ values were found using dog (ϰ = 0,30) and swine (ϰ = 0,35) samples (Silva et al. 2007; Ramos et al., 2016) while substantial agreement (ϰ =0,75) was found in another study with goat samples (Kim et al., 2019). The found seropositivity rates were within the values obtained in other surveys of South American deer. For *T. gondii*, seroprevalence in free-living animals were 27,3% in marsh deer (*Blastocerus dichotomus*), 12,2% in pampas deer (Ozotocerus bezoarticus) (Ferreira et al., 1997), and 40% in brown brocket deer (*Mazama gouazoubira*) and red brocket deer (*Mazama americana*) (De Thoisy et al.,2003). In a captive Brazilian dwarf brocket (*Mazama nana*) population in the south of Brazil, 15,2% of animals were positive. *N. caninum* surveys found positivity in 13% and 75% of pampas deer in Goiás, Central Brazil and in the Pantanal biome respectively (Tiemman *et al., 2005b*). In captivity, 6,2% of Brazilian dwarf brocket tested positive (Zimpel et al., 2015) and a total of 42% of deer from several zoos and breeding centers across Brazil were positive to *N. caninum* (Tiemman et al., 2005a).

The transplacental transmission rate of *N. caninum* was high (81,25%), however, there was no evidence of transplacental transmission of *T. gondii.* We are aware of the interpretation limitation of using samples from adults to estimate transplacental transmission, still, the extremely reactive and elusive behavior of these species makes it nearly impossible to collect blood samples from newborn fawns. Nevertheless, besides the positivity of the offspring from seropositive mothers, some

other patterns can be observed in the family trees that support high rates of congenital transmission of *N. caninum* but not *T. gondii* on these captivity copulations:

- None of the animals positive to *N. caninum*, with known genealogy, had negative mothers. That happened two times with *T. gondii*.
- All the maternal siblings had the same results (all positive/ all negative) to *N.caninum*, but not necessarily to *T. gondii*.
- Only two females positive to *N.caninum* did not seem to have transmitted the parasite to their offspring.

To the author's knowledge, no other studies assessed congenital transmission rate based on serology of living deer, however, the high seroprevalence of *N. caninum* in fawns and the lack of association between seroprevalence of *N. caninum* antibodies and the age of deer observed in other studies suggest that *N. caninum* might be congenitally transmitted in deer, similar to bovine infections (Dubey et al., 1999). Endogenous transplacental transmission is the main mechanism responsible for perpetuation of neosporosis in cattle herds due two different factors. On one hand, the transmission is highly efficient, so that most chronically infected cows give birth to infected calves. On the other hand, neosporosis rarely causes abortion or clinical signs in offspring, so that the infected female calves are often reared for breeding and continue to transmit the infection during future pregnancies (Dubey et al., 2006, Dubey et al, 2007; González-Warleta et al., 2018). The rate found in the present survey is within the range described in cattle, between 40 and 100% (Dijkstra et al, 2008; Dubey et al., 2007). In small ruminants vertical transmission rate of *N. caninum* is discussed with a low rate (15,4%) found in naturally infected animals (Filho et al., 2017). Another study involving three generations of ewes naturally infected by *N. caninum*, demonstrated that endogenous transplacental transmission may also be highly efficient in the ovine host since the congenital infection rate ranged between 66.7% and 93%, with higher rates in the first generation after the infection, and lowest in the subsequent generations (González-Warleta et al., 2018).

Two possible scenarios can be envisaged when it comes of congenital transmission: firstly, primary exposure of the female during pregnancy followed by transmission to the fetus during pregnancy (exogenous transplacental transmission)

for *T. gondii* and *N. caninum*, or secondly, reactivation of chronic infection in the female during pregnancy (endogenous transplacental transmission) for *N. caninum*. There is currently contention as to the importance and relevance of congenital transmission in toxoplasmosis (Williams et al., 2005; Trees and Williams, 2005). Still, vertical transmission of *T. gondii* in sheep is well established and it is reported in other animal species (Hide et al., 2009). Though congenital transmission rate varies according to the study and diagnosis technique (Dubey, 2009) the general acceptance is that less than 4% of sheep persistently transmit it to their offspring (Dubey and Beattie, 1988; Bruxton et al., 2007). Despite that, some studies found a high (31%) congenital transmission in chronically infected sheep (Chiebao et al., 2019) and an incidence of 43-69% in sheep flocks sampled over 3 years (Williams et al., 2005).

Our findings did not demonstrate signs of *T. gondii* congenital transmission in South American deer. Our results support the findings in alpine red-deer (*Cervus elaphus*), wherein high seroprevalence was recorded in older animals but not in calves suggesting horizontal transmission as the main route of infection (Formenti et al., 2015). The same findings were observed in other serological surveys for *T. gondii* in cervids (Vanek et al., 1996; Oksanen et al., 1997; Zarnke et al., 2000; Vikøren et al., 2004). On the other hand, viable *T. gondii* was isolated from 17,4% of white-tailed deer fetuses sampled in a survey from Iowa and Minnesota- US (Dubey et al., 2008b). This suggests that congenital transmission of *T. gondii* in deer may occur with relative frequency.

The discrepancy in these results may be related to the diagnostic technique; some studies showed that serological tests were less efficient at detecting vertical transmission than the PCR, for example (Hide, 2016). Nevertheless, our results agree with other serological surveys in deer species, suggesting that the horizontal route is more likely to be the source of toxoplasmosis in these populations of captive deer.

5 CONCLUSION

The evidence shows that congenital transmission plays an important role in the maintenance of *N. caninum* in South-American deer species, similar to what occurs in cattle. However, there is no evidence of vertical transmission of *T. gondii* in these animals, suggesting that horizontal transmission is probably the main route of

infection of this parasite. The impact of neosporosis and toxoplasmosis in neotropical deer populations is yet to be elucidated.

Credit authorship contribution statement

Maria Helena Mazzoni Baldini: conceived and designed the research; carried out the data collection and laboratory work, wrote and revised the paper and artwork. Eluzai Dinai Pinto Sandoval: carried out statistical analysis, revised writing and artwork. José Maurício Barbanti Duarte: administered and supervised the project, revised all the work and writing.

Declaration of Competing Interest: The authors declare that they have no known competing interests.

Acknowledgments

We thank IMUNODOT Diagnósticos Ltda. and Professor Rosângela Zacarias Machado for the support with lab reagents. We also thank Biol. Natalia Aranha de Azavedo for the artwork used in the figures.

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Capítulo 3- Toxoplasmosis in brown brocket deer (*Mazama gouazoubira*): reproductive and clinical evaluation following experimental infection [2]

Highlights

- Experimental toxoplasmosis did not cause clinical signs in male Brown brocket deer (*Mazama gouazoubira*).

- Two of the four infected animals presented detectable antibodies anti-*T. gondii* by indirect ELISA test.

- Seminal parameters do not seem to be affected by the experimental infection.

- *Toxoplasma gondii* was detected in seminal samples by PCR, suggesting sexual transmission.

Abstract - Little is known about Toxoplasmosis in neotropical deer and its implication in ex-situ conservation efforts. These animals have an elusive behavior and their management in captivity is challenging, which implicates in a lack of studies regarding the impact and distribution of parasitic diseases in these species. This study aimed to assess the effect of experimental *Toxoplasma gondii* infection on brown brocket deer (*Mazama gouazoubira*). clinical and reproductive parameters in male In total, five males were used in the study. Four of them (Male 1 – M1, Male 2 - M2, Male 3- M3 and Male Four – M4) were allocated to the experimental group, whereby each individual has received sporulated oocysts of *T. gondii*, and one (Male 0 - M0) was the control group. We found no evidence of sickness in the experimental group, and two individuals developed antibody responses against *T. gondii* detected by indirect ELISA test. The fluctuation of the seminal parameters such as sperm concentration and motility were not related to the experimental infection, but the PCR analysis revealed that *T. gondii'*s DNA was present in semen samples of both seropositive animals at 35 and 49 days after inoculation. This is the first report of *T. gondii* in cervid semen and this finding suggests that sexual transmission may be a viable route of infection for these animals.

