UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIA ANIMAL

FLAVIANA SANTOS WANDERLEY

ASPECTOS CLÍNICOS, PATOLÓGICOS E REPRODUTIVOS DA INFECÇÃO EXPERIMENTAL POR *Toxoplasma gondii* EM CAPRINOS

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Tese apresentada ao Programa de Pós-Graduação em Biociência Animal, como requisito parcial para obtenção do grau de Doutor.
Área de Concentração: Biotecnologia

Orientador:
Prof. Dr. Rinaldo Aparecido Mota

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ÁREA DE CONCENTRAÇÃO: BIOTECNOLOGIA

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Quando amanhece
Até parece que o sertão
Com alegria
Vai despedindo a escuridão
E a passarada
Em revoada, tão contente
Alcança o espaço
Num grande abraço a toda gente

Quando amanhece
Ao despertar de um novo dia
A natureza
Traz para a mata a alegria
E tudo muda
Com a chegada dessa hora
Cantando todos
Em louvor à nova aurora

Alma do Sertão

Luiz Gonzaga

RESUMO

Objetivou-se estudar os aspectos clínicos, patológicos e reprodutivos da infecção experimental por *Toxoplasma gondii* em caprinos. No primeiro experimento foi avaliada a transmissão de *T. gondii* em cabras inseminadas via vaginal com sêmen contaminado com taquizoítos da cepa CPG (Genótipo III). Foram formados dois grupos experimentais (G1 e G2) constituídos por cinco cabras cada um. As fêmeas do G1 foram inseminadas com sêmen contendo $1x10^5$ taquizoítos e as fêmeas do G2 (controle) com sêmen livre de taquízoitos (inseminação=dia 0). Para confirmar a infecção foram realizados testes sorológicos (reação de imunofluorescência indireta) e detecção do DNA parasitário (reação em cadeia da polimerase) no sangue e órgãos. Para o acompanhamento gestacional foram utilizados exames ultrassonográficos. No G1, a soroconversão ocorreu entre os dias 7 e 14 em 4/5 cabras. Neste mesmo grupo, o DNA de *T. gondii* foi detectado entre os dias 7 e 49 no sangue e vários tecidos de 3/5 cabras e em dois cabritos nascidos a termo. No G2, todas as amostras foram negativas para ambas as técnicas. No segundo experimento, as cabras do G1 do primeiro experimento foram monitoradas nas fases aguda e crônica da infecção; reabsorção embrionária ocorreu em 4/5 cabras na fase aguda, enquanto que na fase crônica foram observados anestro em 2/5 cabras, hidrossalpinge em 1/5 cabras e cisto ovariano em 1/5 cabras. No terceiro experimento pesquisou-se a transmissão venérea de *T. gondii* em cabras acasaladas com o reprodutor infectado experimentalmente por via oral com oocistos da cepa ME-49. Dez cabras foram alocadas em dois grupos (G1 e G2) cada um com cinco animais, sendo as cabras do G1 acasaladas com o bode infectado e G2 (controle) acasaladas com bode sorologicamente negativo. A infecção do reprodutor e das cabras foi confirmada por meio dos achados clínicos, sorológicos, moleculares e histopatológicos. No reprodutor, a soroconversão ocorreu no 7^0 dia pós-infecção (d.p.i.) e o DNA para *T. gondii* esteve presente no sangue e sêmen no 3^0 d.p.i. No G1, 2/5 cabras soroconverteram e em 2/5 cabras o DNA foi detectado no sangue. As cabras do G2 e o reprodutor não infectado foram negativos em todos os testes. Em relação ao desenvolvimento gestacional, nas cabras do G1 ocorreu reabsorção embrionária em 1/5 no 34^0 dia após o acasalamento, 1/5 abortou no 42^0 dia após acasalamento e 3/5 pariram cabritos saudáveis a termo. No G1, o DNA de *T. gondii* estava presente no sangue em 1/5 cabritos ao nascimento e em pelo menos um órgão em 2/5 cabras e 4/5 cabritos nascidos vivos. As cabras do G2 pariram cabritos saudáveis. A partir dos resultados obtidos, conclui-se que a inseminação artificial com sêmen contaminado com taquizoítos de *T. gondii* infecta cabras, causando patologias reprodutivas durante as fases aguda e crônica da infecção. Além disso, constatou-se que a transmissão venérea por *T. gondii* ocorre em cabras acasaladas com reprodutor caprino infectado experimentalmente.

Palavras-chave: Toxoplasmose, caprino, sêmen, taquizoítos, transmissão, distúrbios reprodutivos.

ABSTRACT

The objective was to evaluate the clinical, pathological, and reproductive aspects of the *Toxoplasma gondii* experimental infection in goats. Firstly was studied the transmission *T. gondii* in goats inseminated vaginally with semen contaminated with strain tachyzoites CPG (genotype III). Were divided into two experimental groups (G1 and G2) each with five goats. The females of the G1 were inseminated with semen containing $1x10^5$ tachyzoites and females G2 (control) with semen free tachyzoites (insemination = day 0). To confirm infection were performed serologic tests (indirect immunofluorescence) and detection of parasitic DNA (polymerase chain reaction) in blood and organs. For monitoring pregnancy ultrasound exams were used. In G1, seroconversion occurred between days 7 and 14 at 4/5 goats. In the same group, the DNA of *T. gondii* was present between days 7 and 49 in blood and various tissues of 3/5 goats and two kids born at full term. In G2, all samples were negative for both techniques. In the second experiment, the goats of the G1 in the first experiment were monitored in the acute and chronic phases of infection; embryonic reabsorption occurred in 4/5 goats in the acute phase, while in the chronic phase were observed in anestrus 2/5 goats, 1/5 goats had both hydrosalpinx and ovarian cysts. The third experiment investigated to venereal transmission of *T. gondii* in goats mated with breeder experimentally infected orally with oocysts of the ME-49 strain. Ten goats were allocated into two groups (G1 and G2) each with five animals. The female goats in G1 were mated with a breeder that had been experimentally infected with *T. gondii* oocysts whereas those in G2 (control) were mated with a serologically negative goat. The infection of the breeder and the females was confirmed through clinical, serological, molecular and histopathological findings. Seroconversion ocurred in the breeder infected on the 7th day post-infection (d.p.i.) and *T. gondii* DNA was present in the blood and semen in the 3th d.p.i. In G1, 2/5 goats seroconverted and DNA was present in the blood in 2/5 goats. In G2, all goats were negative in all tests. With regards to gestational development, the goats in G1 ocurred embryonic reabsorption in 1/5 in day 34 after mating, 1/5 aborted in day 42 after mating and 3/5 goats gave birth to full-term healthy kids. One of the kids exhibited PCR-positive blood at birth. In total, 2/5 of the females in G1 and 4/5 of the kids born alive in G1 exhibited PCR-positive in at least one organ. In G2, the goats gave birth goats healthy. In conclusion, artificial insemination with semen containing tachyzoites of *T. gondii* infected goats, causing reproductive pathologies during acute and chronic phases of infection. Furthermore, it was found that venereal transmission of *T. gondii* occurs in goats mated with an infected breeder.

Keywords*:* Toxoplasmosis, goat, semen, tachyzoites, transmission, reproductive disorders.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ºC	Graus Celsius
≥	Maior ou igual
®	Marca Registrada
µL	Microlitro
µM	Micromolar
G1	Grupo 1
G2	Grupo 2
%	Porcentagem
AL	Aglutinação em látex
CBRA	Colégio Brasileiro de Reprodução Animal
CEUA	Comissão de Ética no Uso de Animais
CNPq	Conselho Nacional de Pesquisa
EDTA	Ácido etilenodiamino tetra-acético
DNA	Ácido desoxirribonucleico
d.p.i.	Dias pós infecção
ELISA	Ensaio Imunoenzimático (do inglês: Enzime Linked Immunosorbent Assay)
et al.	Autores colaboradores (Original do latim; “e os outros”)
FACEPE	Fundação de Amparo à Ciência e Tecnologia de Pernambuco
FAPEAL	Fundação de Amparo a Pesquisa do Estado de Alagoas
FITC	Isotiocianato de fluoresceína
FR	Frequência Relativa
HAI	Hemaglutinação Indireta
IBGE	Instituto Brasilleiro de Geografia e Estatística
IgG	Imunoglobulina da classe G
IgM	Imunoglobulina da classe M
IFI	Imunofluorescência Indireta

LTDA	Limitada
MAD	Método de Aglutinação Direta
MHz	Megahetz
MG	Miligrama
Ml	Mililitro
PB	Pares de bases
PCR	Reação em Cadeia da Polimerase
RIFI	Reação de Imunofluorescência indireta
SF	Reação de Sabin e Feldman
T. gondii	*Toxoplasma gondii*
UEL	Universidade Estadual de Londrina
UFAL	Universidade Federal de Alagoas
UFRPE	Universidade Federal Rural de Pernambuco

INTRODUÇÃO

A toxoplasmose é uma zoonose de distribuição mundial causada pelo *Toxoplasma gondii,* um parasito intracelular obrigatório que pode parasitar diferentes tecidos e apresenta ciclo de vida complexo (BEAMAN et al., 1995). Tem importância médica e veterinária por causar doença congênita e aborto (TENTER et al., 2000).

A primeira evidência da toxoplasmose em cabras foi feita por Feldman & Miller (1956). Munday & Mason (1979) foram os primeiros a descreverem a toxoplasmose como importante causa de prejuízos reprodutivos em caprinos. Os caprinos parecem ser mais suscetíveis à toxoplasmose clínica (DUBEY, 1989), acarretando grandes perdas econômicas em rebanhos no mundo (MOYA; SERRATO, 1985; DUBEY et al., 1986; DUBEY et al., 1987; SELLA et al., 1994).

Os caprinos criados extensivamente ou em sistema semi-intensivo são mais acometidos pela toxoplasmose (CAVALCANTE et al., 2008; ARAÚJO NETO et al., 2008; ANDERLINI et al., 2011; GARCIA et al., 2012). e a principal via de infecção é a ingestão de oocistos esporulados do parasito no ambiente (DUBEY; BEVERLEY, 1988). Taquizoítos de *T. gondii* foram isolados na mucosa vaginal, saliva, secreção nasal e urina de caprinos infectados experimentalmente (DUBEY, 1980) e a eliminação desta forma parasitária no leite de cabras naturalmente infectadas também foi relatada por Chiari & Neves (1984). A presença de *T. gondii* no sêmen de caprinos experimentalmente infectados também foi relatada na literatura (DUBEY; SHARMA, 1980; SANTANA et al., 2010).

Embora o ciclo de vida de *T. gondii* seja conhecido desde o final da década de 60, alguns aspectos relacionados à infecção ainda necessitam ser esclarecidos (BASTIEN, 2002), destacando outras prováveis vias de infecção.

Diante dos prejuízos causados pela toxoplasmose nas criações de caprinos no mundo, o estudo de outras vias de transmissão deste parasito é um aspecto relevante na epidemiologia desta doença. O isolamento de *T. gondii* no sêmen de reprodutores caprinos na infecção experimental gera expectativa sobre sua transmissão via venérea.

2. REVISÃO BIBLIOGRÁFICA

2.1 Etiologia e ciclo biológico

Toxoplasma gondii é a única espécie do gênero e foi descoberto quase ao mesmo tempo por Nicolle & Manceaux (1908) em roedores na Tunísia e coelhos no Brasil por Splendore (1908).

Está incluído no REINO Protista, SUBREINO Protozoa, FILO Apicomplexa, CLASSE Sporozoea, SUBCLASSE Coccidia, ORDEM Eucoccidida, SUBORDEM Eimeriina, FAMÍLIA Sarcocystidae, SUBFAMILIA Toxoplasmatinae e GÊNERO *Toxoplasma* (DUBEY, 2010).

Toxoplasma gondii é um coccídeo que pode ser encontrado sob três formas: taquizoítos, cistos teciduais com bradizoítos e oocistos; este último ocorre somente no intestino dos felídeos que são os hospedeiros definitivos (DUBEY et al.,1997).

Os taquizoítos apresentam-se em forma de meia lua e núcleo central. Apresentam extremidade anterior pontiaguda, posterior arredondada e complexo apical (FRENKEL, 1973). **M**ultiplicam-se assexuadamente no interior da célula hospedeira por endodiogenia repetida (SHEFFIELD; MELTON, 1968; HU et al., 2002) durante a infecção aguda no hospedeiro intermediário (MILLER et al., 1972).

Os bradizoítos são semelhantes aos taquizoítos, diferindo por apresentar o núcleo bem próximo à extremidade posterior e apresentarem multiplicação lenta (FRENKEL, 1973). Estão contidos aos milhares dentro dos cistos e podem medir até 100µm de diâmetro. Ocorrem principalmente na musculatura, fígado, pulmão e cérebro e retina (DUBEY et al., 1998).

Os oocistos são encontrados nas fezes dos felinos e medem quando esporulados 12x10 µm. No ambiente, o oocisto contém dois esporocistos, cada um com quatro esporozoítos (DUBEY et al., 1970). São resistentes às condições ambientais e resultam da fase sexuada do ciclo, que é limitada ao epitélio intestinal dos hospedeiros definitivos (DUBEY, 1995).

O parasito se desenvolve em duas fases distintas (Figura 1): assexuada que ocorre nos tecidos dos vários hospedeiros intermediários, incluindo os gatos (DUBEY et al, 1998) e sexuada, que ocorre no epitélio intestinal de felinos jovens

(DUBEY; FEENKEL, 1972). Os gatos e outros felídeos se infectam pela via oral com oocistos e cistos (bradizoítos) ou taquizoítos (FRENKEL et al., 1970). O período pré-patente varia de acordo com o estágio de *T. gondii* ingerido (DUBEY, 2001). O ciclo sexuado ocorre nas células epiteliais do intestino delgado dos felinos, iniciando com a formação dos esquizontes que dividem-se e evoluem para merozoítos que dão origem aos gametas masculinos (microgametas) ou femininos (macrogametas) (DUBEY; FRENKEL, 1972; FERGUSON; DUBREMETZ, 2007). Os microgametas usam seus flagelos para se locomover, penetrar e fertilizar os macrogametas maduros e formar os zigotos. Após a fertilização, uma parede cística é formada em torno do parasito dando origem ao oocisto. Posteriormente, ocorre a ruptura das células epiteliais e os oocistos são eliminados no lúmen intestinal juntamente com as fezes (DUBEY, 2004).

O ciclo assexuado inicia-se com a ingestão dos oocistos maduros, cistos ou taquizoítos pelos hospedeiros intermediários. Cada esporozoíto, bradizoíto ou taquizoíto após rápida passagem pelo epitélio intestinal dão origem aos taquizoítos que se multiplicam intensamente dentro da célula (DUBEY et al.,1998). Estes atingem todos os tecidos e órgãos dos hospedeiros via circulação sanguínea e linfática, multiplicando–se por endodiogenia em células nucleadas, incluindo o endotélio vascular, fibroblastos, células mononucleares e polimorfonucleares (DUBEY et al., 1997; SPEER; DUBEY, 1998), caracterizando a fase inicial (aguda) da doença (NEVES, 2005). Alguns parasitos chegam a diversos órgãos formando os cistos teciduais repletos de bradizoítos, caracterizando a fase crônica da doença (DUBEY, 2004).

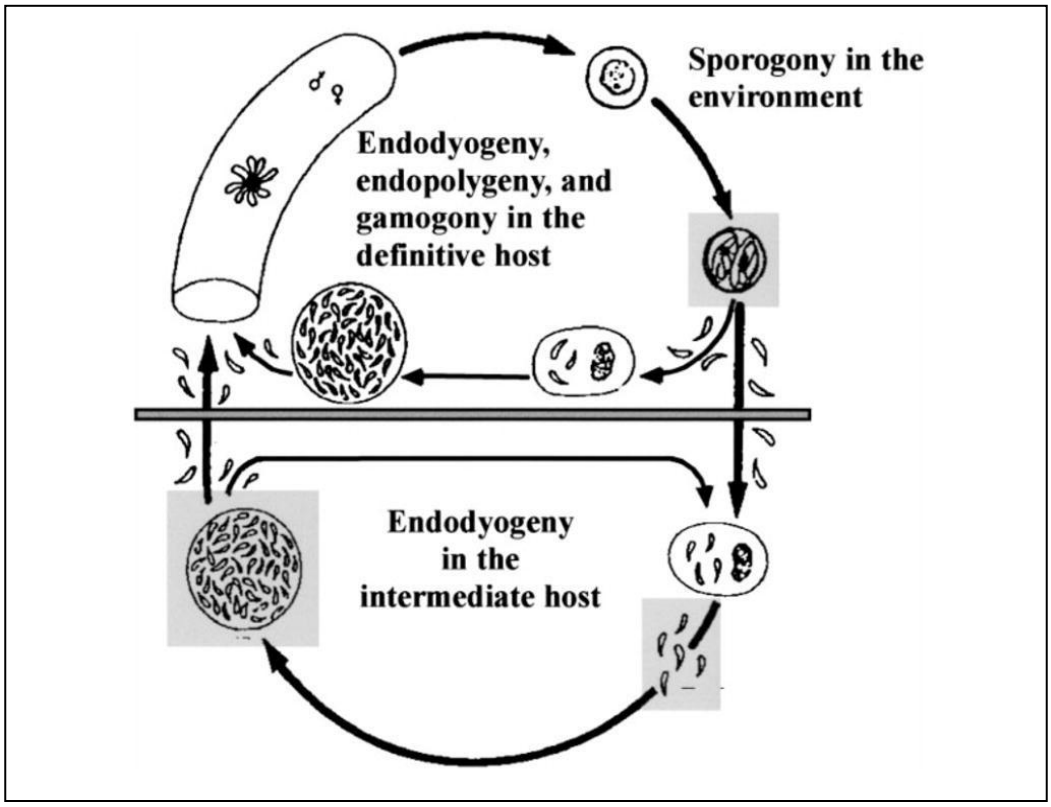

Figura 1- Ciclo biológico de *Toxoplasma gondii*
Fonte: Tenter et al. (2000)

2.2 Epidemiologia da toxoplasmose em caprinos

2.2.1 Distribuição e prevalência

A toxoplasmose é uma das zoonoses mais difundidas no mundo, afetando mamíferos domésticos e selvagens, além das aves (FRENKEL et al., 1970; MILLER et al. 1972; COLE et al., 2000).

Levantamentos sobre a prevalência de anticorpos contra *T. gondii* em caprinos foram relatadas em vários países (MOYA; SERRATO, 1985; DUBEY et al., 1986; RODRÍGUEZ-PONCE et al., 1995; BISSON et al., 2000; TENTER et al., 2000; VAN DER PUIJE et al., 2000; MASSALA et al., 2003; JITTAPALAPONG et al., 2005; SHARIF et al., 2007; TESHALE et al., 2007; SANAD; AL-GHABBAN, 2007; FIALHO et al., 2009), sugerindo que esses animais constituem importantes hospedeiros intermediários (MACHADO; LIMA, 1987).

Dubey (1985) estudou 123 caprinos nos Estados Unidos e observou uma soropositividade de 22,7%. Na Venezuela foi encontrada uma positividade de 17,8% em um total de 438 caprinos avaliados sorologicamente (NIETO; MELENDEZ, 1998).

No Brasil, levantamentos mais recentes realizados no período de 1990 a 2012 demonstraram a infecção por *T. gondii* em vários Estados com positividade variando de 8 a 47% (Tabela 1).

Tabela 1 – Frequência de caprinos positivos para *Toxoplasma gondii* nos diferentes Estados Brasileiros.

REGIÃO	Autor	Ano	Técnica Utilizada	N^0 Animais	F.R. (%)
Nordeste					
Alagoas	ANDERLINI et al.	2011	IFI	454	39
Bahia	GONDIM et al.	1999	AL	439	28,93
Bahia	UZÊDA	2004	IFI	373	16,35
Ceará	CAVALCANTE et al.	2008	ELISA	2362	25,1
Maranhão	SOARES et al.	2010	ELISA	92	36,95
Paraíba	FARIA et al.	2007	IFI	306	24,5
Pernambuco	DA SILVA et al.	2003	IFI	213	40,40
Pernambuco	BISPO et al.	2011	IFI	164	47,6
Pernambuco	PEREIRA et al.	2012	IFI	167	31,7
Rio Grande do Norte	NETO et al.	2008	IFI	366	30,6
Rio Grande do Norte	LIMA et al.	2008	IFI	381	17,1
Centro- oeste					
Goiás	LINHARES et al.	1990	MAD	109	43,1
Sudeste					
Minas Gerais	FIGUEIREDO et al.	2001	MAD	174	19
			IFI	174	19,5
			ELISA	174	19,5
Minas Gerais	CARNEIRO et al.	2009	IFI	767	45,8
			ELISA	767	42,8
Rio de Janeiro	LUCIANO et al.	2011	IFI	206	29,12
São Paulo	SILVA et al.	2002	IFI	100	8
			MAD	100	11
São Paulo	MAINARDI et al.	2003	IFI	442	14,47
São Paulo	FIGLIUOLO et al.	2004	IFI	385	28,7
			IFI	360	30
São Paulo	MODOLO et al.	2008	IFI	923	23,40
Sul					
Paraná	SELLA et al.	1994	IFI	153	30,71
Rio Grande do Sul	MACIEL; ARAÚJO	2004	HAI	360	19,4

F.R. – Frequência Relativa; HAI – Hemaglutinação Indireta; IFI – Imunofluorescência Indireta; ELISA – Ensaio Imunoenzimático; AL – Aglutinação em Látex; MAD – Método de Aglutinação Direta.

2.2.2 Transmissão e fatores de risco

A infecção adquirida por via oral pela ingestão de alimentos e água contaminada com oocistos é considerada a principal forma de infecção nos animais (DUBEY; TOWLE, 1986). Os felídeos, principalmente os gatos, desempenham papel fundamental na transmissão do *T. gondii*, pois são os únicos hospedeiros que eliminam oocistos do parasito nas fezes (DUBEY, 1995).

Gennari et al. (2005) estudaram a frequência de infecção por *T. gondii* em gatos não-domiciliados em 15 municípios no estado de São Paulo e encontraram anticorpos *anti-T. gondii* em 35,4% dos 237 gatos estudados.

A presença de gatos foi comprovada em vários estudos como um importante fator de risco para a disseminação de *T. gondii* em rebanhos de caprinos (DUBEY; LIVINGSTON, 1986; MODOLO et al., 2008; ABU-DALBOUH et al., 2011; GARCIA et al., 2012).

O contato com solo contaminado com fezes de gatos infectados é mais importante que o contato direto com esses animais (DUBEY; BEVERLEY, 1988). A infecção dos hospedeiros intermediários depende não apenas das condições ambientais, mas principalmente da densidade populacional de gatos na área, o que irá determinar o grau de contaminação do solo com oocistos do parasito (RUIZ; FRENKEL, 1980; DIAS; FREIRE, 2005).

A dose mínima estimada para infecção oral em pequenos ruminantes é em torno de 200 oocistos esporulados (DUBEY, 1988). Em estudo com caprinos no Ceará, Cavalcante et al. (2008) concluíram que a probabilidade de infecção é maior onde existem mais de 10 gatos devido a grande contaminação ambiental por oocistos eliminados nas fezes, que na fase aguda eliminam cerca de 100.000 oocistos por grama de fezes. Esses oocistos podem permanecer viáveis no ambiente por até dois anos (FREYRE et al., 1997; DIAS; FREIRE, 2005).

Para os felídeos, a ingestão de restos placentários ou animais infectados por *T. gondii* como roedores e aves também foram considerados importantes fatores de risco (ANDERLINI et al., 2011; ABU-DALBOUH, 2012).

Informações epidemiológicas relacionadas a outros fatores de risco associados à infecção por *T. gondii* em caprinos são relatadas na literatura. Uma

forte relação entre idade e presença de anticorpos anti-*T. gondii* foi relatado em rebanhos caprinos. Animais jovens mostraram prevalências mais baixas de infecção que os adultos, uma vez que esses últimos ficam expostos um maior período de tempo aos oocistos presentes no ambiente (LINHARES et al., 1990; SELLA et al.,1994; TENTER et al., 2000; FIGUEIREDO et al., 2001; MEIRELES et al., 2003; FIGLIUOLO et al., 2004; JITTAPALAPONG et al*.,* 2005; CAVALCANTE et al., 2008; MODOLO et al., 2008; ANDERLINI et al., 2011; ASGARI et al., 2011; GARCIA et al., 2012).