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[2] Este capítulo corresponde ao artigo científico submetido à revista International Journal for Parasitology: Parasites and Wildlife

Keywords: Protozoan; neotropical deer; disease resistance; sexual transmission

1 INTRODUCTION

Medical veterinary sciences have developed a broad knowledge base regarding the pathological effects of parasitism on domestic animal hosts, but this information is rarely transposed to wildlife, and few studies provide knowledge about how parasites affect wildlife and clinical manifestations of infection in those species (Formenti et al. 2015). Experimental infections that evaluate diseases in Neotropical deer are scarce and most of the information we know about these species is due to surveys of naturally infected free-living or captive animals (de Thois et al. 2003; Vieira 2011; Zimpel et al. 2015).

The brown brocket deer (*Mazama gouazoubira*) is a small specie of brocket deer known to occur from southern Mexico to northern Argentina, including much of the Brazilian territory. Despite that, authors still discuss the species' presence in the Amazon forest's region, so more recent geographic distribution studies show its presence south of Amazon (Duarte et al., 2012). The species is classified as Low Concern (LC) by IUCN's Red List (Black-Decima and Vogliotti, 2016). Differently, from most brocket deer species, the brown brocket deer easily adapt to cultivated land, as long as there are small forest areas available for shelter (Pinder and Leeuwenberg 1997). This adaptable behavior brings these deer close to domestic animals, and thereafter, diseases and pathogens. The brown brocket deer is probably the most frequent species of brocket deer found in captivity, with many specimens in zoos and private breeding collections in Brazil.

Toxoplasma gondii is an intracellular protozoan widely spread around the world. Felines including domestic cats act as the definitive host, and various warm-blooded animals like cervids act as intermediate hosts. *T. gondii* is known to cause reproductive disorders in small ruminants (Innes et al., 2009; Lidsay and Dubey, 2020), and despite the effects of reproductive impairment in females being widely reported, only recently some studies have given more importance to toxoplasmosis effects in males, both animal and human (Dalimi and Abdoli, 2013).

Sexual transmission in domestic species has been documented and some works highlight the presence of the parasite in seminal samples of goats (Santana et al., 2010), rams (Lopes et al., 2009b), and bulls (Scarpelli et al., 2009). This transmission route may cause a significant impact on the disease dynamics in

breeding centers for wildlife and free-living populations. Although there are no reports of clinical signs of toxoplasmosis in naturally infected cervids, experimental infections caused acute disease in mule deer (Dubey et al., 1982) and reindeer (*Rangifer tarandus*), (Oksanem et al., 1996). There are also reports of clinical manifestation of toxoplasmosis in pronghorn (*Antilocapra americana*) (Dubey, 1982) and bighorn sheep (*Ovis canadensis canadensis*) (Baszler et al., 2000). Little is known about the consequences of toxoplasmosis neotropical deer species, since only serological studies have been published, and data about disease susceptibility and transmission routes are scarce. In the present study, we evaluated clinical and reproductive parameters of male brown brocket deers (*Mazama gouazoubira*) experimentally infected with *T. gondii* oocysts.

2 MATERIALS AND METHODS

2.1 Ethical approval

The study was approved by the Animal Ethics and Welfare Committee (Comitê de Ética e Bem-estar Animal, CEUA, Protocol number: 06892/19 of the Faculty of Agrarian and Veterinary Sciences (Faculdade de Ciências Agrárias e Veterinárias, FCAV), UNESP, Jaboticabal, SP, Brazil.

2.2 Experimental design

This study was carried out at the Deer Research and Conservation Center, São Paulo State University (UNESP)/Jaboticabal, Brazil. In total, five males (ages 2–7 years old) were used. All animals tested negative for the presence of *T. gondii* antibodies in serum by an indirect ELISA test developed in-house prior to the study.

The animals were housed individually in stalls (4 × 3 m). Animals weighing 16,5–19.5 kg were fed with pelleted equine ration (Essence Traditional – Presence®, 400 g), forage and water *ad libitum*.

From the five animals used in the study, four received the oocysts and one was used as a control.

Clinical parameters were taken from day -7p.i to 40 p.i. every morning. Positive reinforcement using banana pieces was applied to get the animals accustomed to the evaluator’s presence and touch. At days -7, 7, 21, 35 and 49 p.i the animals were chemically restrained with an association of xylazine hydrochloride 2% [Rompum® 1 mg/kg i.m.] and ketamine 10% [Dopalen® 7 mg/kg i.m.] for blood and semen

collection. On days 14, 28, and 42 p.i. the animals were physically restrained for blood collection.

2.3 Experimental infection procedure

Toxoplasma gondii oocysts used for infection were obtained from cat feces from the parasitology laboratory of the State University of Londrina (UEL). A solution of ME49 II strain sporulated oocysts ($5x10^3$) was administered in a piece of banana to four animals, identified as M1, M2, M3 and M4 while the control animal (M0) received a banana piece without the oocyst solution at the same time.

2.4 Clinical Parameters

Animals were monitored daily for clinical signs, febrile response, reactive behavior, respiratory effort, and feed consumption from seven days before infection (-7 p.i.) to forty days after infection (40 p.i.). Food intake was measured by weighing the amount of ration offered minus the leftovers. The respiratory effort was verified by counting chest movements, and febrile response was detected using a non-contact infrared thermometer pointing to the ear’s base.

2.5 Serology

On days -7, 7, 14, 21, 28, 35, 42, and 49 p.i., 3 mL of blood was drawn via venipuncture from the jugular vein in additive-free tubes. The serum was separated by centrifugation at 2000 rpm and stored at -20° C until further analysis.
For the ELISA test, a 96-well microtiter plate was coated with 2.5 µg/ml of crude tachyzoites lysate antigen obtained from cell culture and 0.06M Carbonate buffer at pH 9.6. The plate was blocked by adding 200µl/well of a 6% solution of nonfat dry milk in PBS containing 0.05% of Tween 20 (PBS-T). The plates were incubated at 37°C for 1:30h min, then washed three times with the PBS-T solution. Samples were diluted at 1:100 proportion in PBS-T solution, added to the plate (100 µl/well) and incubated for 1 h at 37°C. The plates were washed again three times with PBS-T, after which 100 µl of rabbit anti-deer HRP-Conjugated IgG (KPL, Gaithersburg, MD, USA) diluted 1/1000 in PBS-T was added to each well followed by another incubation step for 1h at 37°C and then washed three times with PBS-T. Finally, SureBlue Reserve TMB Microwell Peroxidase Substrate (TMB) (KPL, Gaithersburg, MD, USA) was added (100 µl /well) and plates were incubated at 37°C for 15 min in the dark.

The reaction was stopped by adding 50µl of 3 M H2SO4 to each well. Absorbance at 450 nm was determined using a TP-Reader-Basic (Thermo Plate®, China).

2.6 Electroejaculation and semen analysis

On days -7, 7, 21, 35 e 49 p.i., the animals were anesthetized with an association of ketamine 10% [Dopalen® 7mg/kg i.m.] and xylazine 2% [Rompum® 1mg/kg i.m.], for semen collection.