Em alguns trabalhos foi confirmado que os sistemas de criação extensiva ou semi-intensiva também são considerados fatores de risco, pois os caprinos ficam mais expostos aos oocistos eliminados por felinos domésticos e silvestres nas pastagens (FIGUEIREDO et al., 2001; MEIRELES et al., 2003; CAVALCANTE et al., 2008; ARAÚJO NETO et al., 2008; ANDERLINI et al., 2011; GARCIA et al., 2012).

As propriedades localizadas nas áreas periurbanas apresentam maior prevalência da infecção por *T. gondii* do que as localizadas na área rural devido à variação na densidade populacional e à dinâmica dos hospedeiros definitivos e intermediários nos ambientes rurais e urbanos (BISSON et al., 2000; THOMAZ-SOCCOL et al., 2009; KAMANI et al., 2010; GARCIA et al., 2012) . No Brasil, Da Silva et al. (2003) verificaram maior prevalência de infecção em caprinos criados em regiões quentes e úmidas o que também foi relatado na Bahia por Gondim et al. (1999), em Pernambuco por Bispo et al. (2011) e Alagoas por Anderlini et al. (2011). Esse fator foi atribuído à formação de um microambiente mais favorável à manutenção e esporulação dos oocistos (DUBEY, 2010).

Outra importante forma de transmissão do *T. gondii* ocorre quando há parasitemia durante a gestação, pela passagem dos taquizoítos via placenta, causando placentite fetal e propagação de *T. gondii* para o feto caracterizando a transmissão congênita (DUBEY, 2010).

2.3 Patogenia e sinais clínicos em caprinos

Os caprinos são severamente acometidos por *T. gondii* e a infecção durante a gestação pode ocasionar vários transtornos reprodutivos (DUBEY, 1990). Na

infecção natural, cabras que pariram natimortos, abortaram ou cujos neonatos morreram foram sorologicamente positivas para *T. gondii* (DA SILVA et al., 2003; PESCADOR et al., 2007; SILVA FILHO et al., 2008).

Na espécie caprina, o abortamento e a perda neonatal podem ser repetidos em gestações subsequentes (DUBEY, 1982). A ocorrência de mortalidade perinatal, abortos e natimortos estão associados ao período de gestação quando a cabra se infecta (DUBEY, 1981). Vitor et al. (1992) observaram aborto ou nascimento de crias debilitadas com morte após o nascimento em cabras infectadas entre o 52^0 e 67^0 dia de gestação por via subcutânea com taquizoítos. Os animais inoculados entre os 100^0 e 133^0 dias de gestação pariram crias normais que sobreviveram.

Engeland et al. (1996) inocularam bradizoítos por via subcutânea em cabras com aproximadamente 71 dias de gestação e observaram que todas abortaram ou pariram natimortos ou filhotes fracos e tornaram-se sorologicamente positivas.

Em geral, as lesões em tecidos fetais consistem em achados microscópicos, particularmente observados no cérebro, fígado e pulmão (DUBEY; BEATTIE, 1988). Encefalite não supurativa e gliose multifocal têm sido frequentemente descritas na infecção por *T. gondii* em fetos abortados (MUNDAY; MASON, 1979; DUBEY; BEATTIE, 1988). Áreas branco-amareladas de aproximadamente 2mm de diâmetro nos cotilédones, microscopicamente correspondentes a áreas de necrose e mineralização também foram descritas (DUBEY, 1980).

Terpsidis et al. (2009) relataram que *T. gondii* pode comprometer os parâmetros reprodutivos, reduzindo a capacidade de fertilização; este achado foi observado em ratos machos infectados com cistos de *T. gondii* e apresentaram redução do peso do epidídimo, na motilidade dos espermatozoides e na redução da concentração espermática.

Santana et al. (2010) observaram material genético de *T. gondii* no sêmen e em amostras teciduais (pool de próstata, testículo, vesícula seminal e epidídimo) em bodes infectados experimentalmente com oocistos ou com taquizoítos, além de sinais clínicos como hipertermia (5^0 dia pós inoculação), anorexia e letargia entre o 3^0 e o 7^0 dia pós inoculação.

As cabras parecem ser mais suscetíveis à toxoplasmose clínica e podem morrer em consequência da toxoplasmose aguda (DUBEY, 2010). Os sinais clínicos são dose dependente (DUBEY, 1981; DUBEY, 1989) e vários aspectos da

toxoplasmose em caprinos inoculados por via oral com 10, 100, 1000 ou 100.000 oocistos foram relatados por Dubey & Beattie (1988). Todos as cabras apresentaram hipertermia (40°C ou mais) entre o 2^0 e 5^0 dias pós infecção com duração de 2 a 10 dias. Cabras que receberam 100 ou mais oocistos apresentaram letargia, redução de apetite, dispnéia e metade delas apresentou diarréia. Aquelas infectadas com 1.000 ou mais oocistos morreram entre o 7^0 e 20^0 dias pós inoculação. As cabras gestantes que receberam 10, 100 ou 1000 oocistos abortaram ou pariram cabritos infectados.

Animais jovens, imunodeprimidos e com infecção sistêmica geralmente desenvolvem pneumonia intersticial, hepatite necrosante focal, linfadenite, miocardite e meningoencefalite não supurativa (DUBEY et al., 1987; BARKER et al., 1992; REDDACLIFF et al., 1993).

2.4 Diagnóstico

Os sinais clínicos da toxoplasmose são inespecíficos e não podem ser utilizados no diagnóstico definitivo (DUBEY, 2010). A infecção por *T. gondii* pode ser diagnosticada indiretamente por métodos sorológicos e diretamente pela Reação em Cadeia da Polimerase (PCR), hibridação, isolamento, imunohistoquímica (IHQ) e técnicas histológicas (MONTOYA; LIESENFELD, 2004).

A sensibilidade e especificidade dos testes sorológicos variam em função do tipo do teste e da espécie animal (FIGUEIREDO, 2001). As reações sorológicas de Sabin-Feldman, Hemaglutinação Indireta (HAI) (NIETO; MELENDEZ, 1988), Imunofluorescência Indireta (RIFI), Fixação de complemento (FC), Enzime-linked Immunosorbent Assay (ELISA) (HASHEMI-FESHARKI, 1996) e o Immunosorbent Agglutination Assay (ISAGA) (UCHOA et al., 1999; DA SILVA et al., 2002) podem ser realizadas no soro sanguíneo, líquido cefalorraquidiano, humor aquoso e outros fluídos corporais (HINRICHSEN, 2005).

A produção de anticorpos da classe IgM ocorre na fase aguda da infecção, seguida pela elevação de anticorpos da classe IgG que aparece na fase crônica. Os títulos de IgG se mantêm constantes ou ascendentes durante o curso da infecção,

decrescendo ou até desaparecendo em poucos meses no caso da transmissão passiva de anticorpos pelo colostro (DUBEY et al., 1987).

A RIFI é uma das melhores técnicas de diagnóstico sorológico da toxoplasmose, sendo sensível, segura e pode ser usada tanto na fase aguda como na fase crônica. A pesquisa de IgG, em análises sequenciais de amostras de soros têm sido utilizada no diagnóstico da toxoplasmose congênita (CAMARGO, 2001). As análises sorológicas usando a RIFI têm sido amplamente empregadas para detectar diversas espécies animais infectadas por *T. gondii,* incluindo as cabras (VAN DER PUIJE et al., 2000; CONDE et al., 2001; NISHI et al., 2008; GARCIA et al., 2012).

O exame histopatológico não é capaz de concluir o diagnóstico da infecção por *T. gondii*, pois o parasito pode ser confundido com núcleos ou fragmentos nucleares que se coram de forma semelhante devido à ausência de características tintoriais próprias (FARREL et al., 1952; TSUNEMATSU et al., 1964; BARBOSA, 1988). Porém é uma técnica que pode ser utilizada, pois vários autores relataram a observação frequente de taquizoítos e lesões macro e microscópicas na placenta, membranas fetais e tecidos fetais (MCSPORRAN et al., 1985; DUBEY et al., 1986; DUBEY, 1988; UGGLA et al., 1987).

A imunohistoquímica é uma técnica específica que confirma o diagnóstico em algumas horas, sendo capaz de detectar pequenas quantidades de antígeno, tornando-o visível ao microscópio óptico (VON WASIELEWSKI et al.,1997) e marcando os cistos teciduais de *T. gondii* (DUBEY; LIN, 1994; DUBEY, 2010). Através desta técnica foram identificados grupos de taquizoítos e cistos em fragmentos de cérebro de natimorto caprino (PESCADOR et al., 2007).

A alta sensibilidade das técnicas sorológicas pode trazer algumas confusões no diagnóstico devido a presença de IgM residuais no soro. Neste sentido, as técnicas moleculares como a PCR podem auxiliar a obter uma melhor interpretação do estado real da interação parasito/hospedeiro (KOMPALIC-CRISTO et al., 2005).

A PCR foi utilizada para detectar DNA do parasito no sêmen de caprinos (DUBEY; SHARMA, 1980; SANTANA et al., 2010), sangue de ovelhas e cabras com histórico de aborto e tecidos de fetos abortados (MORAES et al., 2010; ABU-DALBOUH et al., 2012). Esta técnica molecular é muito sensível, altamente específica e rápida (YAI et al., 2003). Estudos têm ressaltado a importância da PCR

no diagnóstico complementar da toxoplasmose, inclusive com potencial para sua aplicação na rotina de diagnóstico (MASSALA et al., 2003; SREEKUMAR et al., 2004; PESCADOR et al., 2007; NAVARRO et al., 2009).

Amostras de sangue, fluido cerebroespinhal ou tecidos de animais supeitos de infecção por *T. gondii* podem ser utilizados para o bioensaio (DUBEY, 2010). O isolamento é obtido pela inoculação das amostras suspeitas em camundongos, o que requer de três a seis semanas para a confirmação do diagnóstico (GROVER et al., 1990; HITT; FILICE, 1992; JAMES et al., 1996; KUPFERSCHMIDT et al., 2001; MEI-HUI et al., 2001). O isolamento do parasito no sangue ou outros fluidos corporais caracteriza a infecção aguda (MONTOYA; LIESENFELD, 2004).

2.5 Controle da toxoplasmose em caprinos

A extensão da infecção por *T. gondii* em gatos depende da disponibilidade de aves infectadas e pequenos mamíferos, que por sua vez são infectados pela ingestão de oocistos; dessa forma, a prevalência da infecção em gatos de estimação é menor do que em gatos das zonas rurais e periurbanas (DUBEY, 2010).

Nos rebanhos de caprinos, medidas sanitárias devem ser adotadas para controlar os fatores de risco (PEREIRA et al., 2012). Os gatos devem ser castrados para controlar a população de felinos nas propriedades rurais. Para prevenir a infecção, esses hospedeiros não devem ser alimentados com carne crua, vísceras ou ossos e esforços devem ser feitos para manter os gatos dentro de casa para impedir a caça. Membranas fetais e fetos abortados devem ser enterrados ou incinerados para prevenir a infecção dos felinos e outros animais da fazenda. Os gatos não devem ter contato com cabras prenhes (DUBEY, 2010).

Estas recomendações devem ser repassadas para os criadores, pois o conhecimento dos riscos podem ajudar na implantação de medidas de controle visando reduzir a infecção durante os períodos de gestação (GARCIA et al., 2012).

Várias estratégias para vacinação de hospedeiros intermediários e definitivos para a imunização têm sido discutidas (FISHBACK; FRENKEL, 2001; SCHAAP et al., 2007). Desenvolvimento de vacinas contra o *T. gondii* em humanos não têm sido capazes de desenvolver uma imunidade protetora quando o organismo é desafiado

(JONGERT, et al., 2009) A vacina Toxovax® (Schering-Plough Animal Health Ltd) disponível comercialmente, para a espécie ovina produzida a partir da cepa atenuada S48 de *T. Gondii,* reduz o aborto e a mortalidade neonatal (BUXTON; INNES, 1995), mas não impede a infecção fetal após o desafio com cepa virulenta (STANLEY et al., 2004).

A vacina inativada seria a ideal para a prevenção da infecção por *T. gondii* em gatos evitando a eliminação de oocistos. Tal vacina ainda não está disponível, e tendo em vista o grande número de gatos e a dificuldade de os apanhar, a sua utilização em massa é improvável (FREYRE et al.,1993). Uma vacina oral viva usando bradizoítos de uma cepa mutante (T-263) pode impedir que os gatos eliminem oocistos de *T. gondii*. A produção comercial desta vacina foi descontinuada devido a necessidade de ser mantida congelada, curto prazo de validade, alto custo e falta de interesse por parte dos proprietários de gatos (FRENKEL et al., 1991; FREYRE et al., 1993; GIRALDI et al., 1996).

Mais recentemente, Garcia et al. (2007), ao avaliar uma vacina administrada via nasal em gatos, produzida com proteínas de roptrias incorporadas ao Quil-A no controle da eliminação de oocistos, verificaram uma proteção 65% a mais que no grupo controle.

3. OBJETIVOS

3.1. Geral

Estudar os aspectos clínicos, patológicos e reprodutivos da infecção experimental por *Toxoplasma gondii* via sêmen em cabras.

3.2. Específicos

Estudar a transmissão horizontal (venérea) e vertical (transplacentária) em cabras inseminadas via vaginal com sêmen contaminado com taquizoítos de *T. gondii;*

Descrever os distúrbios reprodutivos na fase aguda e crônica da infecção em cabras experimentalmente infectadas com sêmen contaminado com taquizoítos de *T. gondii;*

Investigar a transmissão venérea e transplacentária do *T. gondii* em cabras acasaladas por monta natural com reprodutor infectado com oocistos;

Identificar as perdas embrionárias e fetais nas cabras infectadas por *T. gondii* por meio de exames ultra-sonográficos;

Pesquisar anticorpos anti-*T. gondii* e DNA parasitário de *T. gondii* em amostras séricas e sangue total em cabras inseminadas via vaginal com sêmen contaminado com taquizoítos ou acasaladas com reprodutor infectado com oocistos como também nos cabritos nascidos vivos;

Detectar a eliminação de taquizoítos de *T. gondii* no sêmen de reprodutor caprino infectado com oocistos por meio da técnica da PCR;

Identificar os órgãos dos caprinos infectados por *T. gondii* mais frequentemente parasitados através da PCR;

Realizar exames histopatológicos nos tecidos dos caprinos experimentalmente infectados por *T. gondii;*

Realizar sequenciamento genético em amostras de órgãos positivos na PCR para *T. gondii,* nos caprinos infectados por oocistos.

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CAPÍTULO 1

Infecção experimental em cabras via vaginal com sêmen contaminado com a cepa "CPG" de *Toxoplasma gondii*

(Artigo submetido e aceito no Periódico The Journal of Parasitology)

Infecção experimental em cabras via vaginal com sêmen contaminado com a cepa "CPG" de *Toxoplasma gondii*

Resumo: Objetivou-se neste estudo avaliar a transmissão de *Toxoplasma gondii* em cabras inseminadas via vaginal com sêmen contaminado com taquizoítos da cepa CPG (Genótipo III). Dez cabras foram alocadas aleatoriamente em dois grupos (G1 e G2) cada um com cinco animais, e inseminadas durante o estro. As cabras do G1 foram inseminadas com sêmen contendo $1x10^5$ taquizoítos e as fêmeas do G2, com sêmen livre de taquízoitos (inseminação =dia 0). Para confirmar a infecção via sêmen foram realizados testes sorológicos (reação de imunofluorescência indireta) e moleculares (reação em cadeia da polimerase) e para o acompanhamento gestacional foram utilizados exames ultrassonográficos. No G1, a soroconversão e a presença de DNA parasitário no sangue estavam presentes em 4/5 e 3/5 cabras respectivamente a partir do dia 7. No G2, todas as cabras foram negativas em ambas as técnicas. Reabsorção embrionária ocorreu em 4/5 cabras do G1 entre os dias 21 e 49. Conclui-se que a inseminação vaginal com sêmen contaminado experimentalmente com taquizoítos de *T. gondii* infecta cabras, levantando a possibilidade da transmissão deste parasito via sêmen.

Termos de indexação: Toxoplasmose, cabra, sêmen, taquizoítos, transmissão

INTRODUÇÃO

A infecção por *Toxoplasma gondii* é relatada mundialmente, tendo importância médica e veterinária, por ser causa de aborto e doença congênita em vários hospedeiros intermediários (Tenter et al., 2000). A primeira evidência da toxoplasmose em caprinos foi descrita por Feldman e Miller (1956) e apesar de menos documentada nesta espécie, aparentemente os danos são maiores, acarretando grandes perdas econômicas aos caprinocultores em vários países do mundo (Dubey et al., 1987; Borde et al., 2006, Pescador et al., 2007).

Munday & Mason (1979) foram os primeiros a descreverem a toxoplasmose como importante causa de prejuízos reprodutivos em caprinos, que são seriamente acometidos ao se infectarem durante a prenhez (Dubey, 1990). Estudos experimentais indicaram que as cabras podem abortar mais de uma vez devido à toxoplasmose e o estágio da gestação durante a infecção primária pode afetar o resultado da mesma (Dubey, 1982).

Toxoplasma gondii já foi identificado no sêmen de caprinos (Dubey e Sharma, 1980; Santana et al., 2010) e de outras espécies domésticas (Blewett et al., 1982; Teale et al., 1982; Scarpelli et al., 2001; Moura et al., 2007;). Moraes, Batista et al. (2010) demonstraram que é possível a transmissão de *T. gondii* via sêmen experimentalmente contaminado quando infectaram dois grupos de ovelhas com doses de 6,5 x 10^4 (G1) e 4 x 10^7 (G2) taquizoítos. Observaram soroconversão de 33.3% no G1 e 100% no G2. No nested PCR detectaram DNA do parasito em 93.3% das amostras nos dois grupos. Também foi observada elevada taxa de reabsorção embrionária G2 (100%).

Devido à importância clínica da toxoplasmose na espécie caprina, principalmente na área reprodutiva, é importante estudar outras possíveis vias de transmissão do parasito. Dessa forma, objetivou-se com este trabalho avaliar a infecção por *T. gondii* em cabras inseminadas com sêmen fresco contaminado com taquizoítos da cepa “CPG” (genótipo III).

MATERIAL E MÉTODOS

O experimento foi realizado nas instalações da Fazenda São Luiz/ Universidade Federal de Alagoas, localizada no município de Viçosa, Alagoas, Brasil (9°22' S; 36°14' W). Todo o procedimento experimental seguiu o International Guiding Principles for Biomedical Research Involving Animals e foi aprovado pelo Comitê de Ética da Universidade Federal Rural de Pernambuco (CEUA-UFRPE – licença 007/2010).

Animais

Neste experimento foram utilizadas 10 cabras multíparas, 3 a 4 anos de idade, distribuídas aleatoriamente em dois grupos de cinco animais (G1 e G2). O sêmen utilizado para inseminação artificial foi proveniente de um reprodutor da raça Saanen com histórico reprodutivo de fertilidade e coletado através de eletroejaculador. O sêmen estava dentro dos padrões estabelecidos pelo Colégio Brasileiro de Reprodução Animal (CBRA, 1998). No início do experimento todos os animais foram vermifugados e vacinados contra Clostridioses e Raiva e estavam sorologicamente negativos para *Toxoplasma gondii e Neospora caninum (*Reação de Imunofluorescência Indireta- RIFI*); Brucella abortus (*Antígeno Acidificado Tamponado) e *Chlamydophila abortus (*Fixação de Complemento). Os animais foram mantidos em baias teladas em sistema intensivo. Foram alimentadas com Capim Buffel (*Cenchrus ciliares L.*), Capim-Corrente (*Urochloa moçambicensis Dandy*) e ração comercial, além de sal mineral e água potável *ad libitum*.

Infecção Experimental através de Inseminação Artificial

A contaminação do sêmen foi realizada com taquizoítos da cepa "CPG" (genótipo III), isolada de um surto de abortos em cabras na cidade de Guarapuava, Paraná, Brasil (Silva Filho et al., 2008). A cepa foi inoculada por via intraperitoneal em camundongo e o lavado obtido foi analisado para determinar uma concentração final de $1x10^5$ taquizoítos/7µL.

A indução do estro das cabras foi realizada através de injeção na submucosa vulvar de 0,15 mg de cloprostenol sódico (Ciosin® Shering Plough, Brasil). Para

identificação das fêmeas em estro utilizou-se o rufião, identificando-se os sinais do estro em até 72 horas após a administração da prostaglandina.

A inseminação artificial das cabras foi realizada via vaginal 12h após a detecção de sinais do estro, utilizando-se aplicador universal e amostras de sêmen acondicionadas em palhetas de 0,25 mL (IMV® Tecnologia, L`Aigle, França). Cada fêmea do G1 foi inseminada com uma dose total de 250 µl, constituída de 243 µl de sêmen fresco diluído em leite desnatado na proporção de 1:9 (v:v) e 7µl de solução contendo $1x10^5$ taquizoítos. As fêmeas do G2 foram inseminadas da mesma forma descrita para o G1 com sêmen sem taquizoítos (grupo controle).

Confirmação da Infecção

Sorologia

Para confirmar a infecção nas cabras, foram realizadas coletas de sangue nos dias 0, 7, 14, 21, 28, 49, 63 e 123 dias pós-inseminação (d.p.i). Os anticorpos anti-*Toxoplasma gondii* foram detectados através da Reação de Imunofluorescência indireta (RIFI), segundo Camargo (1974), usando conjugado IgG anti-caprino (Sigma-Aldrich®) com isotiocianato de fluoresceína (FITC), lâminas sensibilizadas com antígenos de taquizoítos de *T. gondii* (cepa RH). Os soros foram testados na razão dois e nas diluições de 1:16 até 1:4096, considerando-se positiva a amostra com título igual ou maior que 64 conforme Chiari et al. (1985).

Detecção de DNA para *T. gondii*

Para a detecção de DNA de *T. gondii*, amostras de sangue total contendo EDTA foram coletadas nos dias 0, 7, 14, 21, 28, 49, 63 e 123 pós-inseminação. As amostras foram submetidas à extração de DNA utilizando-se Kit comercial “Qiagen DNA Easy Blood and Tissues Kit”(Qiagen® Hilden, Germany),seguindo o protocolo do fabricante. Os pares iniciadores utilizados foram TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) e TOX5 (CGCTGCAGACACAGTGCATCTGGATT) segundo Homan et al. (2000), amplificando uma região de 529 pares de base (pb). As reações de amplificação

foram realizadas em um volume final de 12,5µL contendo: 2,5µL de DNA genômico; 0,5µL de cada primer (TOX4 e TOX5) à 10µM; 2,5µL de Água Mili-Q ultrapura e 6,25µL de Top Taq Master Mix (Qiagen®), de acordo com o protocolo do fornecedor. O perfil térmico das etapas de reações foi feito em um termociclador XP Thermal Cycler (Bioer Technology CO. LTDA, Binjiang, Hangzhou, China), consistindo de uma desnaturação do DNA inicial a 94° C (7min) e seguida de 35 ciclos a 94º C por 1 minuto para a desnaturação, 60º C por 1 minuto para o anelamento, 72º C por 1 minuto para a extensão e extensão final de 10 minutos a 72° C. Os produtos amplificados foram detectados por eletroforese em gel de agarose a 2%, corados com Blue Green (LGC®), visualizados por luz ultravioleta e fotodocumentados. O controle positivo utilizado na reação foi obtido por meio de suspensão de lavados intraperitoneais de camundongos previamente infectados com a cepa RH.

Diagnóstico e acompanhamento da gestação

O diagnóstico e o acompanhamento gestacional foi realizado com auxílio de exames ultrassonográficos semanais (CTS 900V, SIUI, China) entre 15 e 60 dias após a inseminação artificial, utilizando-se transdutor linear multifrequencial e frequência de 10 MHz, via transretal. Após 60 dias de gestação, as avaliações ultrassonográficas foram realizadas em intervalos de 15 dias, via abdominal, até o momento do parto. Além disso, todas as fêmeas foram submetidas à avaliação clínica diária, com aferição de temperatura duas vezes ao dia.

RESULTADOS

Sorologia

Nas cabras do G1, 2/5 soroconverteram no 7^0 d.p.i. e 2/5 no 14^0 d.p.i. Nenhuma das cabras do G2 apresentou anticorpos anti-*T. gondii* (Tabela 1). O maior título observado no G1 foi 1024 aos 28 dias nas duas cabras que soroconverteram aos 7 d.p.i. e 512 aos 21 e 28 d.p.i. nas outras duas cabras (Tabela 2).

Diagnóstico Molecular

O DNA de *T. gondii* foi detectado em 3/5 cabras do G1 a partir do 7º d.p.i.; destas, uma permaneceu positiva até os 49 d.p.i.; DNA de *T. gondii* não foi detectado em nenhuma cabra do G2 (Tabela 1).