The procedure was performed using a standardized electroejaculation protocol described by Duarte and Garcia (1997). A sine-wave electrostimulator and rectal probe (P.T. Electronics, Boring, OR) was inserted with the electrodes ventral to the rectum. Each buck was submitted to electroshocks increasing from 250 mA to 750 mA, with a mean duration of 3 s, and a 3 s delay between each shock (10 sequential shocks). One complete series was performed, then the epididymis and penis were massaged until the semen was completely emitted, collected in a microtube and assessed for color and aspect. A single researcher was responsible for classifying the ejaculate's characteristics to avoid differences in determining sperm color and other seminal variables. After the ejaculate's collection, the aliquot was assessed for volume, wave motion, sperm motility and forward progressive motility. The wave motion was assessed by light microscopy (Olympus CX31, Olympus Optical do Brazil Ltd., São Paulo, Brazil) of three separate fields at X 100 augmentation of fresh non-diluted semen and it was scored on a scale of 0 to 5, where 0 is no movement and 5 is strong wave movement. For sperm motility and forward progressive motility, 10 µl of the semen samples were diluted in TRIS-egg yolk extender at 1:3 or 1:4 proportion depending on semen aspect and placed in a water bath at 37°C. These parameters were assessed by placing 5 µl of the diluted semen between a pre-warmed glass slide and coverslip (37°C) observing it in a light microscope (three separate fields – X 400). Motile spermatozoa were depicted as a percentage while forward progressive motility was scored from 0 to 5 (CBRA, 1998). Both values were used to obtain the Sperm Motility Index (SMI = [motility + (forward progressive motility x 20)] x 0.5) (Howard, 1993).

To determine sperm concentration, 5µl of fresh semen was added to 1000 µl 10% buffered formal saline (1:200) and evaluated using a Neubauer hemocytometer (X 400). Total sperm motility count was calculated by multiplying semen volume, concentration, and motility. Functional test evaluations such as membrane and

acrosome integrity were performed according to the methodology described by Bath and Oko (1989) and Pope et al. (1991).

Sperm morphology was assessed by wet mount technique 10 µl of semen diluted in 10% buffered formol saline were placed in between a microscope slide and a coverslip. 200 spermatozoa were assessed using a phase contrast microscope (Olympus BX60 ®, Olympus Optical do Brasil Ltda, São Paulo, Brazil), in a 1000x magnification. Sperm alterations were classified into major and minor defects according to Blom's (1973) classification.

2.7 PCR analysis

After the semen evaluation was performed, a fresh semen's aliquot was placed in a 0.7 ml cryotube and stored in liquid nitrogen until DNA extraction for PCR analysis.

The DNA was extracted from the semen samples following Bag et al.'s protocol (2016). Quantification of DNA extracted was performed using a NanodropOne spectrophotometer (ThermoFisher Scientific). DNA' amplification was performed by conventional PCR, using primers TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (CGCTGCAGACACAGTGCATCTGGATT), aiming at a repetitive fragment of 529 bp in the genome of *T. gondii* (Homan et al., 2000). The target DNA sequences were amplified in a real-time cycler Rotor-Gene® (Qiagen). The DNA in each reaction was initially melted by incubating at 95°C for 2 min before 30 cycles of amplification. Each amplification cycle consisted of incubations at 95° C for 5 sec, 60°C for 10 sec and 72°C for 20 sec. Each reaction mixture had a final volume of 15,0 µL: 6 µL of SensiFASTTM HRM Kit (1X), 0,6 µL of each oligo (10 uM), 2 µL of DNA (10 ng / µL) and 5,8 µL of H_20 . A positive control (*T. gondii* DNA) was added to each amplification lot together with a negative control. Restriction fragments of the amplified products were analyzed by electrophoresis in 2% agarose gel and observed on a UV transilluminator.

To confirm the results PCR products were sequenced using ABI 3730 DNA Analyzer (Applied Biosystems ™, Foster City, CA) kit, and the final products were analyzed using an BigDye Terminator v3.1 Cycle Sequencing Kit's robust (Applied Biosystems™, Foster City, CA). Finally, all sequences were analyzed by BLAST (http://blast.ncbi.nml.nih.gov/Blast.cgi) in the GenBank database.

3 RESULTS

3.1 Clinical parameters

All deer remained clinically normal throughout the experimental period and none showed any important rise in body temperature, abnormal secretions, or behavioral changes that could be directly related to the experimental infection. Food intake of all animals fluctuated during the experiment, having a drastic reduction on the days that the animals were anesthetized. Food intake of animal M4 decreased significantly in the last 20 days of the experiment. Body temperature measured with an infrared thermometer varied between 36°C and 37°C. The animal M2 showed a temperature peak of 37,7 and 37,9°C at days 23 and 40 p.i. respectively, and 37,6°C of animal M1 in 40 p.i.

3.2 Serology

One of the infected deer (M2) developed detectable antibodies against *T. gondii* by 7 p.i. while the animal (M1) developed a detectable response only on day 42 p.i. Animals M3 and M4 did not developed a detectable antibody response during the period of the experiment.

As expected, the control animal remains negative until the end of the experiment. The percentage of optical density of ELISA test results are expressed in Figure 1.

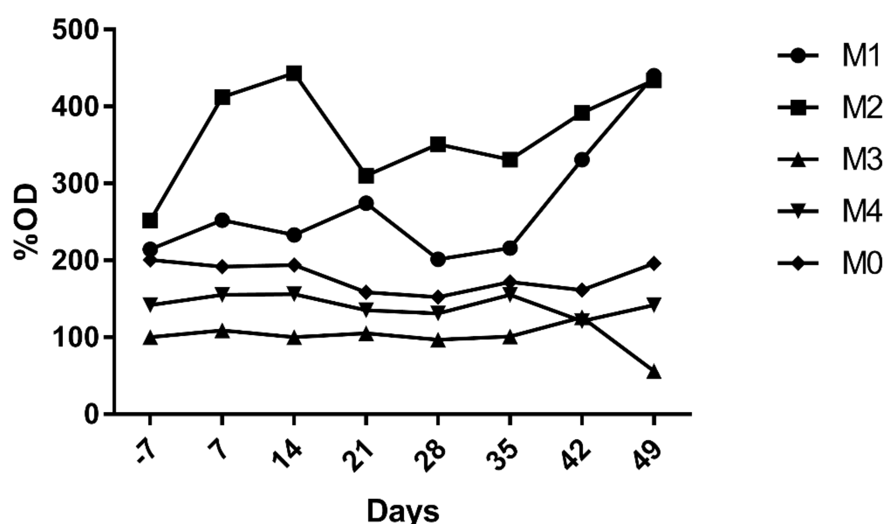

*Figure 1 Percentual of optical density (OD) values obtained in indirect ELISA readings from infected (M1, M2, M3, M4) and uninfected brown brocket deer (*Mazama gouazoubira*) during the experiment.*

3.3 Seminal parameters

M1 showed a decrease in sperm concentration of 25% at day 21 p.i. In M0, this parameter had a reduction of 30% at day 35 p.i.

M2 showed a decrease of membrane integrity at day 49 p.i. The results of the seminal analysis are shown in Figure 2.

3.4 PCR analysis

PCR analyses showed the presence of *T. gondii* DNA in semen samples from M1 and M2 at days 35 and 49 p.i. *T. gondii* sequences were detected in all positive samples with a 100% query cover with a percentage of identity >98%.