Acompanhamento da gestação e avaliação clínica

Em relação ao desenvolvimento gestacional, no G1 observou-se reabsorção embrionária em 4/5 cabras do G1, sendo 2/4 no 21º d.p.i., 1/4 no 30º d.p.i. e 1/4 no 49º d.p.i. Em 1/5 foi observada gestação a termo com nascimento de dois cabritos vivos e clinicamente saudáveis. No G2 todas as cabras gestaram e pariram cabritos a termo vivos e clinicamente saudáveis. Não foram verificadas alterações clínicas nos animais do G1 e G2, exceto a hipertermia (média de 40,5 ^{0}C) observada entre o 3° e 13° d.p.i. em todas as cabras do G1.

DISCUSSÃO

Neste estudo investigou-se os efeitos da infecção experimental em cabras inseminadas via vaginal com sêmen contaminado com *T. gondii* na dose de 1x10^{5} taquizoítos. Diferente de outros estudos realizados em caprinos até o momento, a infecção por *T. gondii* ocorreu no momento da fertilização, possibilitando apresentar os resultados dessa via de infecção em cabras. Estudo similar foi realizado por Moraes, Batista et al. (2010) em ovelhas inseminadas com sêmen contaminado por *T. gondii* diferenciando apenas a dose utilizada para a contaminação do sêmen. Os autores demonstraram que as ovelhas dos dois grupos infectados soroconverteram e apresentaram elevada taxa de reabsorção embrionária. Em outro estudo realizado pelo mesmo grupo de pesquisa (Moraes, Freitas et al., 2010) observou-se que ovelhas infectadas via sêmen apresentaram reabsorção embrionária, aborto, natimortos, além de patologias uterinas e ovarianas como hidrometra, mucometra e cistos foliculares.

Neste estudo, das fêmeas que soroconverteram aos 7 d.p.i., 50% delas apresentaram título máximo de anticorpo de 1024 aos 28 dias; as demais que

soroconverteram no 14^0 d.p.i. apresentaram título máximo 512 aos 21 dias que persistiram até o 123^0 d.p.i. Apesar de variações observadas na resposta imunológica de caprinos relatada nos trabalhos que utilizaram diferentes vias de infecção, os resultados obtidos neste estudo são semelhantes no que se refere ao período da soroconversão. Segundo Conde et al. (2001), anticorpos IgG específicos em caprinos experimentalmente infectados com *T. gondii* foram inicialmente detectados a partir do 14^0 d.p.i., atingindo um pico no 35^0 d.p.i. A resposta imune em cabras também foi detectada no 10^0 d.p.i. na RIFI por Nishi et al.(2001) em animais infectados pela via oral. Santana et al. (2010) relataram que a resposta imune em caprinos infectados com taquizoítos e oocistos iniciou no 11^0 d.p.i. em ambos os grupos. O pico de anticorpos (4096) foi observado no 21^0 d.p.i. para o grupo dos taquizoítos e no 28^0 d.p.i. para o grupo de oocistos. Dubey (2010) também detectou parasitemia entre quatro e oito d.p.i. em caprinos infectados com oocistos, com detecção de IgG na segunda semana de infecção, persistindo até o 441^0 d.p.i. Moraes, Batista et al. (2010) que utilizaram a mesma via de infecção deste estudo (sêmen) observaram soroconversão em ovelhas infectadas entre o 7^0 a 28^0 d.p.i. e título máximo de anticorpos de 1024 aos 28 d.p.i.

O DNA de *T. gondii* no sangue foi detectado na PCR no 7^0 d.p.i. e manteve-se até 49^0 d.p.i.. Moraes et al. (2010) também detectaram o DNA de *T. gondii* no sangue total em ovelhas a partir de 14^0 d.p.i., utilizando a mesma via de infecção.

A única alteração clínica observada nos animais do G1 foi hipertermia que coincidiu com os achados da PCR do sangue. O aumento da temperatura foi verificado entre o 3° e 13° d.p.i., apresentando uma média de $40,5^0$C, sendo o máximo observado de 42^0C no 7^0 d.p.i. Esse achado também coincide com o período de soroconversão em alguns animais. Nishi et al. (2001) observaram além da hipertermia, a anorexia e letargia em cabras infectadas com oocistos. Santana et al. (2010) também observaram hipertermia (40.6 °C) como o sinal clínico mais significativo no 5^0 d.p.i. em bodes inoculados com oocistos de *T. gondii*. Moraes et al. (2010a) relataram hipertermia entre o 4^0 e 6^0 d.p.i. e também entre o 7^0 e 14^0 dias, chegando ao máximo de 42.6◦C no 9^0 e 14^0 d.p.i. e Dubey (1989 e 1981) observou que cabras infectadas com oocistos apresentaram-se febris (40°C ou mais) entre o 2^0 a 5^0 d.p.i e esta se estendeu por um período de 2 a 10 dias.

Outro resultado de destaque observado neste estudo foi a alta taxa de reabsorção embrionária em 80% das cabras do G1. Nesse estudo só foi possível identificar e quantificar a reabsorção embrionária nesta fase da gestação por meio do acompanhamento ultrassonográfico realizado semanalmente. A reabsorção no estágio inicial da gestação confirma que a infecção por *T. gondii* no momento da concepção via sêmen influenciou o desenvolvimento embrionário e culminou com a morte embrionária. Na infecção com oocistos, o parasito invade a placenta das cabras desde os nove dias pós- infecção e os tecidos fetais com 15 dias pós-infecção (Dubey, 2010). Na infecção experimental com oocistos, as cabras frequentemente desenvolvem febre e podem abortar durante a segunda semana pós-infecção (Dubey & Beattie, 1988; Rosa et al., 2001). Acredita-se que na infecção via sêmen, o parasito também se multiplica na placenta e no embrião, causando sua morte e reabsorção embrionária. Moraes et al. (2010b), também observaram a elevada taxa de reabsorção embrionária em ovelhas infectadas com sêmen contaminado com a mesma cepa utilizada neste estudo. Esta cepa foi isolada de um surto de aborto, onde 61 cabras de um mesmo rebanho abortaram aos três meses de gestação e 59 delas apresentaram sorologia positiva para *T. gondii* (Silva Filho et al., 2008).

Com os resultados obtidos neste estudo pode-se afirmar que a inseminação vaginal com sêmen contaminado por taquizoítos de *Toxoplasma gondii* é capaz de infectar cabras, levantando discussão sobre a transmissão via sêmen deste parasito. Como essa via de transmissão ainda não foi comprovada na toxoplasmose caprina, esse aspecto deve ser melhor investigado na infecção experimental com a utilização de reprodutores infectados com oocistos e utilizados na monta natural ou inseminação artificial na fase aguda e crônica da doença.

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Conflito de Interesse

Nenhum

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Tabela 1 - Resultados da PCR e RIFI em cabras inseminadas com sêmen contaminado por *Toxoplasma gondii*

Animal/ Grupo	Dias Pós Infecção (DPI)															
	0 DPI		7 DPI		14 DPI		21 DPI		28 DPI		49 DPI		63 DPI		123 DPI	
G1	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**
1	-	-	-	+	-	+	-	+	-	+	-	+	-	-	-	-
2	-	-	P	-	P	-	P	-	P	-	P	-	P	-	*	*
3	-	-	-	-	P	-	P	-	P	-	P	-	P	-	*	*
4	-	-	-	+	P	-	P	-	P	-	P	-	P	-	*	-
5	-	-	P	+	P	-	P	-	P	-	P	-	P	-	P	-
G2																
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P = positivo no teste sorológico (titulo ≥ 64); + = positivo na PCR

*Animais eutanasiados aos 90 DPI

Tabela 2 - Títulos de anticorpos para *T. gondii* na RIFI em cabras inseminadas com sêmen contaminado por *Toxoplasma gondii*

Animal	Títulos entre 0 e 123 DPI							
	0 DPI	7 DPI	14 DPI	21 DPI	28 DPI	49 DPI	63 DPI	123 DPI
1	-	-	-	-	-	-	-	-
2	-	1:64	1:512	1:512	1:1024	1:1024	1:1024	*
3	-	-	1:64	1:512	1:512	1:512	1:512	*
4	-	-	1:64	1:256	1:512	1:512	1:512	*
5	-	1:64	1:512	1:512	1:1024	1:1024	1:1024	1:1024

*Animais eutanasiados aos 90 DPI

CAPÍTULO 2

Distúrbios reprodutivos em cabras após inseminação artificial com sêmen contaminado com *Toxoplasma gondii*

(Artigo submetido no Periódico PLOS ONE)

Distúrbios reprodutivos em cabras após inseminação artificial com sêmen contaminado com *Toxoplasma gondii*

Resumo: O objetivo deste estudo foi caracterizar as desordens reprodutivas em cabras experimentalmente infectadas com sêmen contaminado com a Cepa “CPG” (genótipo III) de *Toxoplasma gondii*. Dez cabras foram distribuídas aleatoriamente em dois grupos (G1 e G2), cada um com cinco animais, e inseminadas durante o estro. As cabras do G1 foram inseminadas com sêmen contendo $1x10^5$ taquizoítos, enquanto as do G2 (controle) foram inseminadas com sêmen livre de taquizoítos (inseminação = dia 0). No G1, a soroconversão (reação de imunoflourescência indireta) ocorreu entre os dias 7 e 14 em 4 de 5 cabras. Além disso, no G1, o DNA de *T. gondi* estava presente (reação em cadeia da polymerase) no sangue de 3 cabras entre os dias 7 e 49, e em vários tecidos em 3 cabras e 2 nascidos vivos. No G2, todas as cabras foram negativas em todos os testes. As cabras do G1 foram monitoradas nas fases aguda e crônica da infecção; reabsorção embrionária ocorreu em 4 de 5 cabras na fase aguda, enquanto na fase crônica, anestro ocorreu em 2 de 5 cabras, hidrossalpinge em 1 de 5 e cisto ovariano em 1 de 5. A inseminação artificial vaginal com sêmen contendo taquizoítos de *T. gondii* infecta cabras, causando distúrbios reprodutivos durante as fases aguda e crônica da infecção.

Termos de indexação: caprinos; inseminação artificial; toxoplasmose; taquizoítos.

INTRODUÇÃO

A espécie caprina é considerada uma das mais suscetíveis à infecção por *T. gondii* (Dubey & Adams, 1990), sendo a toxoplasmose uma das principais causas de aborto em cabras no mundo (Dubey, 2010; Asgari et al., 2011). A infecção natural por meio da ingestão de oocistos durante a prenhez pode causar infecção placentária e vários transtornos como reabsorção embrionária, mumificação fetal, aborto ou natimorto e mortalidade neonatal em cabras, acarretando grandes perdas econômicas (Dubey, 1982; Blewett, 1983; Dubey et al., 1986; Dubey, 1990; Chanton-Greutmann et al., 2002; Dias e Freire, 2005; Silva Filho et al., 2008).

Na infecção experimental, Moraes et al. (2010b) observaram reabsorção embrionária, anestro, hidrometra, mucometra e cisto folicular em ovelhas infectadas via sêmen contaminado com diferentes doses de taquizoítos de *Toxoplasma gondii* da cepa “CPG” (genótipo III).

Não existem estudos na literatura sobre distúrbios reprodutivos na fase aguda e crônica da infecção por *T. gondii* via sêmen contaminado experimentalmente em caprinos. Desta forma objetivou-se com este estudo diagnosticar e descrever os distúrbios reprodutivos em cabras após inseminação artificial com sêmen fresco contaminado experimentalmente com taquizoítos da cepa “CPG” (genótipo III).

MATERIAL E MÉTODOS

Estação Experimental

O experimento foi realizado nas instalações da Fazenda São Luiz/ Universidade Federal de Alagoas, localizada no município de Viçosa, Alagoas, Brasil (9°22’ S; 36°14’ W). Todo o procedimento experimental seguiu o International Guiding Principles for Biomedical Research Involving Animals e foi aprovado pelo Comitê de Ética da Universidade Federal Rural de Pernambuco (CEUA-UFRPE – licença 007/2010).

Animais e Infecção Experimental

Neste experimento foram utilizadas 10 cabras multíparas, 3 a 4 anos de idade, distribuídas aleatoriamente em dois grupos de cinco animais (G1 e G2). O sêmen utilizado para inseminação artificial foi proveniente de um reprodutor da raça Saanen com histórico reprodutivo de fertilidade e coletado através de eletroejaculador. O sêmen estava dentro dos padrões estabelecidos pelo Colégio Brasileiro de Reprodução Animal (CBRA, 1998). No início do experimento todos os animais foram vermifugados e vacinados contra Clostridioses e Raiva e estavam sorologicamente negativos para *Toxoplasma gondii e Neospora caninum (*Reação de Imunofluorescência Indireta- RIFI*); Brucella abortus (*Antígeno Acidificado Tamponado) e *Chlamydophila abortus (*Fixação de Complemento*).* Os animais foram mantidos em baias teladas em sistema intensivo de criação.

Os taquizoítos utilizados para a contaminação do sêmen eram da cepa “CPG” (genótipo III), isolados de um surto de abortos em cabras na cidade de Guarapuava, Paraná, Brasil (Silva Filho et al., 2008). A cepa foi repassada em camundongo, por meio de inoculação intraperitoneal e aspirada através de lavagem abdominal, utilizando água destilada. Logo em seguida realizou-se a centrifugação e ressuspensão dos taquizoítos de forma a se obter uma concentração final de $1x10^5$ taquizoítos/7µL.

Inicialmente realizou-se a indução do estro das cabras através de injeção de 0,15 mg de cloprostenol sódico (Ciosin® Shering Plough, São Paulo,Brasil) na submucosa vulvar. Para identificação das fêmeas em estro utilizou-se um macho para rufiação duas vezes ao dia, observando-se os sinais do estro em até 72 horas após o tratamento.

A inseminação artificial foi realizada via vaginal 12h após a detecção do estro, utilizando-se aplicador universal e amostras de sêmen acondicionadas em palhetas de 0,25 mL (IMV® Tecnologia, L`Aigle, França). Cada cabra do G1 foi inseminada com uma dose total de 250 µl, composta de 243 µl de sêmen fresco diluído na proporção de 1:9 (v:v) com leite desnatado Molico® (Nestlé Brasil) e 7µl de solução contendo $1x10^5$ taquizoítos. As fêmeas do G2 foram inseminadas da mesma forma descrita para o G1 com sêmen sem taquizoítos (grupo controle).

Confirmação da Infecção

Para confirmar a infecção nas cabras foram realizados exames sorológicos e moleculares. Foram realizadas coletas de sangue nos dias 0, 7, 14, 21, 28, 49, 63 e 123. Os anticorpos anti-*Toxoplasma gondii* foram detectados através da RIFI, (Camargo, 1974), usando conjugado IgG anti-caprino (Sigma-Aldrich®, St. Louis, Missouri, U.S.A) com isotiocianato de fluoresceína (FITC), lâminas (Perfecta®, São Paulo – Brasil) sensibilizadas com antígenos de taquizoítos de *T. gondii* (cepa RH). Os soros foram testados nas diluições de 1:16 até 1:4096, considerando-se positiva a amostra com título igual ou maior que 64, conforme Chiari et al. (1985).

Para a detecção de DNA de *T. gondii*, amostras de sangue total contendo EDTA foram coletadas nos dias 0, 7, 14, 21, 28, 49, 63 e 123 pós-inseminação. A Reação em Cadeia de Polimerase (PCR) também foi realizada em órgãos (fígado, baço, rins, medula, cérebro, pulmão, coração, útero, ovário, testículos) e placentas das cabras e cabritos eutanasiados ao final do experimento. As amostras foram submetidas à extração de DNA utilizando-se Kit comercial “Qiagen DNA Easy Blood and Tissues Kit”(Qiagen®, Hilden, Germany), seguindo o protocolo do fabricante. Para a PCR foram utilizados os pares iniciadores TOX4 (CGCTGCAGG GAGGAAGACGAAAGTTG) e TOX5 (CGCTGCAGACACAGTGCATCTGGATT) segundo Homan et al. (2000), amplificando uma região de 529 pares de base (pb). As reações de amplificação foram realizadas em um volume final de 12,5mL contendo: 2,5µL de DNA genômico; 0,5µL de cada primer (TOX4 e TOX5) à 10µM; 2,5µL de Água Mili-Q ultrapura e 6,25µL de Top Taq Master Mix (Qiagen®), de acordo com o protocolo do fornecedor. O perfil térmico das etapas de reações foi feito em um termociclador XP Thermal Cycler (Bioer Technology Co. Ltd., Binjiang, Hangzhou, China), consistindo de uma desnaturação do DNA inicial a 94°C (7min) e seguida de 35 ciclos a 94ºC por 1 minuto para a desnaturação, 60ºC por 1 minuto para o anelamento, 72ºC por 1 minuto para a extensão e extensão final de 10 minutos a 72°C. Os produtos amplificados foram detectados por eletroforese em gel de agarose a 2%, corados com Blue Green (LGC®, Cotia, São Paulo, SP, Brazil), visualizados através de luz ultravioleta e fotodocumentados. O controle positivo utilizado na reação foi obtido por meio de suspensão de lavados intraperitoneais de camundongos previamente infectados com a cepa RH.

Diagnóstico e acompanhamento da gestação e do parto

O diagnóstico e o acompanhamento gestacional foi realizado com auxílio de exames ultrassonográficos semanais entre 15 e 60 dias após a inseminação artificial, utilizando-se transdutor linear multifrequencial e frequência de 10 MHz (CTS 900V, SIUI, China), via transretal. Após 60 dias de gestação, as avaliações ultrassonográficas foram realizadas em intervalos de 15 dias, via abdominal, até o momento do parto. O nascimento dos animais do G1 e G2 foi acompanhado e as placentas coletadas, identificadas e congeladas para exame molecular (PCR) e em formol tamponado a 10% para exame histopatológico.

Estação de monta Controlada

Todos as cabras do G1 que apresentaram reabsorção embrionária foram submetidas novamente a uma estação de monta controlada. As cabras foram rufiadas por até 60 dias e acasaladas. Após o acasalamento elas foram acompanhadas por exames ultrassonográficos para diagnóstico de gestação ou patologias reprodutivas.

Exame anátomo-histopatológico

Ao final do experimento todas as cabras e cabritos nascidos vivos foram eutanasiados. A eutanásia humanitária foi realizada de acordo com a Resolução nº 1000 do Conselho Federal de Medicina Veterinária (CFMV, 2012). Foram realizadas necropsias de todos os animais e coletados fragmentos do fígado, baço, rins, medula, cérebro, pulmão, coração, útero, ovário e testículos. Os órgãos das cabras do G1 e G2, dos cabritos e das placentas também foram avaliados microscopicamente. As amostras foram fixadas em formol tamponado a 10%. Após fixação, o material foi emblocado em parafina e cortado para exame histopatológico em micrótomo (Model 1512, Leitz Wetzlar, Wetzlar, Hessen, Germany) com 4µm e coradas por hematoxilina e eosina e classificados como ausência de lesões, lesões não relacionadas ou lesões relacionadas à toxoplasmose.

RESULTADOS

Sorologia

No G1, 4 de 5 (80%) fêmeas soroconverteram, sendo que destas, 2 de 4 (50%) no dias 7 e 2 de 4 (50%) no dia 14. Nenhuma das cabras do G2 apresentou anticorpos anti-*T. gondii*. O maior título observado foi 1024 aos 28 dias nas duas cabras que soroconverteram aos 7 d.p.i.; as outras duas cabras apresentaram títulos de 512 no dia 21. A única cabra do G1 que não soroconverteu levou a gestação a termo e seus cabritos nasceram saudáveis e também foram negativos na sorologia (Tabela 1).

Diagnóstico Molecular

O DNA de *T. gondii* foi detectado no sangue em 3 de 5 (60%) das cabras do G1 entre os dias 7 e 49 e no sangue de dois cabritos nascidos vivos. No G2 nenhuma cabra foi positiva**.** O DNA do parasito também foi detectado em vários órgãos das cabras do G1 e dos cabritos (Tabela 2).

Exame Anátomo-histopatológico

As lesões macroscópicas obsevadas nas cabras do G1 foram um cisto ovariano e uma hidrossalpinge bilateral (Tabela 1). Não foram encontradas alterações microscópicas nos órgãos do sistema reprodutor. As principais lesões microscópicas observadas neste grupo foram a presenca de áreas de hemorragia e infiltrado neutrofílico nos pulmões, glomerulonefrite intersticial, presenca de infiltrado neutrofílico no fígado e gliose cerebral.

Distúrbios Pós- Inseminação (Fase aguda)

Na fase inicial da gestação observou-se elevada taxa de reabsorção embrionária em 4 de 5 cabras (80%), entre 21 e 49 dias de gestação no G1; não foi observada perda embrionária em nenhuma fêmea do G2, com nascimento de cabritos saudáveis.

Distúrbios Pós-estação de monta (fase crônica)

Das quatro cabras que apresentaram reabsorção embrionária, duas apresentaram anestro e duas que apresentaram repetição de cio, foram novamente acasaladas e não emprenharam**.** Uma das cabras apresentou repetição de cio em intervalos de sete dias e a outra ciclou em intervalos de 21 dias. Nos exames ultrassonográficos subsequentes à reabsorção embrionária foram identificados cisto ovariano (1/4) e hidrossalpinge (1/4) (Figura 1; Tabela 1), posteriormente confirmados na necropsia. Todas as cabras do G2 pariram cabritos saudáveis e não apresentaram patologias reprodutivas.

DISCUSSÃO

Neste estudo experimental, o sêmen fresco foi contaminado com taquizoítos de *T. gondii* com o objetivo de inseminar cabras e investigar a infecção vertical via sêmen, além de diagnosticar as patologias reprodutivas durante as fases aguda e crônica da infecção. Este estudo é pioneiro no que se refere à infecção de cabras via sêmen no momento da fertilização. Os principais achados observados foram a repetição de cio e reabsorção embrionária nas primeiras semanas da gestação (fase aguda da infecção).

Na infecção experimental, alguns autores demonstraram a presença de *T. gondii* no sêmen por meio de bioensaio (Dubey e Sharma, 1980) onde o parasito foi detectado a partir do 7^0 até 59^0 dia pós-infecção em bodes infectados com oocistos. Santana et al. (2010) também infectaram bodes com taquizoítos e demonstraram o parasito no sêmen por PCR a partir do 5^0 até 70^0 d.p.i. No grupo infectado com oocistos, observaram a eliminação do parasito no 56^0 d.p.i. Em animais naturalmente infectados, Moraes et al. (2010c) detectaram o DNA do parasito no sêmen de reprodutores ovinos por meio da PCR. Contudo, a transmissão do *T. gondii* via sêmen na toxoplasmose naturalmente adquirida em caprinos e ovinos ainda não foi comprovada.

Neste estudo confirmou-se a transmissão de *T. gondii* via sêmen por meio dos achados sorológicos e moleculares observados nas cabras do G1. Achados semelhantes foram obtidos por Moraes et al. (2010a) em ovelhas inseminadas com

sêmen contaminado com diferentes doses de taquizoítos da mesma cepa utilizada neste estudo. Observaram soroconversão a partir do 7^0 dia pós-infecção em 100% das fêmeas inseminadas com dose de 4 x 10^7 taquizoítos e em 33,3% das fêmeas inseminadas com 6,5 x 10^4 taquizoítos. A parasitemia foi observada a partir do 7^0 dia pós-infecção, coincidindo com os achados do presente estudo onde maioria das cabras também apresentou PCR de sangue positiva e soroconversão a partir do 7^0 dia pós infecção.

A elevada taxa de reabsorção embrionária e repetição de cio em intervalos irregulares observados em algumas cabras do G1 demonstram que o parasito inoculado juntamente com o sêmen (momento da fertilização) interferiu na fase inicial da gestação, causando a morte do embrião. Esses achados foram pouco relatados na toxoplasmose naturalmente adquirida em cabras pelo difícil diagnóstico destas alterações nos rebanhos. Na infecção experimental via oral realizada em cabras em diferentes períodos de gestação, Dubey (1981, 1989) relatou a ocorrência de morte e reabsorção fetal em todas as cabras inoculadas após os 50 dias de gestação. Em ovelhas inseminadas com sêmen contaminado com taquizoítos de *T. gondii,* Moraes et al. (2010b) também observaram elevada taxa de reabsorção embrionária.

As cabras são mais sensíveis à infecção por *T. gondii* que as ovelhas e o estágio de gestação na infecção primária pode afetar o resultado da gestação. O aborto é observado com mais frequência na fase aguda da infecção e estudos experimentais demonstraram que cabras infectadas por *T. gondii* podem abortar mais de uma vez (Dubey, 1982).