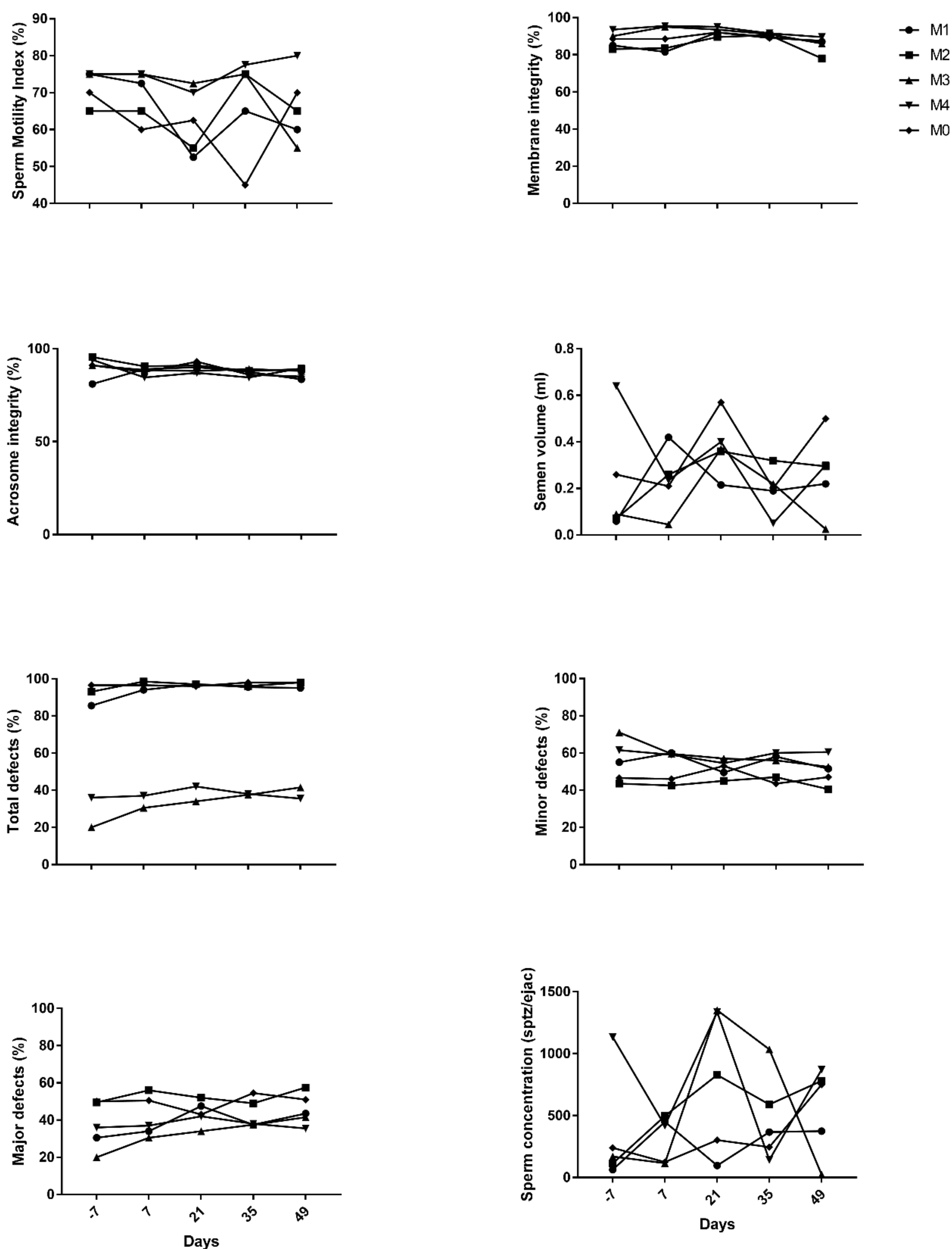

Figure 2. Seminal parameters. Line graphs represent the values of sperm motility index (%), membrane integrity (%), acrosome integrity (%), semen volume (ml), total defects (%), minor defects (%), major defects (%), sperm concentration (sptz/ejaculate) from male 1(●), male 2 (■), and male 3 (▲), male 4 (▼) and male 0 (♦).

4 DISCUSSION

To date, no studies have been performed to assess the implications of *T. gondii* on clinical and reproductive parameters of neotropical deer, and this is the first study that highlights the importance of this protozoan in brown brocket deer males, especially from a reproductive standpoint.

Despite the low number of animals used in the experimental infection, we could observe that the procedure was successful in inducing an infection state in at least two (M1 and M2) of the four infected males *M. gouazoubira*, due to (a) the presence of antibody response measured by ELISA and (b) detection of DNA of *T. gondii* in semen samples. Despite that, two of the animals did not present antibody response, and even the animals with confirmed infection showed only a mild antibody response detected by ELISA test. This result together with the absence of clinical signs may be related to a resistence of the specie to the parasite, it is know that clinical signs is dose dependent of oocysts number, associate to the low virulence of ME49 II strain of *T. gondii*.

T.gondii present a clonal structure consisting of three lineages known astypes I, II, and III. (Howe and Sibley, 1995). Type I strains are usualy lethal to out-bred mice and Type II and III strains are significantly less virulent (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). Studies of *T. gondii* in human and animals in South America suggested that this parasite is genetically diverse (Dubey et al., 2007; Pena et al., 2008) but severe toxoplasmosis in immunocompetent human patients in South

America is often was associated with atypical genotypes (non-Type I, II and III) (Carme et al., 2002; Delhaes et al., 2010). The ME49 strain used in the presente study belong to the Type II genotype, this genotipe was the most prevalente among White-tailed deer (*Odocoileus virgininanus*) from Iowa and Minessota (Dubey et al., 2008). The same strain was used in a experimental infection in reindeer (Oksanen et al., 1996).

The lack of clinical signs following experimental infection was also observed in red deer (*Cervus elaphus*) (Willianson and Willian, 1980) and Elk (*Cervus canadensis*) (Dubey et al., 1980).

Though clinical signs of toxoplasmosis were not reported in naturally infected cervids, acute toxoplasmosis and death were reported in experimentally inoculated mule deer (*Odocoileus heminionus*) (Dubey et al., 1982) and a yearling reindeer

(*Rangifer tarandus*) (Oksanen et al., 1996) however, a high dosis of infection were used. Nevertheless, in most studies involving domestic ruminant species, the major change in clinical parameters consists of a febrile response (rectal temperature > 40°C). This clinical sign was observed between days 4 and 12 p.i. in sheep (Esteban-Redondo and Innes, 1998) and day 2 to 15 p.i. in bovine calves (Costa et al, 1977). The knowledge of clinical manifestations and immune response in wild animals is poorly understood in comparison to information available for domestic species. Formenti et al. (2015) evaluated sera of wild red deer (*Cervus elaphus*) in Italian Alpes and carried out an epidemiological analysis of toxoplasmosis in the population. Their findings confirm high seroprevalence of *T. gondii* infection in red deer with seropositive individuals concentrated in older age groups supporting horizontal transmission as the main route. Moreover, they found a stable prevalence between yearling and adult individuals, with higher serological titers observed in the younger individuals. The same findings were presented by Vikøren et al. (2004) in red deer, roe deer, moose, and reindeer.

Despite the lack of visible clinical signs, studies with experimentally infected cervids have also shown antibody response against *T. gondii*, which is in line with serological results obtained here. In our study, one male presented a detectable antibody response 7 days after infection, and the other one seroconverted only 42 days after inoculation. In experimental infections, sheep presented detectable antibody responses two weeks p.i. with a peak 6 weeks p.i. (Chiebao et al., 2019). In cattle infected with *T. gondii*, seroconversion occurred around 7 days p.i. and peaked between 9 and 21 days (Costa et al., 1977). The reindeer experimentally infected with 5000 oocysts by Oksanen et al. (1996) presented detectable antibodies against *T. gondii* around 14 days p.i. however, the exact time of seroconversion and the titers varied according to the diagnostic technique.

Some studies suggest that *T. gondii* infection can cause temporary impairment on the reproductive parameters of human or animal males as well as impairment of different hormones (Damili and Abdoli, 2013). In rat experiments, findings suggest that Toxoplasmosis can cause temporary impairment on the sperm parameters and a decrease of testes weights, serum testosterone and total antioxidant capacity (Khaki et al., 2011). During the period of the present experiment, seminal evaluation from the infected animals and control showed fluctuation in several parameters including mean sperm motility, membrane integrity and concentration. Nevertheless,

it was not possible to correlate those changes with *T. gondii* infection due to the small number of animals in the experiment and the fact that changes were observed in the control animal's parameters as well. Other experimental infections in rams (Lopes et al., 2009a) and swine (Moura et al., 2007) evidence variations in seminal parameters; however, it was not possible to relate the changes in semen to the infection either. Sexual transmission of *T. gondii* has been described in sheep (Lopes et al. 2013), goats (Santana et al., 2013), rabbits (Liu et al., 2006) dogs (Arantes et al., 2009), and rats (Das et al., 2011) after finding the protozoa's DNA in the semen of experimentally infected male and seropositivity in females after being covered by the inoculated males. Transmission by semen also has been suggested in humans (Flegr et al., 2014; Hlaváčová et al., 2021). Some authors propose that infection during copula is an important route of *T. gondii* transmission and that the parasite may have developed some means of facilitating the transmission that way. Dass et al. (2011) found some interesting results of the sexual transmission of *T. gondii* in rats. Not only did they successfully prove sexual transmission from infected males to uninfected females by recovering *T. gondii* cysts from semen and vaginal lavage after mating, but they also identified that *T. gondii* manipulates mating choice by enhancing the sexual attractiveness of infected animals. Uninfected females would prefer to mate with infected males, and in that way, toxoplasma gained greater opportunities for venereal transmission. *T. gondii* has been extensively studied in the context of parasitic change in host behavior, and the manipulation of mating choice may be an interesting parasite evolutionary adaptation, since, in general, females tend to detect and avoid males infected with an array of bacteria, protozoa and nematodes (Kavaliers et al., 2005). Surprisingly, *T. gondii* infection results in inversion of this innate aversion by females and institutes instead an attraction to parasitized males (Vyas, 2013). Although this behavior change was only observed in rodents, and under a controlled experimental condition, the possibility of those changes occurring in wildlife is relevant. Since sexual transmission of the parasite is likely to occur in cervids, mating choice manipulation is a possibility that should be accessed whereas it may reflect the sexual selection of deer populations in which the disease occurs.

Despite sexual transmission only being proved in a few species, PCR of the semen of infected animals underlines the presence of *T. gondii* DNA in several species such as dogs (Arantes et al., 2009), rams (Lopes et al. 2013), goats (Santana et al., 2013), and bovines (Scarpelli et al, 2009). We were able to detect *T. gondii*

DNA in the inoculated animals' semen on days 35 and 49 p.i., this being the first report of T. gondii in cervid semen.

Those results strongly suggest this may be a viable route of infection in deer as it has been proven to occur in domestic species. These findings need to be taken into consideration in conservation breeding centers since there are several reports of gestational complications in animals infected during copulae, such as fetal reabsorption (de Moraes et al. 2010), abortion (Santana et al., 2013), and fetal maceration (Lopes et al., 2013).

5 CONCLUSION

Neotropical deer do not appear to develop clinical manifestations of infection for *T. gondii* ME49 strain (genotype II), however, the presence of the protozoa DNA in seminal samples suggests that sexual transmission may be a viable route of infection in deer species. We must consider the possibility that recently infected females may have gestational complications due to infection, reducing fetal viability and negatively impacting the fitness of captivity or free-raning populations.

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Capítulo 4- Standardization failure of an indirect ELISA for detection of fecal immunoglobulins to *Toxoplasma gondii* and *Neospora caninum* in deer feces [3]

ABSTRACT- Neotropical deer are elusive animals and their handling both in captivity and in the wild is extremely challenging. Serological survey data are scarce and the development of a non-invasive method of sampling those animals is essential for monitoring the health state of free-running populations of deer in South America. The present study attempted to standardize an indirect ELISA test for detection of anti-*Toxoplasma gondii* and *Neospora caninum* antibodies using fecal samples instead of serum. For *N. caninum*, samples of naturally infected animals were used as control and for *T. gondii*, a pool of samples from experimented infected animals. Both anti deer IgG and Anti-sheep IgA conjugates were used. High background noise reaction was observed in the readings in all ELISA plates, denoting unspecific binding of the antibodies; this problem could not be solved even with several changes in protocols. Therefore, we were unable to continue the optimization of the technique since the results would not be reliable. Despite the negative results, we reinforce the importance of developing a non-invasive method for epidemiological studies in neotropical deer species and hope our study serves as a starting point for other researches with similar aims.

1 INTRODUCTION

Animal health is an important issue for the agricultural industries as well as wildlife conservationists. Both groups have concerns about the impact that diseases may have on animal populations, and each group is apprehensive about disease introduction and transmission interface [1]. The trade-in livestock, wildlife and animal products are enormous and complex and occur on many different scales. There are no overriding rules to control these movements and much of the trade is still based on bilateral agreements between countries [2].

Little is known about the impact of infectious diseases in South-American deer populations and their role in the interface between domestic animals and wildlife,

[3] Este capítulo corresponde ao artigo científico que será submetido submetido à revista Plos One - The missing pieces: a collection of negative, null and inconclusive results

mostly due the difficulty of capturing and handling these animals, caused by their elusive and stressed behavior. Nevertheless, livestock diseases including Toxoplasmosis, Tuberculosis and Foot-and-Mouth disease have been shown to affect wild deer populations in Brazil [3,4,5]. Thereafter a non-invasive diagnostic technique is essential for conducting large-scale epidemiological studies in neotropical deer species. The use of fecal samples in substitution to blood samples presents several benefits, both for the economic and animal welfare point of view. Many studies in genetics, ecology and endocrinology have been performed using fecal samples as non-invasive matrices [6,7,8].

Detection of specific fecal immunoglobulins have been suggested as a non-invasive method for performing epidemiological studies in wildlife, for instance, detection of antibodies to Ebola virus in wild apes samples [9], antibodies to nematodes in Soay sheep (*Ovis aries*) [10] and African swine fever antibodies in Wild boar (Sus scrofa) feces [11].

Adaptive immunity mediated by secretory antibodies is important in the defense against mucosal infections. Specific secretory immunoglobulin A (SIgA) can inhibit initial pathogen colonization by performing immune exclusion both on the mucosal surface and within virus-infected secretory epithelial cells without causing tissue damage [12]. SIgA originates from lymphocytes which migrate to regional lymph nodes and intestinal lymphatic vessels and reach the thoracic duct and enter the bloodstream. These circulating IgA-positive B lymphocytes have an affinity for all body surfaces. Therefore, the gastrointestinal and respiratory tract surfaces are filled with IgA-producing plasma cells. The polymeric immunoglobulins (pIg) are actively transported across the epithelium by the polymeric immunoglobulin receptor (pIgR), found at high levels in the duodenum, jejunum, and colon and at low levels in the lung, kidney, pancreas, and endometrium [13].

In addition to SIgA, significant quantities of IgG can also be detected within the intestinal lumen and in certain tissue locations and may reach levels approximating that observed for SIgA [14] mucosally associated IgG has also been suggested to contribute to host defense [15,16], however, the mechanisms by which IgG gains access to the lumen and the biological functions of IgG once present in this location are uncertain. Receptor-dependent transport pathways for IgG and associated bacterial antigen may exist in epithelial cells of the intestine in adult life which possibly contribute to immune surveillance for luminal and epithelial infections [14].