As alterações reprodutivas não se limitam à fase aguda, o que foi confirmado neste estudo, uma vez que duas cabras que entraram em cio após a reabsorção embrionária foram novamente acasaladas e não conceberam. O diagnóstico de anestro observado em 2 de 4 cabras que tiveram reabsorção embrionária também confirma que na infecção experimental via sêmen o parasito pode causar este tipo de patologia em cabras.

Outro achado de destaque foi a detecção de DNA do parasito em vários tecidos de dois cabritos que nasceram saudáveis em uma cabra do G1. Das duas placentas analisadas, uma também foi positiva na PCR. Este resultado demonstra que o parasito se multiplicou na placenta e se distribuiu nos tecidos do feto sem causar a sua morte. De acordo com Dubey (1989)**,** *T. gondii* se multiplica com maior

intensidade no tecido placentário que em outros tecidos na cabra e em estudo experimental por via oral observou que a partir do 9^0 dia pós-infecção, o parasito já se encontrava na placenta e no 15^0 dia pós-infecção nos tecidos fetais. Isto explica a alta taxa de reabsorção embrionária observada a partir do 21^0 dia pós-infecção.

Em relação aos órgãos dos cabritos e das cabras do G1, os tecidos que mais apresentaram reações positivas na PCR foram o cérebro e coração. Moraes et al. (2010a) também detectaram o DNA do parasito por meio da nested-PCR em vários tecidos fetais de ovinos, cujas mães foram infectadas com sêmen contaminado com taquizoítos de *T. gondii*.

Na fase crônica da infecção foram observados anestro, cistos foliculares e hidrossalpinge. As outras lesões microscópicas observadas foram raras e consideradas em sua maioria inespecíficas para toxoplasmose. Moraes et al. (2010b) também observaram patologias reprodutivas como hidrometra, mucometra e cisto folicular em ovelhas infectadas por *T. gondii* pela mesma via de infecção. Serrano-Martinez et al. (2007) infectaram vacas via sêmen com taquizoítos de *N. caninum*, um protozoário da mesma subfamília do *T.gondii* e também observaram taxas significativas de reabsorção embrionária.

Este estudo é pioneiro no que se refere à infecção experimental de cabras via sêmen no momento da fertilização. Esta via de infecção pode causar a toxoplasmose em cabras e determinar patologias reprodutivas nas fases aguda e crônica da infecção, impedindo novas gestações e confirmando a extrema susceptibilidade dessa espécie a toxoplasmose .

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Conflito de interesse

Nenhum

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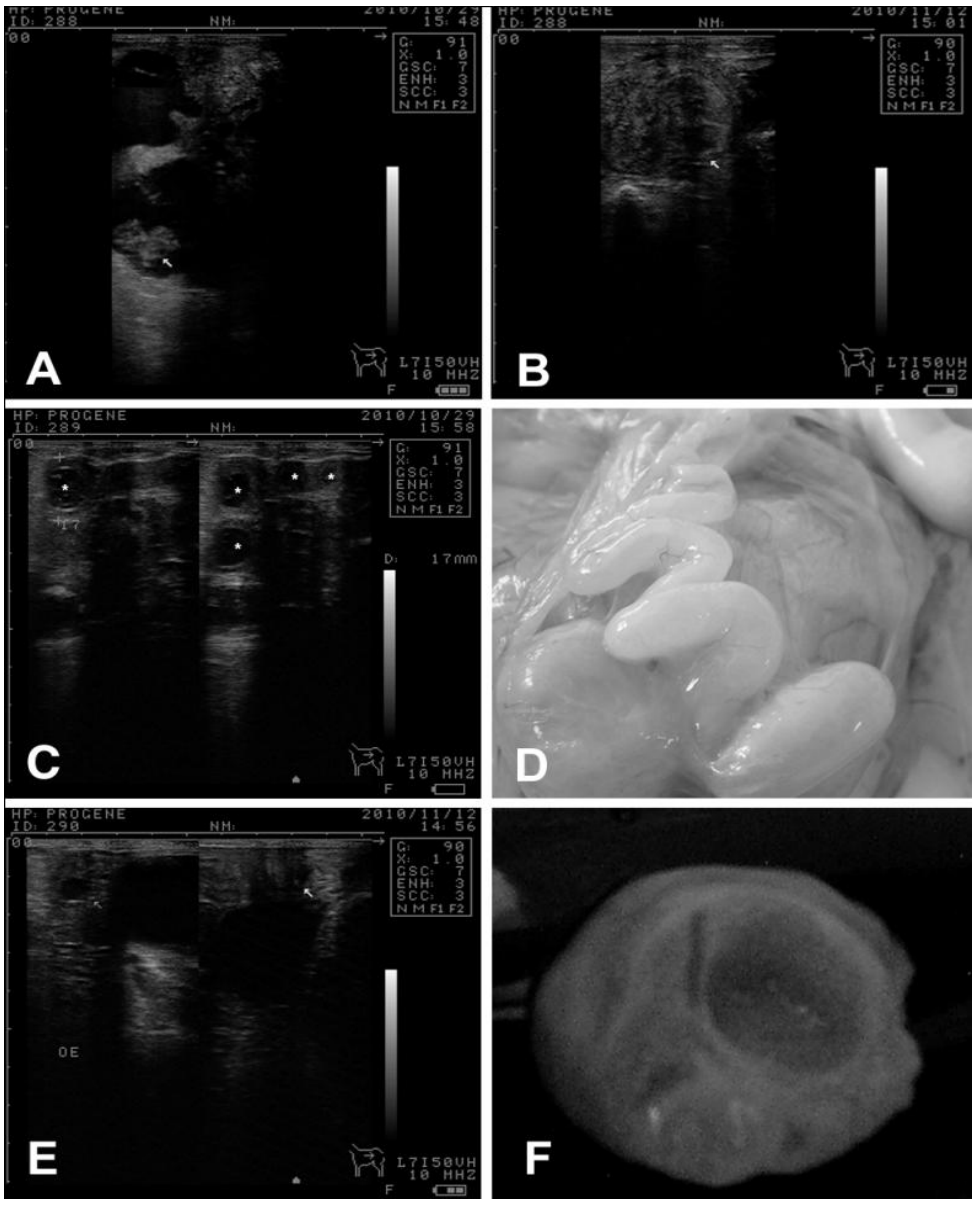

Figura 1 – Confirmação da gestação no Dia 35 na cabra 2 do G1 (observar embrião na seta) **(A)**; Ausência de feto (reabsorção) no Dia 49 d.p.i na cabra 2 do G1 **(B)**; Imagem Ultrassonográfica (frequência 10 MHz) do oviduto da cabra 3 do G1 após inseminação artificial com sêmen contaminado com *Toxoplasma gondii* sendo possível visibilizar a presença de estruturas com aspecto vesicular, apresentando mucosa hipoecogênica de superfície irregular e conteúdo hipoecogênico heterogêneo (asteriscos), medindo aproximadamente 17 mm em seu maior diâmetro. Achados compatíveis com hidrossalpinge **(C)**; Achado de necropsia da cabra 3, confirmando a imagem ultrassonográfica **(D)**; Imagem ultrassonográfica de ovário da cabra 2, compatível com cisto ovariano **(E)**; Achado de necropsia da cabra 2, confirmando a imagem ultrassonográfica **(F).**

Tabela 1 – Resultados da necropsia e exames laboratoriais em caprinos infectados por *Toxoplasma gondii*

Animal/ Grupo	Sorologia	PCR sangue	PCR tecidos	Diagnóstico de Gestação	Gestação Viável	Reabsorção	Alterações Reprodutivas	Necropsia
G1								
1	-	+	-	+	+	-	-	-
1 (C1[a])	-	+	+	-	-	-	-	-
1 (C2[a])	-	+	+	-	-	-	-	-
2	+	-	-	+	-	+	Anestro	Cisto ovariano
3	+	-	+	+	-	+	Repetição de cio	Hidrossalpinge
4	+	+	+	+	-	+	Anestro	-
5	+	+	+	+	-	+	Repetição de cio	-
G2								
6	-	-	-	+	+	-	-	-
6 (C1[a])	-	-	-	-	-	-	-	-
7	-	-	-	+	+	-	-	-
7 (C1[a])	-	-	-	-	-	-	-	-
7 (C2[a])	-	-	-	-	-	-	-	-
8	-	-	-	+	+	-	-	-
8 (C1[a])	-	-	-	-	-	-	-	-
8 (C2[a])	-	-	-	-	-	-	-	-
9	-	-	-	+	+	-	-	-
9 (C1[a])	-	-	-	-	-	-	-	-
10				+	+			
10 (C1[a])	-	-	-	-	-	-	-	-

+= positivo no teste da PCR ou sorologia (titulação≥64); [a]Cabritos nascidos vivos

Tabela 2 - Resultado da PCR em tecidos de cabras infectadas por *Toxoplasma gondii*

Animal	Fígado	Baço	Rim	Medula	Cérebro	Pulmão	Coração	Placenta
1	-	-	-	-	-	-	-	-
1 (C1[a])	**+**	**+**	**+**	**+**	**+**	-	-	-
1 (C2[a])	-	-	-	-	**+**	**+**	**+**	**+**
2	-	-	-	-	-	-	**-**	**-**
3	-	-	-	-	+	-	-	**-**
4	-	-	-	-	+	-	+	**-**
5	-	-	-	-	-	-	+	**-**

[a] Cabritos nascidos vivos

CAPÍTULO 3

Transmissão venérea do *Toxoplasma gondii* em cabras acasaladas com reprodutor experimentalmente infectado com oocistos

(Artigo submetido no Periódico The Journal of Parasitology)

Transmissão venérea do *Toxoplasma gondii* em cabras acasaladas com reprodutor experimentalmente infectado com oocistos

Resumo: Objetivou-se neste estudo comprovar a transmissão venérea de *Toxoplasma gondii* em caprinos. Dez cabras foram alocadas aleatoriamente em dois grupos (G1 e G2), cada um com cinco animais. As cabras do G1 foram acasaladas com um bode infectado experimentalmente com oocistos de *T. gondii* e as do G2 (controle) acasaladas com outro bode sorologicamente negativo. A infecção do reprodutor e das cabras foi confirmada por meio dos achados clínicos, sorológicos, moleculares e histopatológicos. No bode a soroconversão (reação de imunofluorescência indireta) foi observada no 7^0 dia pós-infecção (d.p.i.) e o DNA (reação em cadeia da polimerase) de sangue e sêmen esteve presente a partir do 3^0 d.p.i. No G1, duas cabras soroconverteram e o DNA foi encontrado no sangue de duas delas; no G2 todas as amostras foram negativas nos dois testes. Em relação ao desenvolvimento gestacional, os exames ultrassonográficos mostraram que no G1 houve reabsorção embrionária em 1/5 cabras aos 34 dias pós-acasalamento; 1/5 cabras abortou aos 42 dias pós-acasalamento e 3/5 cabras pariram a termo; 01 dos cabritos apresentou PCR de sangue positiva ao nascimento. No total, 40% das cabras do G1 e 80% dos cabritos nascidos vivos do G1 apresentaram PCR positiva para pelo menos um órgão. Neste estudo comprovou-se a transmissão venérea do *T. gondii* para cabras acasaladas com bode infectado.

Termos de indexação: Toxoplasmose, cabra, sêmen, transmissão

Introdução

A toxoplasmose é uma doença de distribuição mundial causada pelo *Toxoplasma gondii* que tem os felídeos como hospedeiros definitivos e numerosas espécies de vertebrados como hospedeiros intermediários. Apresenta importância veterinária, pois pode provocar abortos e outros problemas reprodutivos em animais (Tenter et al., 2000). A ingestão de oocistos esporulados de *T. gondii* liberados no ambiente nas fezes de gatos é considerada a principal via de transmissão deste parasito (Dubey & Beattie 1988; Dubey & Beverley, 1988).

A doença é considerada a maior causa de problemas reprodutivos em caprinos e ovinos em muitos países do mundo (Skjerve et al., 1998; Borde et al., 2006). A ocorrência de distúrbios reprodutivos em caprinos está mais relacionada ao período de gestação em que o animal se infectou do que com a cepa de *T. gondii* (Dubey, 1981). A infecção experimental com oocistos em cabras gestantes negativas para *T. gondii* no primeiro terço da gestação determinou a ocorrência de retenção ou reabsorção fetal; no segundo e terceiro terços da gestação ocorreram abortamentos, natimortos ou nascimento de cabritos infectados (Dubey and Beattie, 1988; Dubey, 1989; Obendorf et al., 1990).

A presença de *T. gondii* no sêmen de caprinos experimentalmente infectados com oocistos foi comprovada por Dubey e Sharma (1980) e Santana et al. (2010), contudo a transmissão venérea do parasito ainda não foi confirmada.

Considerando a importância desta doença na reprodução de caprinos, objetivou-se neste estudo relatar a transmissão venérea de *T. gondii* em cabras acasaladas com bode infectado com oocistos da cepa ME-49 (Tipo II).

Material e Métodos

O experimento foi realizado nas instalações da Fazenda São Luiz/ Universidade Federal de Alagoas, localizada no município de Viçosa, Alagoas, Brasil. Todo o procedimento experimental seguiu o International Guiding Principles for Biomedical Research Involving Animals e foi aprovado pelo Comitê de Ética da Universidade Federal Rural de Pernambuco (CEUA-UFRPE – licença 007/2010).

Animais

Neste estudo foram utilizados dois bodes da raça Saanen com histórico reprodutivo de fertilidade e sêmen dentro dos padrões estabelecidos pelo Colégio Brasileiro de Reprodução Animal (1998). Para compor o grupo das fêmeas foram utilizadas 10 cabras, alocadas aleatoriamente em dois grupos (G1 e G2), cada um com cinco animais. Esses animais eram sorologicamente negativos para *Toxoplasma gondii e Neospora caninum* (Reação de Imunofluorescência Indireta)*; Brucella abortus (*Antígeno Acidificado Tamponado) e *Chlamydophila abortus (*Fixação de Complemento).

Todos os animais foram alojados em baias teladas em sistema intensivo. Foram alimentadas com Capim Buffel (*Cenchrus ciliares L.*) e ração comercial, sal mineral comercial e água potável *ad libitum*. No início do experimento todos os caprinos foram vermifugados e vacinados contra Clostridioses e Raiva.

Infecção experimental do reprodutor com oocistos

Para a infecção do bode foram utilizados oocistos da cepa ME-49. A dose infectante foi de $2x10^5$ oocistos administrada em dose única por via oral, utilizando-se uma seringa de 5 ml acoplada a uma sonda.

As cabras foram induzidas ao estro em dias diferentes para não entrarem no estro no mesmo dia, o que dificultaria as coberturas pelo bode infectado. Desta forma, quatro dias após a infecção experimental do reprodutor, a primeira cabra foi sincronizada por meio da aplicação por via intramuscular de 0,3 ml de Cloprostenol Sódico (Ciosin® Shering Plough, Brasil). No 6^0 d.p.i., outras três cabras foram sincronizadas e no 10^0 d.p.i., a última cabra foi sincronizada. Desta forma, duas cabras apresentaram estro e foram acasaladas no 8^0 d.p.i.; uma no 9^0 d.p.i.; uma no 10^0 d.p.i. e uma no 13^0 d.p.i. Cada cabra foi acasalada por monta natural (2 a 3 vezes) durante o estro. A mesma estratégia de sincronização e cobertura foi utilizada no grupo controle.

Confirmação da Infecção

Diagnóstico sorológico e molecular

Para confirmar a infecção no bode e nas cabras do G1 e G2 foram realizados exames sorológico e molecular. Nas cabras foram realizadas coletas de sangue nos dias 0, 7, 14, 21, 28, 49, 63 e 123 dias após o acasalamento e no dia do parto; no bode as amostras de sangue e sêmen foram coletadas nos dias 0, 3, 5, 7, 11, 14, 21, 28, 35, 42, 49, 56, 63 e 70 d.p.i. Para os cabritos nascidos vivos foi feita a coleta de sangue logo após o nascimento antes da ingestão do colostro.

Os anticorpos anti-*Toxoplasma gondii* foram detectados na RIFI, usando conjugado IgG anti-caprino (Sigma-Aldrich®,St.Louis,Missouri,U.S.A.) com isotiocianato de fluoresceína (FITC), lâminas sensibilizadas pela técnica "Silk Screen" com antígenos de taquizoítos de *T. gondii* (cepa RH). Os soros foram testados nas diluições de 1:16 até 1:4096.

As amostras de sangue, sêmen e tecidos foram submetidas à extração de DNA utilizando-se Kit comercial "Qiagen DNA Easy Blood and Tissues Kit" (Qiagen®, Hilden, Germany), seguindo o protocolo do fabricante. Os pares iniciadores utilizados foram TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) e TOX5 (CGCTGCAGACACAGTGCA TCTGGATT) segundo Homan et al. (2000), amplificando uma região de 529 pares de base (pb). As reações de amplificação foram realizadas em um volume final de 12,5mL contendo: 2,5µL de DNA genômico; 0,5µL de cada primer (TOX4 e TOX5) à 10µM; 2,5µL de Água Mili-Q ultrapura e 6,25µL de Top Taq Master Mix (Qiagen®), de acordo com o protocolo do fornecedor. O perfil térmico das etapas de reações foi feito em um termociclador XP Thermal Cycler (Bioer Technology Co. Ltd., Binjiang, Hangzhou, China), consistindo de uma desnaturação do DNA inicial a 94°C (7min) e seguida de 35 ciclos a 94ºC por 1 minuto para a desnaturação, 60ºC por 1 minuto para o anelamento, 72ºC por 1 minuto para a extensão e extensão final de 10 minutos a 72°C. Os produtos amplificados foram detectados por eletroforese em gel de agarose a 2%, corados com Blue Green (LGC®, Cotia, São Paulo, SP, Brasil), visualizados através de luz ultravioleta e fotodocumentados. O controle positivo utilizado na reação foi obtido por

meio de suspensão de lavados intraperitoneais de camundongos previamente infectados com a cepa RH.

Amplicons foram purificados empregando comercial GFXTM PCR DNA e um kit de purificação Gel Band (GE Healthcare). Utilizando o sequenciador ABI PRISM 3100 (Applied Biosystems), as reações foram realizadas em ambas as cadeias utilizando iniciadores (TOX4 e TOX5) de acordo com o Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) e as condições de polimerização foram realizadas em 96 poços de acordo com as instruções do fabricante. As sequências foram analisadas por meio BioEdit e MEGA 5 software e comparado com o banco de dados do NCBI utilizando Blastn.

Acompanhamento Clínico

Foi realizada avaliação clínica diária dos bodes e cabras, incluindo aferição de temperatura duas vezes ao dia.

Diagnóstico e acompanhamento da gestação

O diagnóstico e o acompanhamento da gestação (G1 e G2) foi realizado com auxílio de exames ultrassonográficos semanais (CTS 900V, SIUI, China) entre 15 e 60 dias após a monta natural, utilizando-se transdutor linear multifrequencial e frequência de 10 MHz, via transretal. Após 60 dias de gestação, as avaliações ultrassonográficas foram realizadas em intervalos de 15 dias, via abdominal, até o momento do parto. O nascimento dos animais do G1 e G2 foi monitorado, e as placentas foram coletadas, identificadas, e congeladas para realização da PCR ou conservada em formol tamponado (10%) para exame histopatológico.

Exame histopatológico

Ao final do experimento todas as cabras e cabritos nascidos vivos foram eutanasiados. As cabras foram eutanasiadas aos 240 dias pós-acasalamento e os cabritos aos 90 dias após o nascimento e o reprodutor aos 240 d.p.i. A eutanásia humanitária foi realizada de acordo com a Resolução nº 1000 do Conselho Federal

de Medicina Veterinária (CFMV, 2012). Foram realizadas necropsias de todos os animais e coletados fragmentos do fígado, baço, rins, medula, cérebro, pulmão, coração, útero, ovário e testículos. O material foi fixado em formol tamponado a 10%, incluído em parafina, cortadas em seções de 6 µm em um micrótomo (Model 1512, Leitz Wetzlar, Wetzlar, Hessen, Germany) e coradas por hematoxilina e eosina para exame histopatológico.

Resultados

Diagnóstico sorológico e molecular

O bode soroconverteu aos 7 d.p.i. (título de 64); aos 11 d.p.i. apresentou título máximo de 1024, mantido até a última coleta aos 70 d.p.i. No G1, 2/5 cabras soroconverteram aos 123 dias após ao acasalamento com título 32. Nenhum cabrito e cabras do G2 apresentaram anticorpos anti- *T. gondii* (Tabela 1).

O DNA de *T. gondii* foi detectado no sangue e sêmen do reprodutor a partir do 3^0 d.p.i. até a última coleta aos 70 d.p.i., 2/5 cabras do G1 apresentaram PCR positiva no sangue no 7^0 dia após o acasalamento e outra no dia do parto. Nos órgãos examinados na PCR, 2/5 cabras e 4/5 cabritos do G1 foram positivos para pelo menos um órgão**;** o DNA de *T. gondii* não foi detectado em nenhuma cabra e cabritos do G2 (Tabela 2).

A identificação molecular do amplicon foi confirmada através do sequenciamento de fita dupla direta, que indicou 91% de similaridade com sequências de DNA de *T. gondii* armazenados no GenBank.

Sinais Clínicos

O bode infectado apresentou apatia, hiporexia, tosse e hipertermia (máxima de 41,3^0 C), taquicardia e taquipnéia entre o 3^0 e 8^0 d.p.i., recuperando-se após esse período. Neste dia ocorreu o acasalamento da primeira cabra do G1. As cabras do G1 apresentaram tosse seca com extertores no exame clínico após o 9^0 dia após o acasalamento.

Acompanhamento da gestação

Em relação ao desenvolvimento gestacional, no G1 observou-se reabsorção embrionária em 1/5 cabras aos 34 dias após o acasalamento. Abortamento foi observado em 1/5 cabras no 42^0 dia de gestação e 3/5 das cabras pariram a termo. No G2 todas as cabras gestaram e pariram filhotes a termo vivos e clinicamente saudáveis (Tabela 1).

Exame histopatológico

No exame histopatológico foram observadas hemorragia, espessamento de septos alveolares, infiltrado mononuclear perivascular e intersticial compatíveis com pneumonia intersticial; necrose hepática multifocal e presença de infiltrado polimorfonuclear predominantemente neutrofílico; gliose perivascular com hemorragia focal.

Discussão

Neste estudo infectou-se experimentalmente um reprodutor caprino com oocistos de *T. gondii* da cepa ME-49 que acasalou com cabras na fase aguda da infecção. Após a recuperação clínica do reprodutor que ocorreu a partir do 8^0 d.p.i. foram iniciados os acasalamentos com as cabras previamente sincronizadas. Os resultados obtidos neste estudo são inéditos na literatura, uma vez que até o momento não se tem dados sobre a toxoplasmose adquirida via sêmen na monta natural. Neste estudo confirmou-se a eliminação do parasito no sêmen e sua transmissão para as cabras e suas crias e foram observadas algumas alterações reprodutivas em decorrência da infecção como reabsorção embrionária e aborto em duas cabras acasaladas com este bode. Optou-se pelo intervalo entre 7 e 15 d.p.i. para o acasalamento das cabras, considerando-se os resultados obtidos por Dubey e Sharma (1980) que utilizaram um bioensaio em camundongos para demonstrar a presença de *T. gondii* no sêmen de três bodes infectados via oral com 10^4 oocistos da cepa GT-1 (isolado de cabras). Naquele estudo, o parasito foi detectado no sêmen a partir do 7^0 d.p.i. em dois animais e no 12^0 d.p.i. no terceiro animal

infectado. No presente estudo, a eliminação do parasito no sêmen detectado na PCR foi a partir do 3^0 d.p.i.

A infecção do bode foi confirmada pelos sinais clínicos, PCR de sangue e sêmen, sorologia positiva a partir do 7^0 d.p.i. (título de 64) e 1024 no 11^0 d.p.i., sendo mantido até o fim das observações no 70^0 d.p.i. O período de soroconversão foi diferente daquele observado por Santana et al. (2010) que infectaram bodes via oral com dose de 2 x 10^5 oocistos da cepa P e identificaram material genético de *T. gondii* no sêmen no 56^0 d.p.i e detecção de IgG no 11^0 (256) d.p.i., alcançando o maior título (4096) no 28^0 d.p.i. com redução para 1024 a partir do 35^0 d.p.i., mantida até o final do experimento. Nishi et al. (2001) infectaram caprinos com 10^5 oocistos da cepa AS 28 e demonstraram sorologia positiva no 10^0 d.p.i.

Os sinais clínicos observados no reprodutor também estão de acordo com os relatados por Rosa et al. (2001) e Santana et al. (2010). No presente estudo, a hipertermia coincidiu com o período de positividade na PCR e do aumento de título de anticorpos na sorologia.