Toxoplasma gondii and *Neospora caninum* were the pathogens used as a model in the present study. Both are closed related coccidian parasites that are known to affect domestic and wild ruminants [17,18]. Neosporosis and Toxoplasmosis are widespread worldwide and Anti-*T.gondii* and Anti-*N. caninum* antibodies were detected in serological surveys in free-ranging deer [3,19] as well as in captive deers [20,21] from Brazil. A few studies succeed in detecting secretory antibodies to *T.gondii* in intestinal secretions of experimentally infected mice [22,23] and naturally infected humans infants [24]. In the present study we attempted to standardize an indirect ELISA test for detection of antibodies against *N. caninum* and *T. gondii* in deer fecal samples.

2 MATERIALS AND METHODS

2.1 Ethical approval

The study was approved by the Animal Ethics and Welfare Committee (Comitê de Ética e Bem-Estar Animal, CEUA, Protocol number: 003732/18) of the Faculty of Agrarian and Veterinary Sciences (Faculdade de Ciências Agrárias e Veterinárias, FCAV), UNESP, Jaboticabal, SP, Brazil.

2.2 Experimental design

This study was carried out at São Paulo State University (UNESP)/Jaboticabal, Brazil. Prior to the study, 50 deer from the Deer Research and Conservation Center – UNESP/SP) were screened for antibodies against *T. gondii* and *N. caninum* by Indirect fluorescent antibody test (IFAT) and an indirect ELISA test developed “in house”. Individuals that presented positivity by both tests were considered “positive” while individuals that presented negative results in Both techniques were found “negative”. The deer species used in the experiment were: brown brocket deer (*Mazama gouazoubira*), red brocket (*Mazama americana*), small red brocket (Mazama bororo), pampas deer (*Ozotoceros bezoarticus*), and white-tailed deer (*Odocoileus virginianus cariacou*).

For *N. caninum* tests, fecal samples from a pool of six positive animals were used as “positive control”, and feces from six negative animals were used as “negative control”. Otherwise, for *T. gondii* tests, two males brown brocket deer were experimentally infected with 5x10^3 oocysts of *T. gondii* (Me49 strain). Blood and feces

were collected from -7 days post-infection (dpi) until 49 dpi. Serum samples were positive at 14 dpi, and fecal samples from days 14, 21 and 28 dpi were used as "positive control" for *T. gondli*, while feces samples were collected at -7dpi together with samples from an uninfected animal were used as "negative control".

2.3 Fecal extract preparation and storage

Fresh fecal samples were collected from the individual stalls and immediately processed. Approximately 3 g of feces was resuspended in 9 ml of phosphate-buffered saline (PBS) containing Protease Inhibitors Mix (GE Healthcare Bioscience, USA. Cat No: 80-6501-23) at a 1:500 ratio, and EDTA 0,5M pH 8,0 (Thermo Fisher Scientific Inc., Rockford, IL, USA) at 1:200 ratio. The pellets were broken up and the sample thoroughly mixed. The mixture was subjected to a horizontal homogenizer (VDRL shakerTS, 2000 Biomixer®) for 20 min, to allow inhibition of proteases and extraction of antibodies into the fluid. The suspensions were centrifuged at 5000 g for 15 min, and the supernatants were labeled as feces suspensions and stored at -80°C until analysis by ELISA.

2.4 Enzyme-linked immunosorbent assay (ELISA)

For the titration of reagents of indirect ELISA tests to *N. caninum* and *T. gondii* we adapted the guidelines described by Crowther (2009) [25]. For each pathogen were prepared two plates, one for detection of specific IgG and another for detection of IgA. Each plate was divided in two, half received serial dilutions of the positive samples and half serial dilutions of the negative samples in combination with the dilutions of the antigen (Fig 1).

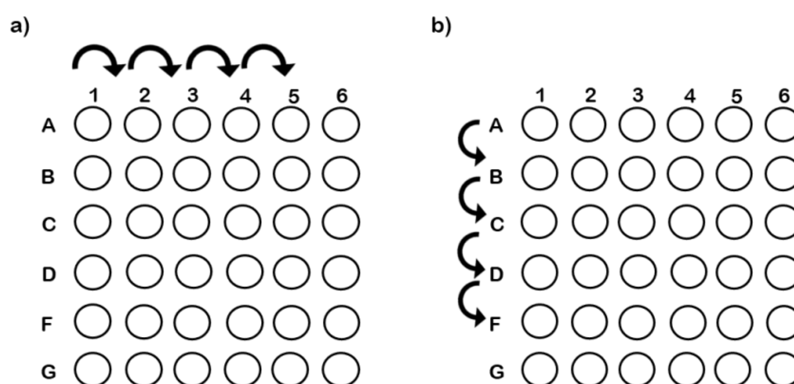

Fig 1. Schematic representation of (A) serial dilutions of antigen in the ELISA plate wells, wells 6 received carbonate-bicarbonate buffer only and (B) serial dilutions of fecal extracts, wells F received blocking buffer (1% ovalbumin in PBS Tween 20) only.

ELISA plates (NUNC MaxiSport®), were coated with serial dilutions of the respective crude soluble antigen (IMUNODOT®, Jaboticabal - São Paulo, Brazil) diluted in carbonate-bicarbonate buffer (pH 9.6; 0.5M) with starting concentration of 10 µg/mL until 0,625 µg/mL. One hundred microliters of the diluted antigen were added to each plate well and then were incubated for 12-14h in a humid chamber at 4 °C. After this period, the excess antigen was discarded and the plates were washed with PBS Tween-20 three times, then, the plate was blocked with 1% ovalbumin (Sigma-Aldrich® Brasil Ltda, Ref: A5253) in carbonate-bicarbonate buffer (pH 9.6; 0.5M). For every plate well, 200 µL of the blocker was added, followed by incubation in the humid chamber at 37 °C for 2:00 hours. After removal of the blocking solution, the plate was washed three times with PBS Tween-20. One hundred microliters of serial dilutions of the fecal extract in blocking buffer (1% ovalbumin), starting with undiluted extract to 1:16 were added in each well. Once again, the plate was incubated in the humid chamber at 4°C overnight, with subsequent washing, as previously described.

For IgG detection, one hundred microliters of anti-deer-IgG peroxidase-labeled (KPL, Gaithersburg, MD, USA), diluted at 1:500 with blocking buffer. For IgA detection, anti-sheep IgA conjugate (Abcam, USA ab112759) was used at 1:10000 since no commercially available anti-deer IgA exists and anti-sheep IgA presents cross-reactivity with cervid Ig (Mackintoch et al 2014; Goñi et al., 2015). Plates were then incubated at 37°C for 1 hour and washed as previously described. After this

step, one hundred microliters of the enzyme-substrate 3,3′, 5,5′-Tetramethylbenzidine (SureBlue™ TMB 1-Component Microwell Peroxidase Substrate) were added to each well. Plates were incubated at room temperature protected from the light for 15 minutes. The reaction was stopped by adding 50µl of 3 M H2SO4 per well. Absorbance at 450 nm was determined using a TP-Reader-Basic (Thermo Plate®, China).

3 RESULTS

Tests results are shown in Fig 2 and 3. The curves that demonstrate the relation between optical density (OD) and dilutions of samples for each concentration of antigen were poorly formed and there was no significant difference between the lines for each antigen concentration, showing a high background, caused by nonspecific binding of antibodies. The background reaction was even higher when an anti-sheep IgA antibody was used, and in the *T. gondii* test, the negative samples OD were higher than the positive ones.

4 DISCUSSION

Results obtained in this study clearly show that we have not succeeded in the efforts to develop an indirect ELISA test for non-invasive diagnosis of *T. gondii* and *N. caninum* in deer samples. It is essential to report that several variations of this protocol were tested and despite the different readings, all of them present the same findings: low OD in low sample concentrations and high background in higher samples concentrations. The variations we tried include: different kinds and concentrations of blocking buffers (BSA, gelatin, non-fat milk powder, fetal bovine serum), different incubation times and temperatures, and conjugate dilutions. Two major hypotheses can explain these poor results: a) deer fecal samples contain a low level of specific antibodies to *T. gondii* and *N.caninum*, and at high samples concentrations, all reactions are nonspecific, and b) the enzyme conjugates that we used bind with other proteins present in deer feces, interfering with specific antibody detection in fecal samples.