Nas cabras, os achados clínicos e histopatológicos caracterizaram um quadro de pneumonia intersticial, compatíveis com as alterações causadas por *T. gondii* (Dubey et al., 1987, Hartley & Dubey 1991, Barker et al., 1992, Reddacliff et al., 1993, McGavin; Zachary, 2012).

A transmissão de *Toxoplasma gondii* para cabras na monta natural foi comprovada neste estudo pelos resultados obtidos em alguns animais do G1. 2/5 cabras apresentaram anticorpos anti-*T. gondii* (título 32) no 123^0 após o acasalamento. Uma dessas cabras também apresentou PCR de sangue positiva no 30^0 dia após o acasalamento e pariu a termo. A outra cabra apresentou reabsorção embrionária detectada no 34^0 dia de gestação. Outra cabra negativa na sorologia e PCR de sangue abortou no 42^0 dia de gestação. Essa cabra apresentou PCR positiva de cérebro e ovário. Ainda observou-se em outra cabra com sorologia negativa, PCR de sangue positiva no dia do parto, além de PCR positiva de fígado, baço e rim e pariu a termo. A última cabra desse grupo apresentou sorologia e PCR de sangue e tecidos negativos e pariu a termo.

Vitor et al. (1992) infectaram cabras gestantes com taquizoítos via subcutânea e confirmaram a transmissão congênita de *T. gondii*. Observaram a presença de anticorpos nos fetos abortados ou naqueles nascidos vivos. Os resultados de

soroconversão das cabras observados neste estudo devem ser melhor investigados uma vez que não se obteve uma resposta com títulos elevados de anticorpos como geralmente se observa em outras vias de infecção. Provavelmente a via de infecção utilizada neste estudo tenha interferido na resposta de anticorpos.

Outro achado de destaque neste estudo foi a PCR positiva no cérebro em 1/5 cabritos, coração em 2/5 cabritos e rim em 2/5 cabritos nascidos vivos. Este resultado indica que *T. gondii* também é transmitido para a prole (transmissão congênita) quando a cabra não apresenta reabsorção embrionária ou aborto. Alguns estudos recentemente realizados em ovinos demonstraram que o parasito foi capaz de infectar ovelhas via sêmen contaminado com *T. gondii* (Moraes et al., 2010 a, b). Observaram elevadas taxas de reabsorção embrionária no grupo de ovelhas infectadas com a maior dose de taquizoítos, além de distúrbios reprodutivos como anestro, cisto folicular, hidrometra e mucometra na fase crônica da infecção. Estes distúrbios não foram observados no presente estudo em cabras provavelmente devido a dose infectante eliminada no sêmen do reprodutor, pois de acordo com Dubey (1981, 1989) que realizaram infecção experimental com oocistos em cabras gestantes, os sinais clínicos observados são dose dependente. Este aspecto também deve ser melhor investigado na infecção experimental e natural de reprodutores quanto ao número de parasitos eliminados no sêmen, além do período de eliminação do parasito.

É importante considerar que neste estudo, o reprodutor acasalou com as cabras na fase aguda da infecção, mesmo com redução da libido, a transmissão ocorreu, pois nesta fase da infecção o número de parasitos provavelmente seja maior no sêmen. Ainda não se pode afirmar sobre a importância real desta via de infecção em condições de campo, pois neste caso o reprodutor na fase aguda da infecção pode não ter interesse no acasalamento das cabras no cio. O impacto desta via de infecção deve ser melhor investigado na reprodução de caprinos em condiçoes naturais.

Os resultados obtidos neste estudo permitem concluir sobre a transmissão de *T. gondii* via sêmen em cabras. Alguns aspectos devem ser melhor esclarecidos em estudos posteriores, principalmente aqueles relacionados com os aspectos imunológicos da infecção por esta via, além da repercussão clínica na reprodução das cabras.

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Tabela 1 – Resultado dos exames laboratoriais em cabritos e cabras acasaladas com reprodutor infectado por *Toxoplasma gondii*

Animal/ Grupo	Sorologia	PCR Sangue	PCR Orgãos	Diagnóstico de Gestação	Reabsorção	Aborto
G1						
1	+	+	-	x	-	-
1*	-	-	+	-	-	-
2	+	-	-	x	x	-
3	-	-	+	x	-	x
4	-	+	+	x	-	-
4 *	-	+	+	-	-	-
4*	-	-	+	-	-	-
5	-	-	-	x	-	-
5*	-	-	+	-	-	-
5 *	-	-	-	-	-	-
G2						
6	-	-	-	x	-	-
6*	-	-	-	-	-	-
7	-	-	-	x	-	-
7*	-	-	-	-	-	-
7*	-	-	-	-	-	-
8	-	-	-	x	-	-
8*	-	-	-	-	-	-
8*	-	-	-	-	-	-
9	-	-	-	x	-	-
9 *	-	-	-	-	-	-
10	-	-	-	x	-	-
10*	-	-	-	-	-	-

+ = positiva na PCR ou sorologia (titulação = 32); * cabritos nascidos vivos

Tabela 2 – Resultado da PCR em tecidos de bodes, cabras e cabritos infectados por *Toxoplasma gondii*

Animal	Fígado	Baço	Rim	Medula	Cérebro	Pulmão	Coração	Placenta	Ovário
1	-	-	-	-	-	-	-	-	-
1 *	-	-	+	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-
3	-	-	-	-	+	-	-	-	+
4	+	+	+	-	-	-	-	-	-
4 *	-	-	-	-	+	-	+	-	-
4 *	-	-	+	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-
5 *	-	-	-	-	-	-	+	-	-
5*	-	-	-	-	-	-	-	-	-
Reprodutor	-	+	+	-	-	-	-	-	-

* Cabritos nascidos vivos

5. CONSIDERAÇÕES FINAIS

Neste estudo comprovou-se a suscetibilidade de cabras à infecção experimental por *Toxoplasma gondii* via sêmen, sendo observadas a soroconversão e problemas reprodutivos na fase aguda e crônica da infecção. Estas patologias podem persistir, causando consequências nas novas gestações ou perdas embrionárias que foi o achado mais relevante nas cabras inseminadas. A transmissão venérea, comprovada neste experimento ainda deve ser melhor estudada e compreendida, principalmente na infecção natural, pois vários aspectos ainda devem ser esclarecidos com destaque para aqueles relacionados à dose infectante no sêmen de reprodutores e a viabilidade do parasito nesta secreção.

APÊNDICES

APÊNDICE A

EXPERIMENTAL VAGINAL INFECTION OF GOATS WITH SEMEN CONTAMINATED WITH THE “CPG” STRAIN OF *Toxoplasma gondii*

(Artigo submetido e aceito no periódico The Journal of Parasitology)

EXPERIMENTAL VAGINAL INFECTION OF GOATS WITH SEMEN CONTAMINATED WITH THE "CPG" STRAIN OF *Toxoplasma gondii*

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STRACT: The objective was to characterize the transmission of *Toxoplasma gondii* in goats experimentally infected vaginally with semen contaminated with the CPG strain (genotype III). Ten female goats were randomly allocated into 2 groups (G1 and G2), each with 5 animals, and inseminated during estrus. Goats in G1 were inseminated with semen containing $1x10^5$ tachyzoites, whereas those in G2 (control) were inseminated with semen free from tachyzoites (insemination = day 0). In G1, seroconversion (indirect immunofluorescence reaction) and DNA (polymerase chain reaction) in the blood was present in 4/5 and 3/5 respectively from the 7th day. In G2, all goats were negative in all tests. Embryonic reabsorption occurred in 4 of 5 goats from G1 between days 21 and 49. In conclusion, artificial vaginal insemination with semen containing tachyzoites of *T. gondii* infected goats, and is a potential transmission route of this parasite through semen.

Toxoplasma gondii infections have been reported in many regions of the world and are of medical and veterinary importance because they lead to congenital diseases and abortion in many intermediate hosts (Tenter et al., 2000). The first evidence of toxoplasmosis in goats was reported by Feldman and Miller (1956). Although the disease is less documented in goats, it apparently causes the greatest damage in this species, leading to large economic losses for goat farmers in many countries worldwide (Dubey et al., 1987; Borde et al., 2006; Pescador et al., 2007).

Munday & Mason (1979) were the first to describe toxoplasmosis as a significant cause of reproductive impairment in goats. These animals can become seriously affected when the infection occurs during pregnancy (Dubey, 1990). Experimental studies have shown that goats may abort more than once due to toxoplasmosis, and that the stage of pregnancy during primary infection can affect the outcome of the pregnancy (Dubey, 1982).

Toxoplasma gondii has already been identified in the semen of goats (Dubey and Sharma, 1980; Santana et al., 2010) and other domestic species (Blewett et al., 1982; Teale et al., 1982;

Scarpelli et al., 2001; Moura et al., 2007). Moraes, Batista et al. (2010) demonstrated that it is possible for *T. gondii* to be transmitted through experimentally contaminated semen to females inseminated with doses of 6.5×10^4 (G1) and 4×10^7 (G2) tachyzoites. Seroconversion was observed in 33.3% of the females in G1 and in 100% of those in G2. In the "nested" PCR, DNA of the parasite was detected in 93.3% of the samples analyzed in both groups. An elevated embryonic reabsorption was also recorded in G2 (100%).

Due to the clinical significance of toxoplasmosis in goats, particularly in terms of reproduction, it is important to study other possible transmission routes of the parasite. Therefore, the aim of the present study was to assess *T. gondii* infection in goats inseminated with fresh semen that had been contaminated with tachyzoites of the "CPG" strain (genotype III).

MATERIALS AND METHODS

The present study was conducted on the São Luiz farm and in the Universidade Federal de Alagoas, located in the municipality of Viçosa, Brazil. All of the experimental procedures followed the guidelines set down in the International Guiding Principles for Biomedical Research Involving Animals. The present study received approval from the Ethics Committee of the Universidade Federal Rural de Pernambuco under protocol number CEUA - UFRPE - 007/2010.

Animals

Ten multiparous crossbred goats were used in the present study. They were randomly allocated divided into 2 groups (G1 and G2), each with 5 animals. The animals were confined in an intensive screened bay system. They were fed with Buffel grass (*Cenchrus ciliares L.*), hay grass (*Urochloa moçambicensis Dandy*) and commercial foodstuffs, as well as mineral salt and drinking water *ad libitum*. All of the animals were dewormed at the beginning of the experiment.

The semen to be used for insemination was provided by a male of the Saanem breed with a history of reproductive fertility and complied with the standards established by the Colégio Brasileiro de Reprodução Animal (1998). The animal was serologically negative for *T. gondii* and *Neospora caninum* (Indirect Immunofluorescence) as well as for *Brucella abortus* (Buffered Acidified Antigen) and *Chlamydophila abortus* (Complement Fixation).

Experimental infection through artificial insemination

Semen contamination was performed with tachyzoites of the "CPG" strain (genotype III), isolated from an outbreak of abortions in goats in the city of Guarapuava, Paraná, Brazil (Silva Filho et al., 2008). The strain was inoculated intraperitoneally in mice and the lavage obtained was analyzed to determine a final concentration of $1x10^5$ tachyzoites/7µL.

Estrus was induced in the goats by injecting 0.15 mg of cloprostenol sodium (Ciosin® Shering Plough, (São Paulo, SP, Brazil) into the vulvar submucosa. A teaser was used to identify the females in estrus for a period of up to 72 hours after administration of the prostaglandin.

Artificial insemination of goats was performed vaginally 12 hours after detecting signs of estrus, using a universal applicator and samples of semen packed into straws of 0.25 ml (IMV® Tecnologia, L`Aigle, France). Each female in G1 was inseminated with a total dose of 250 µl, containing 243 µl of fresh semen diluted in skimmed milk in a ratio of 1:9 (v: v) and 7µl of solution containing $1x10^5$ tachyzoites. The females in G2 were inseminated in the same way with semen containing no tachyzoites (control group).

Confirmation of infection

Serology:

In order to confirm infection in the goats, blood samples were collected after 0, 7, 14, 21, 28, 49, 63 and 123 d.p.i. Antibodies to *Toxoplasma gondii* were detected using the Indirect Immunofluorescence Reaction (RIFI), using anti-goat IgG conjugated (Sigma-Aldrich®) with

fluorescein isothiocyanate, as well as slides sensitized by "Silk Screen" with antigens of tachyzoites of *T. gondii* (RH strain). The sera were tested at a ratio of four and dilutions of 1:16 to 1:4096, considering a sample to be positive when the titers were equal to or greater than 64 (Camargo, 1974).

Detection of T. gondii *DNA:*

In order to detect *T. gondii* DNA, blood samples of whole blood containing EDTA were withdrawn at 0, 7, 14, 21, 28, 49, 63 and 123 d.p.i. Samples were submitted to PCR. The samples were then submitted to DNA extraction using the "Qiagen DNA Easy Blood and Tissues Kit" (Qiagen®, Hilden, Germany), following the manufacturer's instructions. The primer pairs used were TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (CGCTGCAGACACAGTGCATCTGGATT) as described by Homan et al. (2000), amplifying a region of 529 base pairs (bp). The amplification reactions were carried out in a final volume of 12.5µL containing: 2.5µL of genomic DNA; 0.5µL of each primer (TOX4 and TOX5) to 10µM; 2.5µL of Milli-Q ultrapure water and 6.25µL of Top Taq Master Mix (Qiagen®), following the manufacturer's instructions. The thermal profile of the reaction steps was conducted in an XP Thermal Cycler (Bioer Technology Co. Ltd., Binjiang, Hangzhou, China), consisting of a denaturation of the initial DNA at 94 C (7 min) and followed by 35 cycles at 94 C for 1 min for denaturation, 60 C for 1 min for annealing, 72 C for 1 min for extension and a final extension of 10 min at 72 C. The amplified products were detected by agarose gel electrophoresis (2%), stained with Blue Green (LGC®, Cotia, São Paulo, SP), visualized under ultraviolet light, and finally photodocumented. The positive control used in the reaction was obtained through the suspension of washed intraperitoneal mice that had been previously infected with the RH strain.

Diagnosis and monitoring of pregnancy

The diagnosis and monitoring of pregnancy was performed using weekly ultrasound examinations (CTS 900V, SIUI, China) between the 15th and 60th days after artificial insemination. These examinations were carried out transrectally with a multi frequency linear transducer and a frequency of 10MHz. After 60 days of pregnancy, the ultrasound assessments were performed abdominally at intervals of 15 days until the time of birth. All of the females were submitted to daily clinical assessments and their temperature was measured twice a day.

RESULTS

Serology

In G1, 2/5 goats seroconverted on the 7th d.p.i. and 3/5 on the 14th d.p.i. None of the goats in G2 seroconverted (Table I). The greatest titer in G1 was 1,024 at 28 days in the 2 goats that seroconverted after 7 d.p.i. and 512 at 21 d.p.i. in the other 2 goats (Table II).

Molecular Diagnosis

Toxoplasma gondii DNA was detected in 3/5 goats in G1 from the 7^{th} d.p.i. Of these three animals, one remained positive until the 49^{th} d.p.i. In G2, all goats were negative (Table I).

Pregnancy monitoring and clinical assessment

In terms of gestational development, embryonic reabsorption ocurred in 4/5 goats in G1, with 2 on the 21^{st} d.p.i., 1on the 30^{th} d.p.i., and 1 on the 49^{th} d.p.i. With regards to the remaining goat (1/5), a full-term pregnancy occurred with the birth of 2 live and clinically healthy progeny. All of the goats in G2 gave birth (full-term) to live and clinically healthy kids. There were no clinical abnormalities discovered in the animals of G1 and G2, other than hyperthermia (mean of 40.5 C) between the 3^{rd} and 13^{th} d.p.i. in all of the goats in G1.

DISCUSSION

The present study investigated the effects of experimental vaginal infection of goats through semen contaminated with *T. gondii* ($1x10^5$ tachyzoites). The animals were infected with *T. gondii* at the time of fertilization, which differs from all previous studies with goats, enabling the presentation of results specific to this infection route in goats. A similar study was conducted by Moraes, Batista et al. (2010) in sheep that were inseminated with semen that had been contaminated with *T. gondii*, with a different dose used in the contamination of the semen. The authors demonstrated that sheep from the two infected groups seroconverted and presented elevated rates of embryonic reabsorption. A separate study performed by the same authors (Moraes, Freitas et al., 2010) reported that sheep infected through semen exhibited embryonic reabsorption, abortion, stillbirths, and uterine and ovarian pathologies such as hydrometra, mucometra and follicular cysts.

Among the females who seroconverted on the 7th d.p.i. in the present study, 50% presented a maximum antibody titer of 1,024 at 28 days. The goats that seroconverted on the 14th d.p.i. exhibited a maximum antibody titer of 512 at 21 days which persisted until the 123rd d.p.i. In spite of the differences in immunological responses related to time of seroconversion of goats reported in studies using different infection routes, the results obtained in the present study are similar in terms of the period of seroconversion. According to Conde et al. (2001), specific IgG antibodies in goats that were experimentally infected with *T. gondii* were detected from the 14th d.p.i., and peaked on the 35th d.p.i. The immune response in goats was also detected on the 10th d.p.i. of the RIFI by Nishi et al. (2001) with animals that had been orally infected. Santana et al. (2010) reported that the immune response of goats that had been infected with tachyzoites and oocysts began on the 11th d.p.i. in both groups. The peak of antibodies (4,096) was recorded on the 21st d.p.i. in the tachyzoite group and on the 28th d.p.i. in the oocyst group. Dubey (2010) also observed parasitemia between

the 4th and 8th d.p.i. in goats that had been infected with oocysts, with the IgG detected in the second week of infection, and persisting until the 441st d.p.i. Moraes, Batista et al. (2010) used the same infection route as the present study (semen) and reported seroconversion in infected sheep between the 7th and 28th d.p.i., as well as a maximum antibody titer of 1,024 on the 28th d.p.i.

The response of antibodies to *T. gondii* is individual and depends on the inoculation route. The vaginal route was studied for the first time in the present study and a number of responses still require investigation. The authors of the present study believe that the goat that did not seroconvert was affected by an insufficient quantity of tachyzoites to stimulate a detectable humoral immune response in the Indirect Immunofluorescence. However, the quantity was sufficient for detection using the molecular technique, which is more sensitive and can detect small quantities of the parasite in blood. Canadá et al. (2006) and Moraes, Batista et al. (2010) also obtained negative results in the serology and positive results in the PCR for females infected with *T. gondii* through the intrauterine route.

The only clinical abnormality in G1 was hyperthermia, which coincided with the PCR findings in the blood. An increase in temperature was confirmed between the 3rd and 13th d.p.i., with a mean temperature of 40.5 C and the maximum temperature of 42 C recorded on the 7th d.p.i.. This finding also coincided with the period of seroconversion in a number of animals. Santana et al. (2010) also reported hyperthermia (40.6 C) as the most significant clinical sign (5th d.p.i.) in goats that had been inoculated with oocysts of *T. gondii*. Nishi et al. (2001) reported anorexia, lethargy and hyperthermia in goats that were infected by oocysts. Moraes, Batista et al. (2010) reported hyperthermia between the 4th and 6th d.p.i. and also between the 7th and 14th d.p.i., reaching a maximum of 42.6 C on the 9th and 14th d.p.i. Dubey (1981, 1989) related that goats which had been infected with oocysts exhibited fever

(40 C or more) between the 2nd and 5th d.p.i. and this condition lasted for a period of between 2 and 10 days.

Another significant result of the present study was the high rate of embryonic reabsorption in 3/5 goats in G1. In the present study, the identification and quantification of embryonic reabsorption was possible due to the weekly ultrasound examinations. Reabsorption in the initial stage of pregnancy confirms that the infection with *T. gondii* at the time of conception affected the development of the embryo and culminated in its death. When the infection is with oocysts, the parasite invades the placenta of goats from the 9th day after infection and the fetal tissues after 15 days (Dubey, 2010). In cases of experimental infection with oocysts, goats often develop fever and may abort during the second week after infection (Dubey, 2010; Rosa et al., 2001). It is believed that when semen is used to cause the infection, the parasite also multiplies in the placenta and in the embryo, leading to embryonic reabsorption and death. Moraes, Freitas et al. (2010) also reported high rates of embryonic reabsorption in sheep that were infected with semen contaminated with the same strain used in the present study. This strain was isolated from an outbreak of abortions, in which 61 goats from the same herd aborted after three months of pregnancy and 59 of them exhibited positive serology for *T. gondii* (Silva Filho et al., 2008).

The results of the present study confirm that vaginal infection using semen contaminated with tachyzoites of *Toxoplasma gondii* can infect goats. Since this transmission route has still not been proven in terms of toxoplasmosis in goats, further studies should be conducted with naturally infected goats.

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Table 1 – PCR and serology results from goats inseminated with semen contaminated with *Toxoplasma gondii*

Animal/ Grupo	Days post-infection (DPI)															
	0 DPI		7 DPI		14 DPI		21 DPI		28 DPI		49 DPI		63 DPI		123 DPI	
G1	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**	**IFI**	**PCR**
1	-	-	-	+	-	+	-	+	-	+	-	+	-	-	-	-
2	-	-	P	-	P	-	P	-	P	-	P	-	P	-	*	*
3	-	-	-	-	P	-	P	-	P	-	P	-	P	-	*	*
4	-	-	-	+	P	-	P	-	P	-	P	-	P	-	*	-
5	-	-	P	+	P	-	P	-	P	-	P	-	P	-	P	-
G2																
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P =positive based on serology test (titration≥64); **+ =** positive based on PCR test.

*****Animals euthanized (90 DPI)

Table 2 – Antibody titers for *T. gondii* in the IFI of goats inseminated with semen contaminated with *Toxoplasma gondii*

Animal	Títers between 0 to 123 Days Post-infection (DPI)							
	0 DPI	7 DPI	14 DPI	21 DPI	28 DPI	49 DPI	63 DPI	123 DPI
1	-	-	-	-	-	-	-	-
2	-	1:64	1:512	1:512	1:1024	1:1024	1:1024	*
3	-	-	1:64	1:512	1:512	1:512	1:512	*
4	-	-	1:64	1:256	1:512	1:512	1:512	*
5	-	1:64	1:512	1:512	1:1024	1:1024	1:1024	1:1024

*****Animals euthanized (90 DPI)

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Review articles: Only invited reviews are published. Unsolicited reviews should not be submitted, but topics may be suggested to the editor or members of the editorial board.
Critical comments: Critical comments are for correcting errors of published fact, providing alternative interpretations of published data, or presenting new ideas based on published information.
Book reviews: Books having a broad interest to the membership of the Society are reviewed by invitation.

GUIDELINES FOR AUTHORS

Electronic submission

The *Journal of Parasitology* accepts papers submitted online using the **PeerTrack** system provided by Allen Press (http://www.edmgr.com/jparasitology). The only software required is Adobe Acrobat Reader (available free from www.adobe.com). First time users of the system are required to register for an account and will be assigned an account login and password. The account login and password are required for subsequent use of the system. The system allows authors to check the status of their manuscript and to add updated files. Access to manuscripts submitted electronically is strictly controlled by login and user privileges, thus assuring data security and confidentiality.
Text files should be submitted in Microsoft Word files (doc or docx), text, Postscript, and rich text. Use of the line numbering and page numbering features of your word-processing software will greatly facilitate review. **Do not submit the text of your manuscript as a PDF file**. Figure legends must be submitted in the manuscript and not as part of the figures themselves. Plates must not be embedded in the manuscript, but rather submitted as individual files uploaded into PeerTrack. After successful submission to PeerTrack, the manuscript will be assigned a tracking number and to an Associate Editor.

Articles

Manuscripts are to be organized in the following format and sequence, beginning with that for the running head, numbered consecutively.
Running head: Provide the last names of authors (use et al. for more than 2) and a shortened title. The entire running head may not exceed 60 characters and spaces. Style: RH: JONES ET AL.—LIFE CYCLE OF *H. DIMINUTA*, or RH: JONES AND SMITH – LIFE CYCLE OF *H. DIMINUTA*
Title: Immediately after the running head give the title of the article (in all caps), names of authors, and address of the first author. Include the *e-mail* address, in italics, of the corresponding author only. The manuscript title and authors‘ names should be in bold type, and the same font size (preferably 12 pts) as the text. All other information should be in roman type, but not in bold font. Titles should be short and descriptive. Avoid ‗‗empty words‘‘ such as —preliminary studies on . . . and biology or ecology of . . . ‖. Do not use author and date citations with scientific names in the title. In the title only, numbers less than 11 are spelled out; numbers indicating papers in a series will not be accepted. Present addresses and addresses for remaining authors, if different from that of the first author, are given as footnotes, and are to follow the figure legend(s). Footnote designations are as follows: *, †, ‡, §, ‖, #, ¶, **, ††. (See examples at end of guidelines.) Please note that while author names are in bold font, symbols denoting footnotes are not.
Abstract: The abstract must not exceed 400 words. The abstract should be factual (as opposed to indicative) and should outline the objective, methods used, conclusions, and significance of the study. The abstract is headed with the word abstract in capital letters, ending with a colon. Text is run in after the colon,

is not subdivided into paragraphs, and does not contain literature citations.
Introduction: The introduction should **immediately** (no space) follow the abstract and should be un-headed. The introduction should establish the context of the paper by stating the general field of interest, presenting findings of others that will be challenged or developed, and specifying the specific question or hypothesis to be addressed. Accounts of previous work should be limited to the minimum information necessary to give an appropriate perspective. Do not use extra spacing between paragraphs in the Introduction, or throughout the text.
Materials and methods: This section should give sufficient information to permit repetition of the study by others. Methods and apparatus used should be indicated, but specific brand names and models need to be mentioned only if significant. The source, e.g., city and state (if in the U.S.A.), both spelled in full, of special equipment or chemicals should also be given. If the source is outside the U.S.A., then the city and country should be given. Previously published or standard techniques are to be referenced, but not detailed. Generic descriptions should be given for unusual compounds used.
The primary heading for this section should be typed in all bold capital letters, starting at the left-hand margin of the page. The heading is unnumbered and ends without punctuation. Second-level headings in bold type should be on a separate line beginning at the left-hand margin. The initial letter of the first word is the only capital letter except as needed for proper nouns. These headings are unnumbered and end without punctuation. Third-level headings are indented for a paragraph, italicized, and end with a colon, also italicized. The initial letter of the first word is the only capital letter, except capitals needed for proper nouns. Text runs in immediately following this heading. Further subdivision should not be needed. If the materials and methods section is short, it should not be subdivided; it is unnecessary to provide headings, beyond the primary head, for a series of subsections comprising single paragraphs.
Results: This section should contain a concise account of the new information. Tables and figures are to be used as appropriate, but information presented in them should not be repeated in the text. Avoid detailing methods and interpreting results in this section. The results section may be subdivided and headed
as for the materials and methods section.
Discussion: An interpretation and explanation of the relationship of the results to existing knowledge should appear in the discussion section. Emphasis should be placed on the important new findings, and new hypotheses should be identified clearly. Conclusions must be supported by fact or data. The headings and subdivisions, if needed, in this section are as described for the materials and methods section. *Acknowledgments:* These should be concise. Ethics require that colleagues be consulted before being acknowledged for their assistance in the study. The heading for this section is as for the primary head described for the materials and methods section. Subdivisions are not used in this section.
Literature cited: Citations are arranged alphabetically. All references cited in the text must appear in the literature-cited section, and all items in this section must be cited in the text. Citation of unpublished studies or reports is not permitted, i.e., a volume and page number must be available for serials and a publisher, city, state, and full pagination for books. Abstracts not subjected to peer review may not be cited in the text or in the literature cited sections. Work may be cited as ‗‗in press'' only when the paper has been accepted for publication. If absolutely necessary, a statement may be documented in the text of the paper by ‗‗pers. comm.''. The citation is indicated in the style: (X. Y. Smith, pers. comm.).
Personal communications do not appear in the literature-cited section.
Style in the text:
Allen (1989)
(Allen, 1989)
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(Jones, 1987; Allen, 1989)—chronological
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Multiple authors with the same year of publication should be (Smith, Jones et al., 1988; Smith, Walker, and Jones, 1988), **not** (Smith et al., 1988a, 1988b)
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Journal article, 1 author
Nollen, P. M. 1990. Chemosensitivity of *Philophthalmus megalurus* (Trematoda) miracidia. Journal of Parasitology **76:** 439–440.
Journal article, 2 authors
Edwards, D. D., and A. O. Bush. 1989. Helminth communities in avocets: Importance of the compound community. Journal of Parasitology **75:** 225–238.
Book
Schmidt, G. D., and L. S. Roberts. 1989. Foundations of parasitology, 4th ed. Times Mirror/Mosby College Publishing Company, St. Louis, Missouri, 750 p.

Chapter in edited book
Nesheim, M. C. 1989. Ascariasis and human nutrition. *In* Ascariasis and its prevention and control, D. W. T. Crompton, M. C. Nesbemi, and Z. S. Pawlowski (eds.). Taylor and Francis, London, U.K., p. 87–100.
Thesis or dissertation
Monks, W. S. 1987. Relationship between the density of *Moniliformis moniliformis* and distribution within the definitive host population. M.S. Thesis. University of Nebraska-Lincoln, Lincoln, Nebraska, 64 p.
Number of authors
If there are more than 10 authors, then include names of the 10, followed by et al.
Note that abbreviations are not used for titles or serial publications and that spaces appear between initials.
The literature cited section has a primary heading as described for materials and methods.
Footnotes: Footnotes are used only for the title page of regular articles to indicate authors' addresses and to whom correspondence should be sent. Those for tables are typed directly under the table to which they pertain. Footnotes appear at the end of the manuscript directly after the figure legends (see example at end of guidelines).
Tables: Tables are used only to present data that cannot be incorporated conveniently into the text. Ordinarily, values from statistical tests are not published as tables; tests employed and probability accepted for significance can be stated in the materials and methods section with significant differences indicated in tables by footnotes or in the text by a statement.
Tables must be designed to fit in 1 or 2 columns. Only rarely may they be designed to fit the height of a printed page. Generally, if the width does not fit the height of a typed page, the table is too wide. Tables may be continued on following pages to accommodate length, but pages cannot be photoreduced, single-spaced, oversized, or otherwise modified to contain additional material.
Tables are numbered with Roman numerals in a continuous series and so referenced, in sequence, in the text. Captions are typed above the data on the same page. Species names are spelled out in full (and italicized) the first time used in each caption. All columns in a table must have headings, with the first letter of the first word and proper nouns capitalized, e.g., Number sampled, % Recaptured.
Horizontal lines should be avoided in the body of the table; vertical lines are not permitted. If symbols are necessary, the table must be prepared as a line drawing and treated as a figure. Use of letters and numbers as superscripts or subscripts is not permitted. Table footnotes must be used in the sequence that follows: *, †, ‡, §, ||, #, ¶, **, ††.
Figures: All figure captions are to appear consecutively after the literature cited section. Do not place figure captions on the same page as the figures. Each figure or plate of figures must have a caption. The caption is written in paragraph style, beginning with the word ‗FIGURE.'' Captions are typed in roman, except when italic type is required, e.g., a genus and species. For plates, a summary statement should precede the specific explanation of each figure. Avoid repeating information for each figure that can be placed in the summary statement. Genus and species names are spelled out in full the first time used in each caption. The caption must contain an explanation of all abbreviations used on the figures and indicate the value of lines or scale bars used to show size. Moreover, size should not be indicated by magnification in the caption because the figure might not be printed at the size calculated.
Figures & Plates
Plates should be submitted composed of one or more figures ready for final publication. **Please note** that all figures should be submitted as separate files and **not** as part of the text. Figure legends should not be placed on the plate, they should be at the end of the manuscript file directly after the literature cited section. With the initial submission for review, each plate must conform to *Journal* specifications for publication. Plates must be sized to 1 column (88 mm) or 2 column (182 mm) width, and must be no longer than 220 mm in height to leave sufficient room beneath for a figure legend (in case of very long figure legends, plates must be adjusted to allow for the legend). When composing plates, sizing of individual figures should be proportional to information content of the individual figure. Plates are to be arranged fully rectangular, with a 1.4mm white space between all abutted images in plates creating a full rectangle. Plates must be submitted in any one of the following styles: black and white line art, grayscale, or color. Plates submitted as black and white line art, including line drawings, black and white graphs, and charts, must have a minimum resolution of 1200 dpi and must be submitted as TIFF, GIF, or EPS (postscript); bitmapped images will be returned to authors. Plates submitted as grayscale (halftone) photographs, charts, or graphs must have a minimum resolution of 300 dpi and must be submitted as TIFF, GIF, JPEG (quality level >9), or EPS. Plates submitted as color photographs, charts, or graphs, must have a minimum resolution of 450 dpi and must be submitted in CMYK. If color figures are submitted, please note that the cost of printing a color plate is $500, and is $75 for a version online. This is the responsibility of the authors. **Plates not intended for printing in color must be submitted in grayscale.**
All letters and numbers appearing on a plate must be in a sans-serif font, e.g., Helvetica, Ariel, Geneva, Gills-Sans, Lucida, or Verdana. All species binomena, such as those on phylogenetic trees, must

be italicized. Non-scientific names, e.g., —sp.‖ locality, strain, or reference label, must not be italicized. All figures in a plate are to be identified with a figure number or capital letter in the upper or lower left-hand corner, applied directly within the figure and without an added background, shadowing, outline, circle, period, or parentheses. At final print size, font height for numbers/letters identifying figures must be at least 4 mm and not more than 8 mm high. The font size used for labeling structures therein, must be at most two-thirds of the size of the numbers/letters used for identifying the figure and must be at least 2 mm high.

If possible, scale bars should be situated at the lower right-hand corner of the figure. Scale bars should be no thicker than 4 pts (or 1.4 mm at printed size) and normally would be at least 10 mm long and no longer than one-half the width of the figure being scaled. Scale bars (including those for substitutions on phylogenetic trees) may be labeled directly on the figure with their dimensions, bearing in mind that scale bars as generated by microscopic devices typically do not meet *Journal* standards for resolution or font specifications; alternatively, they may be defined in the figure legends. Separate figures in a plate may not share a single scale bar, except in the special circumstance in which all figures in the plate share the same scale.

Prior to review, the Graphics Associate Editor will examine the submitted paper to be certain that all figures conform to *Journal* specifications as described above. The Graphics Associate Editor will also evaluate the level of professional quality relative to what is expected for final publication. Manuscripts with figures that do not conform to *Journal* standards will be returned promptly to the author(s) for revision. **PLEASE NOTE:** Resampling poor resolution images to a larger size does not improve resolution. Resubmitted manuscripts that have merely re-sampled images to a larger size will be returned to the author again.

Taxonomic (Systematics) papers

Taxonomic papers have a distinct style that must be adhered to in preparing a manuscript. Single taxon papers will be considered only if presented in a comprehensive manner. A taxonomic paper should follow instructions given for a regular article, i.e., the title page, abstract, introduction, and materials and methods. In taxonomic papers, however, the results section is e replaced by a section headed **DESCRIPTION** (or **REDESCRIPTION**, as the case may be), beginning at the left-hand margin and the DESCRIPTION is followed in the next line by the italicized scientific name in bold type; it begins at the left-hand margin. Synonyms and reference to figures follow, each as a separate line at the left-hand margin (these are not in bold font). The text of the Description follows as a new paragraph beginning with *Diagnosis*. The DESCRIPTION section must be written using a telegraphic style, i.e., do not employ prepositions, start sentences with the subject, and refrain from using verbs except as absolutely necessary. This DESCRIPTION is followed by a **Taxonomic summary** section, headed as described for second-level headings in the instructions for the materials and methods section. The Taxonomic summary section comprises a listing of the type host, other hosts, site, locality, and specimens deposited. Each of these topics is italicized, but not indented. The *Host* subsection must include the full scientific name of the host, the authority‘s name, and an indication if *Symbiotype* specimens were deposited in a vertebrate museum along with accession numbers. The *Locality* should include map coordinates (e.g., 95°5'11"N, 48°3'15"W) as well as the name of the locality, e.g., ocean, river, etc., and the geopolitical region. *Prevalence and density* data are included when known. Museum accession numbers for appropriate type material (new taxa) and for voucher specimens (surveys) are required; the accession number of the museum must be preceded by the acronym of the appropriate museum. It is recommended that authors consider depositing one, or more, type or voucher specimens in the U.S. National Parasite Collection, Beltsville, Maryland, although this not a requirement for publication in the *Journal.* Appropriate photographic material should be deposited for descriptions of coccidia. Frozen tissues must also include accession numbers if deposited in a museum. In the case of phylogenetic studies involving, or based upon, molecular sequence data, novel nucleotide, or protein sequence data must be deposited in the GenBank Museum, and GenBank numbers must be obtained and added to the manuscript no later than the proof stage**.** The Taxonomic summary is followed by a **Remarks** section, headed as described for second level headings in the instructions for the materials and methods section. The Remarks section must include comparisons to all similar taxa so as to definitively distinguish new taxa from existing taxa; the section heading is typed in bold font and begins at the left margin. . The final section of a taxonomic paper should be the **DISCUSSION**, which includes a thoughtful dialogue regarding important considerations of phylogenentic position, molecular survey information, etc. It should be a synthesis section, placing the new findings in a phylogenetic and/or ecological context as appropriate.

Research notes

Manuscripts are to be organized in the following format and sequence with all pages, numbered consecutively. *Title page:* Research Notes do not have a running head, but should be identified as such on the title page, i.e., RH: RESEARCH NOTE. On first page, give the title of the note in bold type and capitalize the first letter of all principal words. On a separate line, give the names of the authors, also in bold type, and capitalize only the authors‘ initials and first letter of the last name. The addresses currently are run in after

the last author‘s name, with a comma in bold type separating the names and beginning address. Follow with addresses of the authors in roman type, joined by semicolons, matched to authors other than the first one by symbols like those used for regular articles. Begin all lines at the left-hand margin. Identify the corresponding author with an acceptable symbol; this is the person with whom the editor will correspond and to whom page proofs will be sent.

Abstract: An abstract is to be provided as described for articles.

Text: The text of a research note is written without sections and without extra spacing between paragraphs. Acknowledgments may be given, without heading, as the last paragraph. Literature is cited in the text as described for articles.

Literature cited, tables, figure captions, and figures: These items are in the form and sequence described for articles.

Review articles

Invited review articles should be submitted using the format described for articles, except that other section names may be used in place of the materials and methods, results, and discussion sections. Headings should be restricted to major headings and no more than 2 sublevels. Use of tabular data or figures from the work of others must be consistent with copyright law, and it is the responsibility of the author to supply appropriate permissions when the manuscript is submitted.

Critical comments

Manuscripts are to be organized in the following format and sequence with all pages, beginning with the title page, numbered consecutively.

CRITICAL COMMENT…

Title: Starting at the left-hand margin, give the title of the article in bold type and capitalized. Immediately following title, give the name of the author(s) in bold type.

Names and addresses of authors: These follow the title. The style is as described for research notes.

Text: The text is written without subdivision. Literature citations are made as for articles. Acknowledgments may be included as an un-headed final paragraph.

Literature cited: If citations have been used in the text, the literature cited section is as described for articles.

Tables, figure captions, and figures: When present, these are as described for articles.

Book reviews Manuscripts are organized in the following format and sequence with all pages, beginning with that for the title, numbered consecutively.

Title: Give the title of the book being reviewed, and other critical information, in the following style:

Toxoplasmosis of Animals and Man, by J. P. Dubey and C. P. Beattie. CRC Press, Boca Raton, Florida. 1988. 220 p. ISBN Number. Hardcover $124.95.

Note that, unlike in the literature cited section, the first letters of principal words are capitalized. The words ‗‗edited by‘‘ are substituted for ‗‗by‘‘ when appropriate. The book title, etc., should begin at the left-hand margin.

Text: Begin as a new paragraph immediately following the book title. The text usually is not subdivided. If literature must be cited, a headed literature cited section follows the text in the style described for articles. Figures and tables should not be used.

Name and address of author: This information follows the text or, if present, the literature cited section. The reviewer‘s name should be in bold type; the address should follow, but not in bold type.

General points of style

Scientific names: The full binomen is written out at the first use of a species name. At subsequent use, the genus is abbreviated by use of the first letter, except at the beginning of a sentence where it is written out. Genera and species should be italicized, not underlined, throughout the manuscript. Author and date citations for scientific names need not be used in non-systematic papers. In systematic papers, author and date citations are used the first time a taxon is mentioned in the abstract and the text, but not subsequently except as described for tables and figures. Use must be according to the International Code of Zoological Nomenclature and should be consistent for all parasite and host species mentioned. Author and date citations used only as authorities for scientific names do not appear in the literature cited section.

Authors are reminded that names of taxa are not names of organisms, e.g., *Fasciola* is the name of a genus (a group of related species) and as such it does not lay eggs, ingest cells, possess a sucker, etc. These are properties of organisms.

Ecological terms: The terms prevalence, incidence, intensity, mean intensity, density, relative density, abundance, infrapopulation, suprapopulation, site, niche, and habitat are to be used as recommended by the ASP Ad Hoc Committee on the Use of Ecological Terms in Parasitology (1982, *Journal of Parasitology* **68:** 131–133); also see Bush et al. (1997, *Journal of Parasitology* **83:** 575–583) for an expanded and updated treatment of ecological terminology.

Mathematical and chemical notations: Authors should attempt to write mathematical equations so

that they can be set in 1 line of type. When 1 unit appears in a denominator, use the solidus, e.g., g/m^2; for 2 or more units in a denominator, use negative exponents, e.g., g·m^{-2}·day^{-1}. Manuscripts submitted to the *Journal of Parasitology* should conform to the same conventions as those used for chemical and biochemical/molecular nomenclature.

All chemical structures not accommodated by a single line of type must be drafted and reproduced as figures.

Use of numbers: In the text, numbers should be Arabic numerals except when beginning a sentence. Naked decimals are not permitted in the text, tables, legends, or on figures, i.e., 0.1, **not** .1. Numbers greater than 999 must have commas. Metric units are to be used in all articles. The 24-hour system is used to indicate time, e.g., 1500 hr.

Acronyms and abbreviations: At first use, acronyms are placed in parentheses following the name written out in full. At subsequent use, the acronym alone is used. An acronym may begin a sentence. Sentences may not begin with an abbreviation, and abbreviations are as recommended in the Council of Science Editors (CSE) style manual. The *Journal* uses all International System of Measurement (SI) metric unit abbreviations. Common CSE and SI abbreviations include the following (the same abbreviation is used for plural form):

wk (week)
hr (hour; use 0–2400 hr for time)
sec (second)
min (minute)
mo (month)
day (not abbreviated)
n. sp. (new species)
n. gen. (new genus)
L (liter; but ml for milliliter)
RH (relative humidity)
p.o. (per os)
s.c. (subcutaneous)
i.pl. (intrapleural)
i.p. (intraperitoneal)
PI (post-inoculation, or post-infection)
p. (page)
ad lib. (ad libitum) U.S.A. (as a noun)
U.S. (as an adjective)
sp. gr. (specific gravity)
t-test
U-test
P (probability)
(arithmetic mean) x
r (correlation coefficient)
n (sample size)
SD (standard deviation of the mean)
SE (standard error of the mean)
df (degrees of freedom)
NS (not significant)

Basic SI units

Meter m
Kilogram kg
Second sec
Ampere A
Volt V
Mole mol

Prefixes for SI units

Factor	Prefix	Symbol
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	µ
10^{-9}	nano	n
10^{-12}	pico	p
10^{1}	deca	da
10^{2}	hecto	h
10^{3}	kilo	k
10^{6}	mega	M
10^{9}	giga	G
10^{12}	tera	T

Miscellaneous: Unless stated otherwise, U.S.A. is understood for locations, including addresses of authors, and is not stated.

Words and abbreviations in Latin and other non-English languages, except for genus and species names, are not italicized. American spelling supersedes English spelling.

No and none are treated as singular, e.g., no worm was found. If this form is not satisfactory, avoid use of the words.

The suffix -like is hyphenated only in combination with a name in italic type or to avoid a triple l.

Studies involving sacrifice of animals are outside the scope of the *Journal*; however, many appropriate studies involve killing of animals.

Because manuscripts are accepted only with the understanding that the work was conducted in compliance with all relevant laws and within the ASP policy on animal care and use, a separate statement regarding animal care and use is not printed as a part of each paper. Likewise, use of the word euthanasia is redundant.

Papers are not dedicated to individuals. Dedication is only at the direction of the ASP Council and it is in the form of an entire issue.

Revising manuscripts

When manuscripts are returned for revision, a cover letter from the editor provides directions that must be followed carefully. A point-by-point statement of what has been revised and a brief rebuttal of those criticisms not addressed should be provided. All suggestions of the reviewers and the associate editor and editor must be addressed individually. Reviewers are usually kept anonymous and assigned numbers to retain anonymity. The revised manuscript and the author‘s comments may be reviewed again (by the same referee[s]), subject to the discretion of the editor.

Retain a complete and exact copy of the manuscript, tables, and figures for reference.

Correcting proof and ordering reprints

Authors are responsible for the accuracy of their proofs and, therefore, what ultimately is printed in the *Journal.* **Corrected proofs must be returned to the editor promptly**, ideally on the same day as received. Proofs are sent by e-mail and corrections can be sent by e-mail to Dr. Gerald W. Esch at: esch@wfu.edu. Proofs are to be corrected, not revised. Additions usually are disallowed except to correct errors made in typesetting and by the editor. Correction of errors made by the author may be billed to the author at the rate of $5.00 each. Queries on the proof are to be answered by ‗‗yes‘‘ or ‗‗no‘‘; and if there is no change to be made you should write ―ok as set‖; otherwise do not use ‗‗ok‘‘ or ‗‗stet.‘‘ Reprints can now be ordered online; a link is provided. Only the author designated to receive correspondence receives proof and reprint order forms. It is the responsibility of this author to clear the proof with other authors and to provide the opportunity for them to order reprints. Reprint orders are to be sent to Allen Press using the standardized form provided.

SCHEDULE FOR PRINTING INSTRUCTIONS

These instructions can be found online at: http://asp.unl.edu

ACKNOWLEDGMENTS

These instructions are a revision of policies and practices formulated by previous editors. The staff at Allen Press, especially Annielaurie Seifert, Mary Reilly, and Valerie Pierce, contributed ideas and advice for the revision.

Gerald W. Esch, Department of Biology, Wake Forest University, P.O. Box 7629, Winston-Salem, North Carolina 27109.

Name /PARA/2008_guide 04/14/2008 03:51PM Plate # 0-Composite pg 8 # 8 **EXAMPLE FOR FULL-SIZED ARTICLE**

The following presents a sample layout of the way in which your manuscript should appear for a full-sized article.

RH: NADLER ET AL.- GENETICS OF GEOGRAPHIC VARIATION IN *A. SUUM* (not to exceed 60 characters and spaces)

GENETIC STRUCTURE OF MIDWESTERN *ASCARIS SUUM* POPULATIONS: A COMPARISON OF ISOZYME AND RAPD MARKERS

Steven A. Nadler, Rachel L. Lindquist*, and Thomas J. Near

Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115. *e-mail: snadler@ucdavis.edu* (the e-mail address is of the corresponding author ONLY)

ABSTRACT: Isozyme and random amplified polymorphic DNA (RAPD) markers were used to characterize the genetics of geographic variation among population samples of *Ascaris suum.* . . .

Molecular markers are of great potential utility for revealing intraspecific variation among parasite populations. (**Note:** This was the first sentence of the Introduction, that it is not preceded by a heading.) By studying the genotypes or haplotypes of individuals. . . .

MATERIALS AND METHODS

Ascaris suum adults were collected from the intestines of pigs at a local meat processing plant. . . .

RESULTS

Isozyme data

Six of the 13 loci surveyed showed evidence of polymorphism; however, only 3 of the 6 variable loci showed sufficient enzymatic activity

and resolution on gels to score. . . .

RAPD data and comparative analysis of markers

Of the 29 RAPD primers surveyed, 9 yielded amplification products that met both subjective. . . .

DISCUSSION

Isozyme data

Geographic variation: Relatively few studies have focused on the genetics of geographic variation among populations of parasitic helminths. In part, this is due to. . . .

Polymorphisms: A paradigm of parasite population structure is that parasitic organisms are characterized by small populations with high levels of inbreeding. . . .

Genetic drift

Genetic drift among *A. suum* infrapopulations may be promoted by their small effective population size and founder effects. The overall sex ratio. . . .

ACKNOWLEDGMENTS

We are grateful to Mr. Don Temperly for assistance at FDL. . . .

LITERATURE CITED

ANDERSON, R. M., AND R. M. MAY. 1978. Regulation and stability of host-parasite population interactions. I. Regulatory process. Journal of Animal Ecology **47:** 219–247.

ANDERSON, T. J. C., M. E. ROMERO-ABAL, AND J. JAENIKE. 1993. Genetic structure and epidemiology of *Ascaris* populations: Patterns and host affiliation in Guatemala. Parasitology **107:** 319–334.