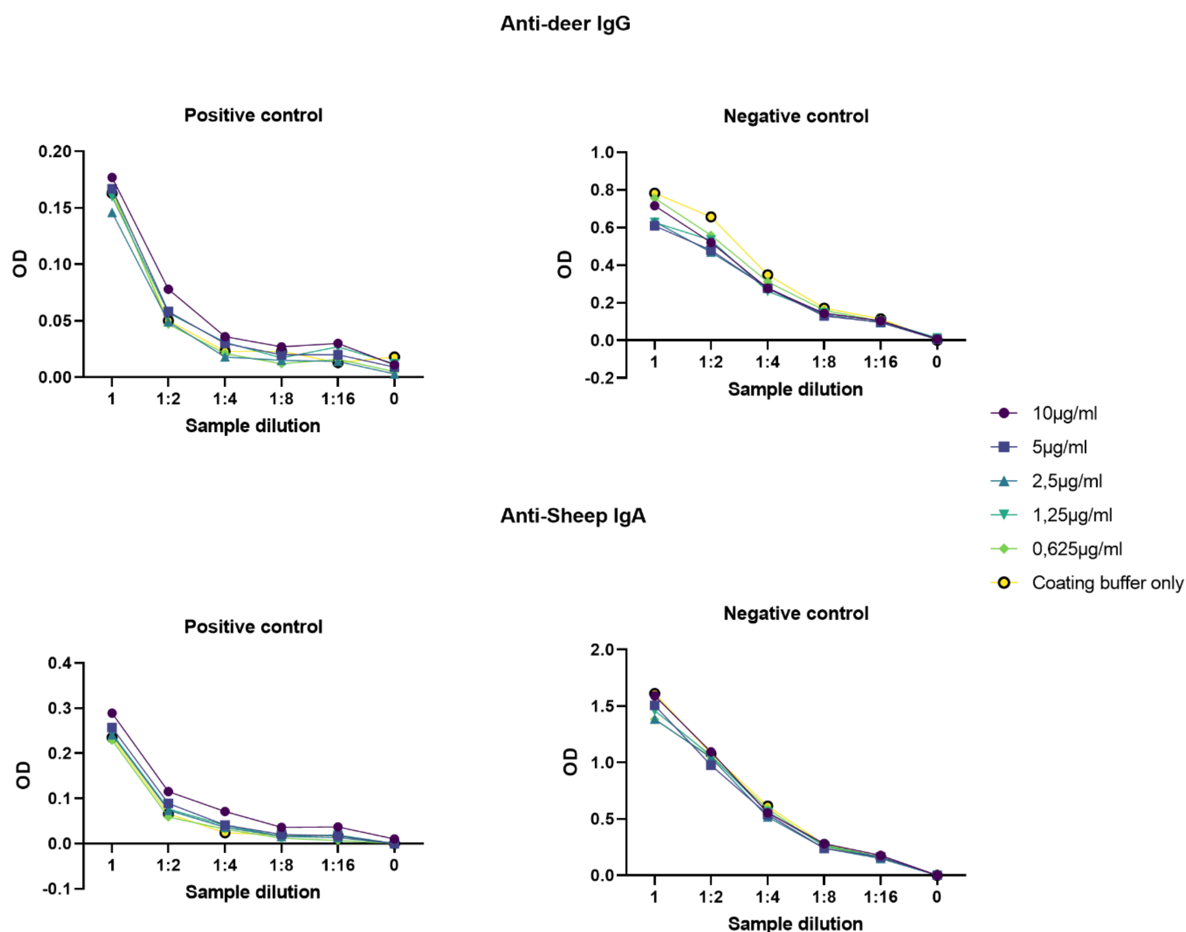

Fig 2. Graph relating OD values for different *T. gondii* antigen concentrations against dilutions series of samples in carbonate-bicarbonate buffer. Curves show titration of antiserum at different dilutions from wells 1-5. Wells 6 received a carbonate-bicarbonate buffer only.

The absence of a commercially available anti-deer IgA conjugate may contribute to the difficulty of detecting secretory immunoglobulins in cervids samples, nevertheless, some studies achieved good results using anti-sheep IgA for detection of specific immunoglobulins in deer feces [26] and saliva [27].

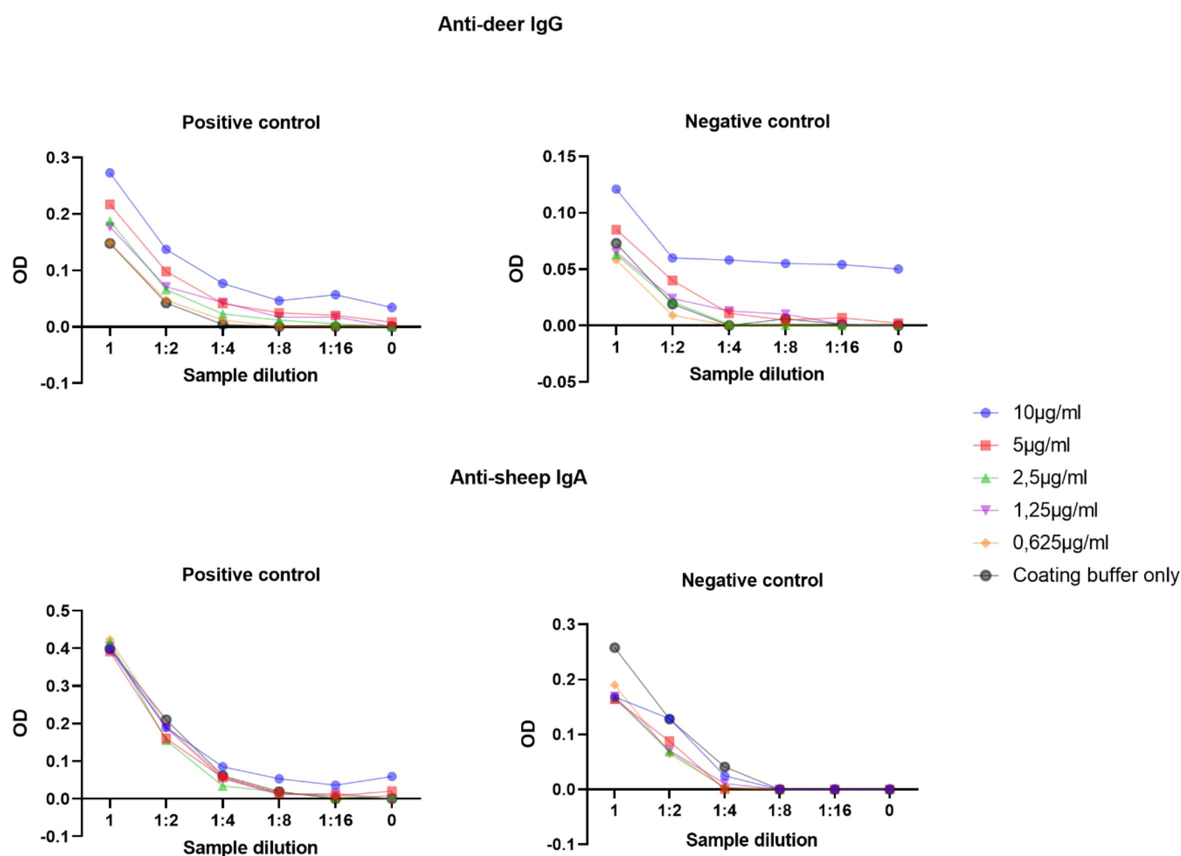

Fig 3. Graph relating OD values for different *N. caninum* antigen concentrations against dilutions series of samples in the carbonate-bicarbonate buffer. Curves show titration of antiserum at different dilutions from wells 1-5. Well 6 received a carbonate-bicarbonate buffer only.