_____, _____, AND J. JAMES. 1995. Botulism. Journal of Parasitology **81:** 1–10.

MONKS, W. S. 1987. Relationship between the mean density of *Moniliformis moniliformis* and distribution within the definitive host population. M.S. Thesis. University of Nebraska-Lincoln, Lincoln, Nebraska, 64 p.

NESHEIM, M. C. 1989. Ascariasis and human nutrition. *In* Ascariasis and its prevention and control, D. W. T Crompton, M. C. Mescheim, and Z. S. Pawlowski (eds.). Taylor and Francis, London, U.K., p. 87–100.

SCHMIDT, G. D., AND L. S. ROBERTS. 1989. Foundations of parasitology, 4th ed. Times Mirror/Mosby Publishing Company, St. Louis, Missouri, 750 p.

FIGURE 1. Random amplified polymorphic DNA fingerprints for 11 *Ascaris suum* individuals. . . .

FIGURES 2–4. Isozymes and infrapopulations. (**2**) Gels showing the genetic make-up of. . . . (**3**) Comparison of 4 infrapopulations. . . . (**4**) Isozymes from *Ascaris suum.*. . . .

*Department of Biology, Southeast Missouri State University, Cape Girardeau, Missouri 63701. **EXAMPLE FOR RESEARCH NOTE**

The following represents a sample layout of the manner in which the file on your diskette should appear for a RESEARCH NOTE.

RH: RESEARCH NOTE

HEPATIC SARCOCYSTOSIS IN A HORSE

C. R. Davis, B. C. Barr*, J. R. Pascoe†, H. J. Olander‡, and J. P. Dubey§, Department of Anatomy, Physiology and Cell Biology, University of California, Davis, California 95616; *University of California, California Veterinary Diagnostic Laboratory, Davis, California 95616; †Department of Surgical and Radiological Sciences, University of California, Davis, California 95616; ‡Department of Pathology, Microbiology and Immunology, University of California, Davis, California 95616; and §Parasite Biology and Epidemiology Laboratory, Livestock and Poultry Sciences Institute, Agriculture Research Service, United States Department of Agriculture, Beltsville, Maryland 20705-2350. *e-mail: jdubey@anri.barc.usda.gov* (the e-mail address is of the corresponding author ONLY)

ABSTRACT: This report examines the phylogenetic relationships of *Tetrabothrium* spp. . . .

The various species of *Tetrabothrium* are examined within an evolutionary context (this constitutes the first sentence of the text).

If you wish to acknowledge, add without a title as an indented sentence immediately following the last paragraph of the RESEARCH NOTE.

LITERATURE CITED

(See **EXAMPLE** for full-sized article.)

Figure legends will follow.

APÊNDICE B

REPRODUCTIVE DISORDERS IN GOATS AFTER ARTIFICIAL INSEMINATION WITH SEMEN EXPERIMENTALLY CONTAMINATED WITH *Toxoplasma gondii*

(Artigo submetido para o Periódico PLOS ONE)

Reproductive disorders in goats after artificial insemination with semen experimentally contaminated with *Toxoplasma gondii*

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Abstract

The objective was to describe reproductive disorders in goats experimentally infected with semen contaminated with the CPG strain of *Toxoplasma gondii* (genotype III). Ten female goats were randomly allocated into two groups (G1 and G2), each with five animals, and inseminated during estrus. Goats in G1 were inseminated with semen containing $1x10^5$ tachyzoites, whereas those in G2 (control) were inseminated with tachyzoite-free semen (insemination = Day 0). In G1, seroconversion (indirect immunofluorescence reaction) occurred between Days 7 and 14 in 4 of 5 goats. Furthermore, in G1, *T. gondi* DNA was present (polymerase chain reaction) between Days 7 and 49 in the blood of 3 does, and in various tissues of 3 does and 2 two newborn progeny. In G2, all goats were negative in all tests. The goats from G1 were monitored in the acute and chronic phases of infection; embryonic reabsorption occurred in 4 of 5 goats in the acute phase, whereas in the chronic phase, 2 of 5 goats were anestrus, and 1of 5 had both hydrosalpinx and ovarian cysts. In conclusion, artificial insemination with semen containing tachyzoites of *T. gondii* infected goats, causing reproductive pathologies during acute and chronic phases of infection.

Keywords: Goats; artificial insemination; toxoplasmosis; tachyzoites.

1. Introduction

Toxoplasmosis is a common cause of abortion in goats worldwide [1], [2] since this species is susceptible to *Toxoplasma gondii* [1]. Natural infection during pregnancy can cause placental infection, embryonic reabsorption, fetal mummification, abortion, stillbirth, and neonatal mortality, which together lead to significant economic losses [4]-[10].

A previous study [11] reported embryonic reabsorption, anestrous, hydrometra, mucometra, and follicular cysts in ewes experimentally infected with semen contaminated with varying concentrations of tachyzoites of the CPG strain of *T. gondii* (genotype III).

However, no studies have examined the acute nor the chronic phase of reproductive disorders in goats experimentally contaminated with *T. gondii.* Thus, the aim of the present study was to diagnose and describe reproductive disorders in goats after artificial insemination with fresh semen experimentally contaminated with *T. gondii* tachyzoites of the CPG strain (genotype III).

2. Materials and methods

2.1 Experimental details

The study was conducted at the São Luiz farm, an experimental unit of the Federal University of Alagoas located at Viçosa-Alagoas, Brazil (9°22’ S; 36°14’ W). All experimental procedures followed the guidelines of the International Guiding Principles for Biomedical Research Involving Animals and were approved by the Ethics Committee of the Federal Rural University of Pernambuco (CEUA-UFRPE – protocol 007/2010).

2.2 Animals and Experimental Infection

This study used 10 mixed-breed multiparous goats, 3 to 4 years old, randomly allocated into two groups of five animals (G1 and G2). Semen was donated by one Saanen buck, 2 years of age, previously deemed sound for breeding, and routinely used for semen collection via electroejaculation. Semen was within the established

standards of the Brazilian College of Animal Reproduction [12]. At the beginning of the experiment, animals were dewormed and vaccinated against clostridial diseases and rabies, and were determined to have negative serology for *Toxoplasma gondii* and *Neospora caninum* [indirect immunofluorescence reaction (RIFI)] *Brucella abortus* (buffered acidified antigen); and *Chlamydophila abortus* (complement fixation). The animals were raised in confinement with uniform feeding, housing and lighting conditions.

Tachyzoites used for semen contamination were of the CPG strain (genotype III) isolated from an outbreak of goat abortions in the city of Guarapuava, Paraná, Brazil [10]. The strain was transferred to mice via intraperitoneal inoculation, and abdominal lavage was performed with distilled water. Subsequently, the tachyzoites were centrifuged and re-suspended to obtain a final concentration of 1×10^5 tachyzoites/ 7 µL.

For experimental infection by artificial insemination, estrus was induced in the does using an injection of 0.15 µg of cloprostenol sodium (Ciosin® Shering Plough, São Paulo, SP, Brazil) into the vulvar submucosa. A male teaser was used twice a day to identify females in estrus, and twelve hours after estrous detection, vaginal artificial insemination was performed.

Each goat in G1 was inseminated with 243 µl of fresh semen (previously diluted in a skim milk base extender, 1:9, v:v), which were added $1x10^5$ tachyzoites in a 7 µl solution. The goats in G2 were inseminated by the same technician and under similar conditions, with the exception that no tachyzoites were added to semen destined for the control group.

2.3 Confirmation of Infection

Serologic and molecular examinations were conducted on goats to confirm infection. Blood was collected on Days 0, 7, 14, 21, 28, 49, 63 and 123. Antibodies to *Toxoplasma gondii* were detected using RIFI [13] and an anti-goat IgG conjugate (Sigma-Aldrich Co. ®, St. Louis, Missouri, U.S.A.) with fluorescein isothiocynate, as well as sensitized slides (Perfecta®, São Paulo – Brasil) with antigens to tachyzoites of *T. gondii* (RH strain). The sera were tested and placed in dilutions of 1:16 to 1:4096. The sample was considered positive when the titer was equal to or greater than 64 [14].

In order to detect *T. gondii* DNA, samples of whole blood were withdrawn on Days 0, 7, 14, 21, 28, 49, 63 and 123 after insemination into glass tubes containing EDTA and subjected to Polymerase Chain Reaction (PCR). Polymerase Chain Reaction was also performed on the organs (liver, spleen, kidneys, medulla, brain, lung, heart, uterus, ovaries, testicles) and on the placenta of goats and kids at euthanization at the end of the experiment. The samples were subjected to DNA extraction using the "Qiagen DNA Easy Blood and Tissues Kit (Qiagen®, Hilden, Germany) following manufacturer instructions. In PCR, the primer pairs TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (GCTGCAGACACAGTGCATCTGGATT) were used, as described [15], amplifying a region of 529 base pairs. Amplification reactions were carried out in a final volume of 12.5µL containing: 2.5 µL of genomic DNA; 0.5 µL of each primer (TOX4 and TOX5) at 10 µL; 2.5 µL of Milli-Q ultrapure water, and 6.25 µL of Top Taq Master Mix (Qiagen®), following manufacturer instructions. The thermal profile of the reaction steps was conducted in an XP Thermal Cycler (Bioer Technology Co. Ltd., Binjiang, Hangzhou, China), consisting of denaturation of the initial DNA at 94°C (seven minutes) and followed by 35 cycles at 94ºC for one minute for denaturation, 60ºC for one minute for annealing, 72ºC for one minute for extension, and a final extension of ten minutes at 72°C. The amplified products were detected using agarose gel electrophoresis (2%), stained with Blue Green (LGC®, Cotia, São Paulo, SP, Brasil), visualized under ultraviolet light, and finally photodocumented. The positive control used in the reaction was obtained through the suspension obtained from intraperitoneal lavage of mice that had been previously infected with the RH strain.

2.4 Diagnosis, monitoring of pregnancy and birth

Diagnosis and monitoring of pregnancy was conducted by means of weekly transrectal ultrasound examinations between Days 15 and 60, using a multi-frequency linear transducer (CTS 900V, SIUI, China). After 60 days of pregnancy, ultrasound assessments were performed transabdominally at intervals of 15 days until the time of birth. The births of animals in G1 and G2 were monitored, and the placentas were collected, identified, and frozen for molecular examination (PCR) or placed in buffered formalin (10%) for histopathological examination.

2.5 Controlled mating season

All of the goats in G1 that exhibited embryonic reabsorption were subjected to another controlled mating season. Does were teased for up to 60 days and then mated. After mating, they were monitored using ultrasound examinations to diagnose pregnancy or reproductive pathologies.

2.6 Anatomo-pathological examination

At the end of the experiment, all does and newborn kids were euthanized. Euthanasia was performed according to Resolution 1000 of the Federal Council of Veterinary Medicine [16]. Necropsies were performed on all animals, and sections of the liver, spleen, kidneys, medulla, brain, lung, heart, uterus, ovaries and testicles were collected. The organs of does and kids, as well as the placentas from G1 and G2 were assessed microscopically, and samples were fixed in 10% buffered formalin. After fixation, the material was paraffin-embedded and sectioned for histopathological examination in a microtome (Model 1512, Leitz Wetzlar, Wetzlar, Hessen, Germany) at 4 µm, stained with hematoxylin and eosin, and classified as “injury-free”, “non-related injuries” or “toxoplasmosis-related injuries”.

3. Results

3.1 Serology

In G1, 4 of 5 (80%) of the goats seroconverted, 2 of 4 (50%) on the Day 7 and the other 2 of 4 (50%) on Day 14. None of the goats in G2 exhibited antibodies to *T. gondii*. The greatest titer was 1024 after 28 days in the two goats that seroconverted on Day 7. The other two does exhibited titers of 512 on Day 21. The one goat in G1 that did not seroconvert completed a full term pregnancy, and the kids also had negative serology (Table 1).

3.2 Molecular diagnosis

Between Days 4 and 49, *T. gondii* DNA was detected in the blood of 3 of 5 (60%) of the goats in G1, as well as in the blood of the kids born to them. This DNA was not found in any of the goats in G2. The DNA of the parasite was also detected in a number of organs of the goats and kids in G1 (Table 2).

3.3 Anatomo-pathological examination

Macroscopic injuries found in the goats of G1 included an ovarian cyst and a bilateral hydrosalpinx (Table 1). The main microscopic injuries in this group were neutrophilic infiltration of the lungs, interstitial glomerulonephritis, neutrophilic infiltration of the liver and mild cerebral gliosis.

3.4 Post-insemination disorders (acute phase)

In the early stages of pregnancy, there was a high rate of embryonic reabsorption in 4 of 5 goats in G1 (80%), between the 21st and 49th days of pregnancy. There were no such embryonic difficulties in G2, in which all kids were born healthy.

3.5 Post mating season disorders (chronic phase)

Of the four goats that exhibited embryonic reabsorption, two were acyclic, and the other two exhibited successive heats and natural mating, but without getting pregnant. One of the latter animals presented clinical signs of estrus for seven days, whereas the second one had an abnormal 21 d estrous cycle. In subsequent ultrasound examinations for embryonic reabsorption, an ovarian cyst (1/4) and hydrosalpinx (1/4) were identified and later confirmed during post-mortem examination (Figure 1; Table 1). All of the goats in G2 gave birth to one or two healthy kids with no signs of reproductive pathologies.

4. Discussion

In this study, fresh semen was contaminated with tachyzoites of *T. gondii* in order to perform vaginal artificial insemination in goats and to investigate vertical infection through the semen, as well as reproductive pathologies associated with acute and chronic phases of infection. This is a pioneer study on infection of goats through the reproductive tract. The main findings were repeat breeding and embryonic reabsorption in the first weeks of pregnancy (acute phase of infection).

A number of authors have demonstrated the presence of *T. gondii* in semen using bioassays [17], with detection of the parasite from Days 7 to 59 in goats infected with oocysts. In one study [18], goats were infected with tachyzoites, and it

was reported that the parasite was present in semen (PCR) from Days 5 to 70. In the group infected with oocysts, the authors reported elimination of the parasite on Day 56. Also, in naturally infected animals, it was reported [19] that the DNA of the parasite was present in the semen (PCR) of breeding sheep. However, transmission of *T. gondii* through semen in naturally acquired cases of toxoplasmosis in goats and sheep has not been confirmed.

In the present study, the serologic and molecular results of goats in G1confirmed the transmission of *T. gondii* through the semen. Similar findings were reported [20] in sheep that were inseminated with semen that had been contaminated with different concentrations of tachyzoites of the strain used in the present study. The same authors reported seroconversion beginning at Day 7 in 100% of goats that were inseminated with a concentration of 4×10^7 tachyzoites and in 33% of the animals inseminated with a concentration of 6.5×10^4 tachyzoites. Parasitemia was detected beginning at Day 7, coinciding with the findings of the present study, in which most goats also exhibited positive blood results (PCR) and seroconversion beginning at Day 7.

High rates of embryonic reabsorption and repeat breeding at irregular intervals in some goats in G1 of the present study demonstrated that the parasite, introduced through semen, affected the initial phase of pregnancy and caused death of the embryo. These findings are not common in cases of naturally acquired toxoplasmosis in goats because it is difficult to diagnose this result in herds. In two studies [21], [22], goats were experimentally infected orally in different periods of gestation, and death and fetal reabsorption were reported in all of the goats after 50 days of pregnancy. It was also reported [9] that high rates of embryonic reabsorption occurred in sheep that were inseminated with semen that had been contaminated by tachyzoites of *T. gondii*.

Goats are more sensitive to *T. gondii* infection than sheep, and the stage of pregnancy at the time of primary infection could affect the overall result of the pregnancy. Abortion is more common in the acute phase of infection in goats. Experimental studies reported that goats infected by *T. gondii* may abort more than once [4].

Reproductive abnormalities were not limited to the acute phase. This was confirmed in the present study by the two goats that presented clinical signs of estrus

after embryonic reabsorption and were subsequently subjected to natural mating for a second time, but with no development of pregnancy. The diagnosis of anestrus in 2 of 4 of the goats that experienced embryonic reabsorption was also a significant finding, confirming that this type of pathology can be caused by experimental infection of goats through semen.

Another significant finding was the detection of DNA of the parasite in various tissues of two kids that were born healthy to a goat in G1. The placentas of both were analyzed and one of them was positive in PCR. This result demonstrated that the parasite multiplied in the placenta and then spread in the fetal tissues without causing death. According to one study [22], *T. gondii* multiplies with a greater intensity in placental tissue than any other type of tissue in goats. The same study examined oral infection and found the parasite in the placenta from as early as Day 9 and in fetal tissues beginning at Day 15. This explains the high rates of embryonic reabsorption discovered in the present study beginning at Day 21.

With regard to the organs of kids and does in G1of our study, the tissues that exhibited the highest degree positive reactions for *T. gondii* in PCR were the brain and the heart. Another study [20] also found DNA of the parasite using PCR in a number of fetal tissues of lambs whose mothers had been infected with semen contaminated with tachyzoites of *T. gondii*.

Anestrus, follicular cysts, and hydrosalpinx were detected in the chronic phase of infection. Other microscopic injuries were rare and considered unrelated to toxoplasmosis. One study [11] also reported reproductive pathologies such as hydrometra, mucometra and follicular cysts in sheep that were infected with *T. gondii* using the same infection route as that of the present study. In an additional study [23], cows were infected with semen containing tachyzoites of *N. caninum*, a protozoan of the same subfamily as *T. gondii,* and significant rates of embryonic reabsorption were also reported.

This is a pioneer study in which does were infected with *Toxoplasmosis gondii* in semen at the time of artificial insemination. This infection route appeared to cause toxoplasmosis in goats with concurrent reproductive pathologies in the acute and chronic phases of infection, preventing subsequent pregnancies and confirming the extreme susceptibility of this species to toxoplasmosis.

Acknowledgements

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Author Contributions

Conceived and designed the experiments: RAM, FSW, WJNP. Performed the experiments: FSW, WJNP, DRC, EPBXM. Contributed reagents/materials/analysis tools: AAO, PCK, OLSN. Wrote the paper: FSW. Revised the manuscript: RAM.

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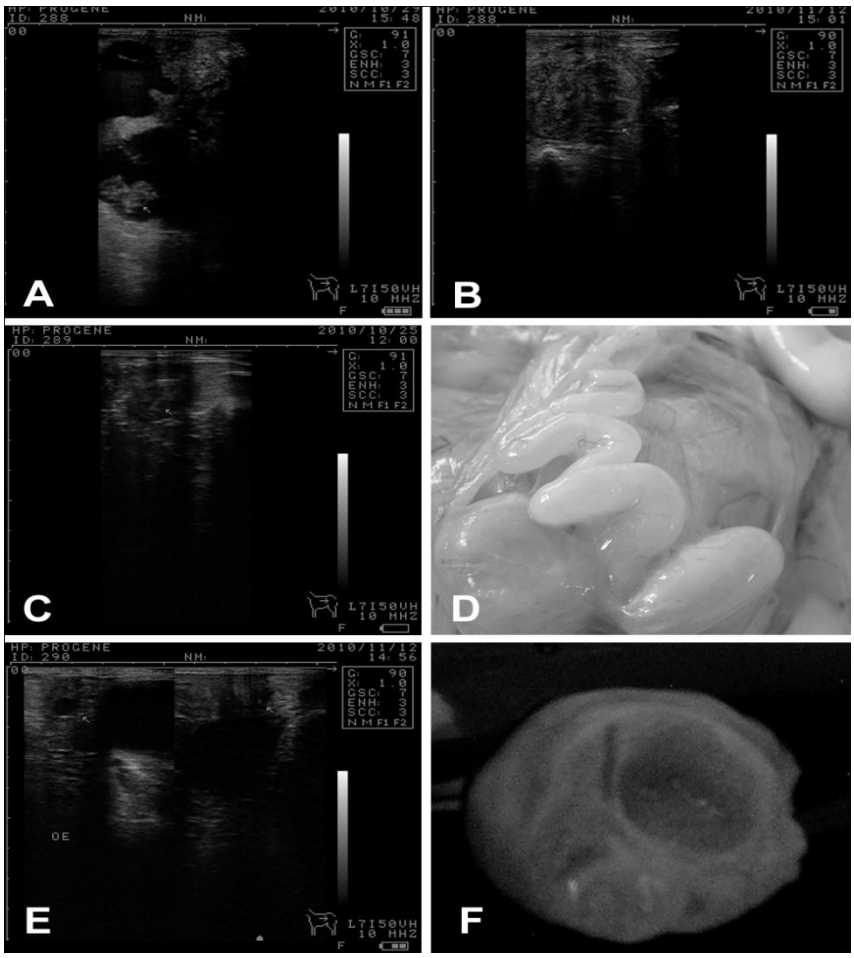

Figure legends

Figure1. Reproductive disorders in goats in the acute and chronic phase after infection with *T. gondii*

Confirmation of pregnancy on Day 35 in goat 2 of G1 (arrow indicates the embryo) **(A)**; Absence of fetus (reabsorption) on Day 49 in goat 2 of G1**(B)**; Ultrasound image (frequency 10 MHz) of the oviduct of the goat 3 of G1 after artificial insemination with semen contaminated with *Toxoplasma gondii.* Note the presence of vesicular structures exhibiting hypoechoic mucosa with an irregular surface and hypoechoic heterogonous content (asterisks), the largest these measuring approximately 17mm in diameter. Findings compatible with hydrosalpinx **(C)**; Necropsy photograph goat 3 confirming ultrasound findings **(D)**; Ultrasound image of the ovary in goat 2 indicating an ovarian cyst **(E)**; Necropsy photograph of the goat 2 confirming ultrasound findings **(F)**.

Table 1. Results of necropsy and laboratory examinations in goats infected with *Toxoplasma gondii*

Animal/ Group	Serology	PCR Blood	PCR organs	Gestation Diagnosis	Viable pregnancy	Reabsorption	Reproductive disorders	Necropsy
G1								
1	-	+	-	+	+	-	-	-
1 (C1[a])	-	+	+	-	-	-	-	-
1 (C2[a])	-	+	+	-	-	-	-	-
2	+	-	-	+	-	+	Anestrus	Ovarian cyst
3	+	-	+	+	-	+	Repeat breeding	Hydrossalpinx
4	+	+	+	+	-	+	Anestrus	-
5	+	+	+	+	-	+	Repeat breeding	-
G2								
6	-	-	-	+	+	-	-	-
6 (C1[a])	-	-	-	-	-	-	-	-
7	-	-	-	+	+	-	-	-
7 (C1[a])	-	-	-	-	-	-	-	-
7 (C2[a])	-	-	-	-	-	-	-	-
8	-	-	-	+	+	-	-	-
8 (C1[a])	-	-	-	-	-	-	-	-
8 (C2[a])	-	-	-	-	-	-	-	-
9	-	-	-	+	+	-	-	-
9 (C1[a])	-	-	-	-	-	-	-	-
10	-	-	-	+	+	-	-	-
10(C1[a])	-	-	-	-	-	-	-	-

+ = positive based on PCR test or serology test (titration≥64); [a] Viable newborns

¶

Table 2. Results of PCR in tissues from does infected with *Toxoplasma gondii*

Animal	Liver	Spleen	Kidney	Spinal Cord	Brain	Lung	Heart	Placenta
1	-	-	-	-	-	-	-	-
1 (C1[a])	**+**	**+**	**+**	**+**	**+**	-	-	-
1 (C2[a])	-	-	-	-	**+**	**+**	**+**	**+**
2	-	-	-	-	-	-	**-**	**-**
3	-	-	-	-	+	-	-	**-**
4	-	-	-	-	+	-	+	**-**
5	-	-	-	-	-	-	+	**-**

[a] Viable newborns

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Manuscript File Type Requirements

Authors may submit their manuscript files in Word (as .doc or .docx), LaTeX (as .pdf), or RTF format. Only RTF and .doc files can be used during the production process.

LaTeX Submissions. If you would like to submit your manuscript using LaTeX, you must author your article using the *PLOS ONE* LaTeX template and BibTeX style sheet. Articles prepared in LaTeX may be submitted in PDF format for use during the review process. After acceptance, however, .tex files and formatting information will be required as a zipped file. Please consult our LaTeX guidelines for a list of what will be required.

Submissions with equations. If your manuscript is or will be in .docx format and contains equations, you must follow the instructions below to make sure that your equations are editable when the file enters production.

If you have not yet composed your article, you can ensure that the equations in your .docx file remain editable in .doc by enabling "Compatibility Mode" before you begin. To do this, open a new document and save as Word 97-2003 (*.doc). Several features of Word 2007/10 will now be inactive, including the built-in equation editing tool. You can insert equations in one of the two ways listed below.