The elimination period of specific secretory antibodies may also play a role in the outcome of diagnosis tests, since most studies detect specific immunoglobulins after experimental infection or vaccination [11,28,29]. Our study used samples of animals experimentally infected with *T. gondii* oocysts and despite that, the OD of the negative sample pool was even higher than the infected animals proving the high background reaction. The background noise is caused by hydrophobic binding of immunoglobulin components in sample specimens to solid surfaces. This background reaction is unique to individual samples and varies significantly, sometimes even exceeding the true antibody-antigen reaction [30]. The tests we performed in our laboratory in the attempt to optimize the technique showed that samples of some particular animals presented higher background reactions than others, and even though we don't know exactly which proteins are the source of the nonspecific binding, its concentration in the samples appear to vary according to the individual.

The total amount of immunoglobulins in bovine serum samples is around 11,2 mg/ml for IgG1 and 0,37 mg/ml for IgA. Otherwise, concentrations found in intestinal secretions were 0,25 mg/ml for IgG1 and 0,24 mg/ml for IgA [31]. Those differences may explain why detection of immunoglobulins in fecal samples by immunoassays can be much more challenging than detection in serum. The promising results with secretory antibodies against nematodes in naturally infected animals [10,27] may be related to constant stimulation of intestinal mucosa which maintains higher levels of antibodies on intestinal secretions and saliva compared to pathogens that stimulate the mucosa for a shorter period.

Immunoglobulins classes and their source in the organism can also interfere in the concentrations in the intestinal mucosa and consequent success of the immunoassay. In bovines, IgG subclass immunoglobulin, especially IgGl, in large amounts into mammary and perhaps other exocrine secretions, is without parallel except among other ruminants [31]. Mucosal IgA, IgM, produced locally at the lamina propria, and IgG, produced either locally or systemically, are transported by the Polymeric Immunoglobulin Receptor (pIgR) or by the Neonatal Fc-Receptor (FcRn), respectively [32]. For any given secretion, both pathways are presumably operative but apparently to differing extents in different organs and for different immunoglobulin isotypes. Experiments in bovines concluded that nearly l00% of the colostral IgG1 was serum-derived [33]. In sheep, 25% of the IgG1 and IgG2 and 90% of the IgA in intestinal lymph is of local origin [34]. The large amount of serum-derived IgG1 found in exocrine secretions including intestinal lymph suggests that specific immunoglobulins from serum can also be transported through the mucosal epithelium and consequently be detected in secretions. Experiments with humans following intranasal administration of live influenza A virus vaccine have concluded that nasal wash antibodies appeared to be mainly derived from the serum by a process of passive transudation [35]. Nevertheless, the concentrations of these serum-originated IgG in secretions are supposedly very low, and efficient detection would rely on a very sensitive technique.

The low concentrations of fecal-specific antibodies in addition to high background reactions and non-homogeneous distribution in feces appear to be the main obstacles in using fecal samples in indirect ELISA. Fergunson et al. (1995) [36] strongly discouraged the use of fecal extracts in immunological tests, according to the authors intestinal transit has an important influence on the recovery of secretory

immunoglobulins due to the time available in the lumen on the gastrointestinal tract for molecules of interest to be destroyed by digestive enzymes and bacterial proteases. Thus, immunological results using fecal samples would be unreliable.

Even though our negative results are discouraging, we emphasize the importance of developing non-invasive techniques for epidemiological studies in elusive wild species. Due to some promising results obtained in other studies [10,11,29], we suggest new approaches for detecting specific antibodies in deer feces, changings in the ELISA types such as sandwich ELISA, in the immunological technique like western blotting [9] and expanding the range of target pathogens may come out with better results.

Funding: This research was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

Acknowledgments:

Authors want to thank to IMUNODOT Diagnósticos Ltda. and Professor Rosângela Zacarias Machado for the support with lab reagents together with Professor Hélio José Montassier for help in developing the protocols.

Competing interests:

The authors have declared that no competing interests exist.

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CAPÍTULO 5 – Considerações finais

O presente trabalho teve como principal objetivo desenvolver e aplicar métodos que gerem mais informações sobre a transmissão e manifestação da toxoplasmose e neosporose em cervídeos neotropicais, já que os dados sobre patologias e parasitos são escassos nessas espécies e a maior parte do que sabemos são informações extrapoladas de ruminantes domésticos e cervídeos de zonas temperadas.

Durante o desenvolvimento dos experimentos foi realizada a padronização de ELISA para a detecção de anticorpos anti-*T. gondii* e anti-*N.* caninum em soro de cervídeos com subsequente avaliação da taxa de transmissão congênita das duas doenças. Esse foi o primeiro estudo, de nosso conhecimento, que utilizou técnicas sorológicas para estimar a taxa de transmissão congênita da neosporose e toxoplasmose em cervídeos. Os resultados obtidos mostraram que a transmissão nesses animais se assemelha às observadas em bovinos (Dubey et al, 2007; Dijkstra et al, 2008), apresentando uma alta taxa de transmissão congênita de *N. caninum* (81,25%) enquanto para *T. gondii* a estimativa dessa via de tranmissão em animais cronicamente infectados foi nula. Com esses resultados podemos identificar as fontes de infecções em animais de cativeiro e tomar as medidas profiláticas necessárias para controlar o parasita em animais de cativeiro.

O ELISA padronizado foi utilizado também para avaliar a resposta de anticorpos em veado-catingueiro (*Mazama gouazoubira)* após uma infecção experimental com 5000 oocistos exporulados de *T. gondii* da cepa ME49. Dos quatro animais que receberam os oocistos por via oral, aprenas dois desenvolveram resposta de anticorpos detectáveis. Apesar de nenhum animal ter apresentado sinais clínicos ou alteração em parâmetros seminais decorrente da infecção, o DNA do *T. gondii* foi identificado por meio de PCR no semên dos animais que positivos aos 35 e 49 dpi. Esses resultados sugerem a possibilidade de transmissão sexual do parasito nessa espécie, sendo o primeiro estudo a achar evidências desse modo de transmissão em cervídeos.

Além da pradronização de ELISA para amostras de soro, também foi tentada a padronização da técnica utilizando amostras de fezes. Os dados epidemiológicos em cervídeos de vida-livre no Brasil são escassos, pela dificuldade que envolvem o processo de captura e o desenvolvimento de técnicas que utilizem amostras não

invasivas é imprescindível para que seja possível monitorar a sanidade dos cervídeos neotropicais. Fezes já foram utilizadas para amostragem não invasivas em estudos sobre genética e endocrinologia de cervídeos (González et al., 2009; González e Duarte, 2007; Oliveira e Duarte, 2013), por esse motivo buscou-se avaliar a viabilidade de um método não invasivo de amostragem epidemiológica. As amostras fecais utilizadas foram provenientes de animais naturalmente infectados com *N. caninum* e experimentalmente infectados com *T. gondii.* Foram realizadas diversas alterações de protocolo, porém não foi possível contornar o problema de elevado ruído e baixas OD% obitidas nas leituras. Mesmo com os resultados desencorajadores recomendamos que alterações na técnica sejam tentadas visando a detecção de imunoglobulinas secretoras, já que outros estudos, utilizando espécies e patógenos diferentes, tiveram resultados mais promissores (Reed et al., 2014; Watt et al., 2016).

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