If you have already composed your article as .docx and used its built-in equation editing tool, your equations will become images when the file is saved down to .doc. To resolve this problem, re-key your equations in one of the two following ways.

1. Use MathType to create the equation (recommended)
2. Go to Insert > Object > Microsoft Equation 3.0 and create the equation

If, when saving your final document, you see a message saying "Equations will be converted to images," your equations are no longer editable and PLoS will not be able to accept your file.

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2. Guidelines for Standard Sections

Title

Manuscripts must be submitted with both a full title and a short title, which will appear at the top of the PDF upon publication if accepted. Only the full title should be included in the manuscript file; the short title will be entered during the online submission process.

The full title must be 150 characters or fewer. It should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).

Examples:

- Impact of Cigarette Smoke Exposure on Innate Immunity: A *Caenorhabditis elegans* Model
- Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial

The short title must be 50 characters or fewer and should state the topic of the paper.

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Authors and Affiliations

All author names should be listed in the following order:

- First names (or initials, if used),
- Middle names (or initials, if used), and
- Last names (surname, family name)

Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.

This information cannot be changed after initial submission, so please ensure that it is correct.

To qualify for authorship, a researcher should contribute to all of the following:

1. Conception and design of the work, acquisition of data, or analysis and interpretation of data
2. Drafting the article or revising it critically for important intellectual content
3. Final approval of the version to be published

All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author must have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments.

When a large group or center has conducted the work, the author list should include the individuals whose contributions meet the criteria defined above, as well as the group name.

One author should be designated as the corresponding author, and his or her email address or other contact information should be included on the manuscript cover page. This information will be published with the article if accepted.

See the *PLOS ONE* Editorial Policy regarding authorship criteria for more information.

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Abstract

The abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

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Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

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Materials and Methods

This section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references. We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information. These are published online only, but are linked to the article and are fully searchable. Further information about formatting Supporting Information files, can be found here.

Methods sections of papers on research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the Reporting Guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Methods sections of papers with data that should be deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.

Methods sections of papers using cell lines must state the origin of the cell lines used. See the Reporting Guidelines for cell line research for more information.

Methods sections of papers adding new taxon names to the literature must follow the Reporting Guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

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Results, Discussion, and Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn. Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Publication Criteria for more information.

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Acknowledgments

People who contributed to the work but do not fit the *PLOS ONE* authorship criteria should be listed in the acknowledgments, along with their contributions. You must ensure that anyone named in the acknowledgments agrees to being so named.

Funding sources should not be included in the acknowledgments, or anywhere in the manuscript file. You will provide this information during the manuscript submission process.
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References

Only published or accepted manuscripts should be included in the reference list. Manuscripts that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only as "unpublished data."
References must be listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, citations should be indicated by the reference number in brackets. Journal name abbreviations should be those found in the NCBI databases. A number of reference software companies supply PLOS style files (e.g., Reference Manager, EndNote).
Proper formatting of the references is crucial; some examples are shown below.

- Published papers. Hou WR, Hou YL, Wu GF, Song Y, Su XL, et al. (2011) cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res 10: 1576-1588.
  Note: Use of a DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.

- Accepted, unpublished papers. Same as above, but "In press" appears instead of the page numbers.

- Electronic journal articles. Huynen MMTE, Martens P, Hilderlink HBM (2005) The health impacts of globalisation: a conceptual framework. Global Health 1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14. Accessed 25 January 2012.

- Books. Bates B (1992) Bargaining for life: A social history of tuberculosis. Philadelphia: University of Pennsylvania Press. 435 p.

- Book chapters Hansen B (1991) New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health. pp. 21-28.

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Tables

Tables should be included at the end of the manuscript. All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above.
Tables occupying more than one printed page should be avoided, if possible. Larger tables can be published as Supporting Information. Please ensure that table formatting conforms to our Guidelines for table preparation.
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Figure Legends

Figures should not be included in the manuscript file, but figure legends should be. Guidelines for preparing figures can be found here.
Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations.
Further information about figure legends can be found in the Figure Guidelines.
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3. Specific Reporting Guidelines

Human Subject Research

Methods sections of papers on research using human subject or samples must include ethics statements that specify:

- The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

  For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:
- Explicitly describe their methods of categorizing human populations

- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subject research, see the Publication Criteria and Editorial Policies.

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Clinical Trials

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement website. Before the paper can enter peer review, authors must:

1. Provide the registry name and number in the methods section of the manuscript
2. Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as Supporting Information (which will be published alongside the paper, if accepted)
3. Include the CONSORT flow diagram as the manuscript's "Figure 1"

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

For more information about *PLOS ONE* policies regarding clinical trials, see the Editorial Policies.

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Animal Research

Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s) (where ethical approval is not required, the manuscript should include a clear statement of this and the reason why)
- Relevant details for efforts taken to ameliorate animal suffering

For example:

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title.

We encourage authors to use the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines as a reference.

For more information about *PLOS ONE* policies regarding animal research, see the Publication Criteria and Editorial Policies.

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Observational and Field Studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

For more information about *PLOS ONE* policies regarding observational and field studies, see the Publication Criteria and Editorial Policies.

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Cell Line Research

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

For more information about *PLOS ONE* policies regarding observational and field studies, see the Publication Criteria.

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Systematic Review/Meta-Analysis

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as Supporting Information and provide the registry number in the abstract.

If your article is a Systematic Review or a Meta-Analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flowchart as Figure 1 (required where applicable)
- Include the PRISMA checklist as Supporting Information

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Paleontology and Archaeology Research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

See the *PLOS ONE* Editorial Policies for more information regarding manuscripts describing paleontology and archaeology research.

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Software Papers

Manuscripts describing software should provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

See the *PLOS ONE* Editorial Policies for more information about submitting manuscripts.

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Database Papers

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

See the *PLOS ONE* Editorial Policies for more information about submitting manuscripts describing databases.

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New Zoological Taxon

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni Fisher sp. nov. urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the Methods section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

All *PLOS ONE* articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

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New Botanical Taxon

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). In association with the International Plant Names Index (IPNI), the following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, "the description or diagnosis required for valid publication of the name of a new taxon" can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of a *PLOS ONE* article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

PLOS ONE staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the Methods section, include a sub-section called "Nomenclature" using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a *PLOS ONE*

article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].
All *PLOS ONE* articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.
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New Fungal Taxon

When publishing papers that describe a new fungal taxon name, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific fungal name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.
Effective January 2012, "the description or diagnosis required for valid publication of the name of a new taxon" can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.
Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of a *PLOS ONE* article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
Additional information describing recent changes to the Code can be found here.
For proper registration of the new taxon, we require two specific statements to be included in your manuscript.
In the Results section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:
Hymenogaster huthii. Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]
You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.
In the Methods section, include a sub-section called "Nomenclature" using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):
The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a *PLOS ONE* article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MycoTaxo.aspx?Link=T&Rec=. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].
All *PLOS ONE* articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.
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Qualitative Research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.
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APÊNDICE C

VENEREAL TRANSMISSION BY *Toxoplasma gondii* IN GOATS MATED WITH A BREEDER THAT WAS EXPERIMENTALLY INFECTED WITH OOCYSTS

(Artigo submetido para o Periódico The Journal of Parasitology)

VENEREAL TRANSMISSION BY *Toxoplasma gondii* IN GOATS MATED WITH A BREEDER THAT WAS EXPERIMENTALLY INFECTED WITH OOCYSTS

Flaviana Santos Wanderley, Wagnner José Nascimento Porto*, Diogo Ribeiro Câmara*, Vinicius Vasconcelos Gomes de Oliveira, João Luiz Garcia†, Pedro Paulo Feitosa de Albuquerque‡, Andréa Alice da Fonseca Oliveira‡, and Rinaldo Aparecido Mota‡**

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STRACT: The objective was to prove the venereal transmission of *Toxoplasma gondii* in goats. Ten female goats were randomly allocated into two groups (G1 and G2), each with five animals. The female goats in G1 were mated with a breeder that had been experimentally infected with *T. gondii* oocysts whereas those in G2 (control) were mated with a serologically negative goat. The infection of the breeder and the females was confirmed through clinical, serological, molecular and histopathological findings. Seroconversion (indirect immunofluorescence reaction) ocurred in the breeder infected on the 7th day post-infection (d.p.i.) and *T. gondii* DNA was present (polymerase chain reaction) in the blood and semen in the 3th d.p.i. In G1, 2 of 5 goats seroconverted and DNA was present in the blood in 2 of 5 goats. In G2, all goats were negative in all tests. With regards to gestational development, the goats in G1 ocurred embryonic reabsorption in 1 of 5 in day 34 after mating, 1of 5 goats aborted in day 42 after mating and 3 of 5 goats gave birth to full-term healthy kids. One of the kids exhibited PCR-positive blood at birth. In total, 40% of the females in G1 and 80% of the kids born alive in G1 exhibited PCR-positive in at least one organ. The present study proved that venereal transmission of *T. gondii* occurs in goats mated with an infected breeder.

Toxoplasmosis is a disease of worldwide distribution caused by *Toxoplasma gondii* which uses felids as definitive hosts and various species of vertebrates as intermediary hosts. This disease has veterinary significance as it can cause abortions and other reproductive problems in animals (Tenter et al., 2000). Ingestion of sporulated oocysts of *T. Gondii,* released into the environment in cat feces, is considered to be the main tranmission route of this parasite (Dubey, 2010).

Toxoplasmosis is considered the main cause of reproductive problems in goats and sheep in many countries worldwide (Skjerve et al., 1998; Borde et al., 2006). Reproductive disorders in goats are more commonly associated with the time of the pregnancy at which the animal was infected than the strain of *T. gondii* (Dubey, 1981). Experimental infection with

oocysts in pregnant goats, negative for *T. gondii* in the first trimester of the pregnancy, defined the occurrence of fetal retention or resorption. In the second and third trimesters of pregnancy, abortions, stillbirths or birth of infected kids are common (Dubey, 1989; Dubey, 2010; Obendorf et al., 1990).

The presence of *T. gondii* in the semen of goats that were experimentally infected with oocysts has been proven by Dubey and Sharma (1980) and Santana et al. (2010), although venereal transmission of the parasite has not yet been proven.

Bearing in mind the significance of this disease in terms of goat reproduction, the aim of the present study was to report venereal transmission of *T. gondii* in goats mated with a breeder that had been infected with oocysts of the ME-49 strain (Tipe II).

MATERIALS AND METHODS

The present study was conducted on the São Luiz farm and in the Universidade Federal de Alagoas, located in the municipality of Viçosa, Brazil. All of the experimental procedures followed the guidelines set down in the International Guiding Principles for Biomedical Research Involving Animals. The present study received approval from the Ethics Committee of the Universidade Federal Rural de Pernambuco under protocol number CEUA - UFRPE - 007/2010.

Animals

Two Saanen breeders, 2 years of age, with a history of reproductive fertility and semen which met the established standards of the Brazilian College of Animal Reproduction (1998) and ten multiparous crossbred goats, 3 to 4 years old, randomly allocated into two groups of five animals (G1 and G2) were used in the present study.

At the beginning of the experiment, animals were dewormed and vaccinated against clostridial diseases and rabies, and were determined to have negative serology for *T. gondii* and *Neospora caninum* [Indirect Immunofluorescence Reaction (RIFI)] *Brucella abortus*

(Buffered Acidified Antigen); and *Chlamydophila abortus* (Complement Fixation). The animals were raised in confinement with uniform feeding, housing and lighting conditions.

Experimental infection of the breeder with oocysts

Oocysts of the ME-49 strain were used to infect the goats. The dosage used was $2x10^5$ oocysts, administered orally in a single dose, using a 5 ml syringe, coupled with a catheter.

The females were synchronized to not be in estrus on the same day, which would impede mating with the infected male. Four days after the infection of the breeder, the first female was synchronized by the intramuscular application of 0.3 ml of Sodium Cloprostenol (Ciosin® Shering Plough, São Paulo, SP, Brazil). On the 6th day post-infection (d.p.i.), three other females were synchronized and on the 10th d.p.i., the last female was synchronized. Using this method, two females came into estrus and were mounted on the 8th d.p.i.; one female on the 9th d.p.i.; one on the 10th d.p.i. and one on the 13th d.p.i. Each female mated naturally (2 to 3 times) during estrus. The same synchronization and mating strategy was used in the control group.

Confirmation of the Infection

Serological and molecular diagnosis:

Serological and molecular examinations were conducted to confirm the infection of the breeders and the goats in both G1 and G2. Blood was collected from the females on days 0, 7, 14, 21, 28, 49, 63 and 123 after natural mating and on the day of birth. Blood and semen were collected from the breeders on days: 0, 3, 5, 7, 11, 14, 21, 28, 35, 42, 49, 56, 63 and 70 d.p.i. Blood samples were taken from the kids that were born alive straight after the birth and before ingestion of the colostrum.

Antibodies to *Toxoplasma gondii* were detected using RIFI, and anti-goat IgG conjugate (Sigma-Aldrich Co.®, St. Louis, Missouri, U.S.A.) with fluorescein isothiocynate, as well as sensitized slides (Perfecta®, São Paulo – Brasil) with antigens to tachyzoites of *T.*

gondii (RH strain). The sera were tested and placed in dilutions of 1:16 up to 1:4096. The sample was considered positive when the titer was equal to or greater than 64 (Camargo, 1974).

The blood, semen and tissue samples were submitted to DNA extraction using the Qiagen DNA Easy Blood and Tissues Kit (Qiagen®, Hilden, Germany), following the instructions of the manufacturer. The primer pairs used were TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (CGCTGCAGACACAGTGCA TCTGGATT), as described by Homan et al. (2000), amplifying a region of 529 base pairs (bp). The amplification reactions were carried out in a final volume of 12.5µl containing the following: 2.5µL of genomic DNA; 0.5µL of each primer (TOX4 and TOX5) at 10µM; 2.5µL of ultrapure Milli-Q water and 6.25µL of Top Taq Master Mix (Qiagen®), following the instructions of the manufacturer. The thermal profile of the reaction stages was conducted in an XP Thermal Cycler (Bioer Technology Co. Ltd., Binjiang, Hangzhou, China). This involved denaturation of the initial DNA at 94 C (7 minutes), followed by 35 cycles at 94 C for 1 minute for denaturation, 60 C for 1 minute for annealing, 72 C for 1 minute for extension and a final extension of 10 minutes at 72 C. The amplified products were detected using agarose gel electrophoresis (2%), stained with Blue Green (LGC®, Cotia, São Paulo, SP, Brasil), visualized under ultraviolet light and photodocumented. The positive control used in the reaction was obtained through the suspension obtained from intraperitoneal lavage of mice that had been previously infected with the RH strain.

Amplicons were purified by employing commercial GFXTM PCR DNA and a Gel Band Purification kit (GE Healthcare). Using the sequencer ABI PRISM 3100 (Applied Biosystems), reactions were carried out in both strands using primers (TOX4 e TOX5) according to the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the polymerization conditions were performed in 96-well places according to the

manufacturer's instructions. Sequences were analyzed through Bioedit and MEGA 5 software and compared with the NCBI database using Blastn.

Clinical monitoring

Clinical monitoring of the breeders and females, including two temperature measurements, was performed on a daily basis.

Diagnosis and monitoring of pregnancy

Diagnosis and monitoring of pregnancy was conducted by means of weekly transrectal ultrasound examinations between 15 and 60 days after natural mating, using a multi-frequency linear transducer (CTS 900V, SIUI, China). After 60 days of pregnancy, ultrasound assessments were performed transabdominally at intervals of 15 days until the time of birth. The births of animals in G1 and G2 were monitored, and the placentas were collected, identified, and frozen for molecular examination (PCR) or placed in buffered formalin (10%) for histopathological examination.

Histopathological examination

At the end of the experiment, all animals were euthanized. The goats were euthanized 240 days after mating and the kids were euthanized 90 days after their birth. The breeder was euthanized on the 240th d.p.i. Euthanasia was performed according to Resolution number 1000 of the Federal Council of Veterinary (CFMV, 2012). Necropsies were performed on all animals and sections of the liver, spleen, kidneys, medulla, brain, lung, heart, uterus, ovaries and testicles were collected. The material was fixed in buffered formalin at 10%, placed in paraffin, cut into 6µm sections in a microtome (Model 1512, Leitz Wetzlar, Wetzlar, Hessen, Germany) and stained with hematoxylin and eosin.

RESULTS

Serological and molecular diagnosis

The breeder seroconverted on the 7th d.p.i. (titer of 64). On the 11th d.p.i., it exhibited a maximal titer of 1024, which was maintained until the final collection on the 70th d.p.i. In G1, 2 of 5 females seroconverted on the 123rd day after mating with a titer of 32. None of the goats or kids in G2 exhibited antibodies to *T. gondii* (Table 1).

DNA of *T. gondii* was detected in the blood and semen of the breeder from the 3th d.p.i. until the last collection on the 70th d.p.i. Two females from G1 exhibited PCR-positive blood on the 7th day after mating and one other female did so on the day of the birth. In the organs examined in the PCR, 2 of 5 females and 4 of 5 kids in G1 were positive in at least one organ. DNA of *T. gondii* was not found in any of the goats or kids in G2 (Table 2).

The molecular amplicon identities were confirmed through direct double-strand sequencing, which indicated 91% similarity with the *T. gondii* DNA sequences stored in GenBank.

Clinical signs

The infected breeder exhibited apathy, hyporexia, coughing, hyperthermia (maximal temperature of 41.3^0 C), tachycardia and tachypnea between the 3rd and 8th day after mating, with a recovery after this period. On this day, the first mounting occurred in G1. The females in G1 exhibited a dry cough with rales in the clinical examination after the 9th d.p.i.

Monitoring of the pregnancy

With regards to gestational development, embryonic reabsorption occurred in 1 of 5 females in G1 on the 34th day after mating. Abortion was recorded in 1 of 5 females on the 42nd day of pregnancy and 3 of 5 of the females gave birth at full term. In G2, all of the females gave birth to full-term clinically healthy kids (Table 1).

Histopathological examination

The main microscopic injuries in this group were: hemorrhage, thickening of the alveolar septa, perivascular mononuclear and interstitial infiltration compatible with

interstitial pneumonia; multifocal necrosis of the liver and the presence of predominantly neutrophilic polymorphonuclear infiltration; perivascular gliosis with focal hemorrhage.

DISCUSSION

In the present study, a breeder sheep was experimentally infected with oocysts of the ME-49 strain of *T. gondii* and mated with females during the acute phase of the infection. After the clinical recovery of the breeder on the 8th d.p.i., the mating with previously synchronized females began. The results obtained in the present study are unpublished in the literature. No data previously existed related to toxoplasmosis acquired via semen in natural mating. The present study confirmed the elimination of the parasite in the semen and its transmission to the females and kids. A number of reproductive abnormalities were observed in the females resulting from the infection (embryonic reabsorption and abortion). An interval between the 7th and 15th d.p.i. was selected for the mating. This decision was based on the results obtained by Dubey and Sharma (1980) who used a bioassay in mice to demonstrate the presence of *T. gondii* in the semen of three goats infected orally with 10^4 oocysts of the GT-1 strain (isolated in goats). In that study, the parasite was detected in the semen from the 7th d.p.i. in two animals and on the 12th d.p.i. in the third infected animal. In the present study, elimination of the parasite in semen was detected in the PCR from the 3rd d.p.i.

Infection of the breeder was confirmed by clinical signs, PCR of the blood and semen, as well as positive serology from the 7th d.p.i. (titer of 64). In total, 1024 titers were recorded on the 11th d.p.i., which was maintained until the end of the experiment on the 70th d.p.i. The seroconversion period differed from that found by Santana et al. (2010), who infected goats orally with a dose of 2×10^5 oocysts of the P strain. The same author identified genetic material of *T. gondii* in the semen on the 56th d.p.i and detected IgG on the 11th (256) d.p.i., reaching the greatest titer (4096) on the 28th d.p.i., which then reduced to 1024 titers from the 35th d.p.i. and remained stable until the end of the experiment. Nishi et al. (2001) infected

goats with 10^5 oocysts of the AS-28 strain and demonstrated positive serology on the 10th d.p.i.

The clinical signs observed in the breeder were in agreement with the findings of Rosa et al. (2001) and Santana et al. (2010). In the present study, hyperthermia coincided with the period of positivity in the PCR and the increase of antibody titers in the serology.

In goats, the clinical and histopathological findings characterized interstitial pneumonia compatible with the abnormalities caused by *T. gondii* (Dubey et al., 1987, Hartley & Dubey, 1991, Barker et al., 1992, Reddacliff et al., 1993, McGavin; Zachary, 2012).

Transmission of *Toxoplasma gondii* to goats through natural mating was proven in the present study by the results obtained in certain animals in G1. Two of the females exhibited antibodies to *T. gondii* (32 titers) on the 123th day after mating. One of these goats also exhibited PCR-positive blood on the 30th day after mating and gave birth at term. The other goat exhibited embryonic reabsorption which was detected on the 34th day of the pregnancy. Another female, which was negative in the serology and in the PCR, aborted on the 42th day of the pregnancy. This female was PCR-positive in the brain and ovaries. In another goat, the serology was negative but the blood was PCR-positive on the day of the birth, and PCR-positivity was also discovered in the liver, spleen and kidney. This goat gave birth at term. The final goat of this group exhibited negative serology and PCR of the blood and tissues, and also gave birth at term.

Vitor et al. (1992) infected pregnant goats subcutaneously with tachyzoites and confirmed a congenital transmission of *T. gondii*. These authors observed antibodies in the aborted fetus as well as in the kids born alive. The seroconversion results found in the present study need to be investigated further since no response was achieved with elevated titers of antibodies, as is generally found with other infection routes. The infection route used in the present study probably affected the antibody response.

Another important finding of the present study was the positive PCR in the brain of 1/5 kids, the heart of 2/5 kids and the kidney of 2/5 kids that were born alive. This indicates that *T. gondii* was also transmitted to the offspring (congenital transmission) when the goat did not experience embryonic reabsorption or abortion. A number of recent studies with sheep have demonstrated that the parasite was capable of infecting sheep via semen contaminated with *T. gondii* (Moraes, Batista et al., 2010; Moraes, Freitas et al., 2010). High rates of embryonic reabsorption were recorded in the group of sheep infected with the greater dose of tachyzoites, as well as reproductive disorders such as anestrous, follicular cyst, hydrometra and mucometra during the chronic stage of the infection. These disorders were not recorded in the present study with goats, probably due to the infective dose being eliminated in the breeder's semen. According to Dubey (1981, 1989), who experimentally infected pregnant goats with oocysts, the clinical signs observed are dose-dependent. This aspect should also be investigated further in both experimental and natural infection of breeders, particularly in terms of the number of parasites eliminated in the semen and the timing of the elimination.

It is important to remember that in the present study, the breeder mated with the females in the acute stage of the infection while experiencing a reduced libido. The transmission took place because the number of parasites in the semen is probably higher during this stage. It is not yet possible to confirm the true importance of this infection route in field conditions, since the breeder may not be interested in mating with the goats that are in heat during the acute stage of infection. The impact of this infection route should be further investigated in relation to goat reproduction in natural conditions.

The results obtained in the present study allow the authors to conclude that *T. gondii* is transmitted via goat semen. Further studies are recommended to investigate this subject in more detail, particularly in relation to immunological aspects of infection by this route, as well as the clinical repercussions for goat reproduction.

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Table 1 – Results of laboratory examinations in goats and viable newborns mated with a breeder infected with *Toxoplasma gondii*

Animal/ Group	Serology	PCR Blood	PCR organs	Gestation diagnosis	Reabsorption	Abortion
G1						
1	+	+	-	x	-	-
1(C1*)	-	-	+	-	-	-
2	+	-	-	x	x	-
3	-	-	+	x	-	X
4	-	+	+	x	-	-
4 (C1*)	-	+	+	-	-	-
4(C2*)	-	-	+	-	-	-
5	-	-	-	x	-	-
5 (C1*)	-	-	+	-	-	-
5 (C2*)	-	-	-	-	-	-
G2						
6	-	-	-	x	-	-
6 (C1*)	-	-	-	-	-	-
7	-	-	-	x	-	-
7 (C1*)	-	-	-	-	-	-
7 (C2*)	-	-	-	-	-	-
8	-	-	-	x	-	-
8 (C1*)	-	-	-	-	-	-
8 (C2*)	-	-	-	-	-	-
9	-	-	-	x	-	-
9 (C1*)	-	-	-	-	-	-
10	-	-	-	x	-	-
10 (C1*)	-	-	-	-	-	-

+ = positive in the PCR or serology (titration = 32); * Viable newborns

Table 2 – Results of PCR in tissues from breeders, females and kids infected with *Toxoplasma gondii*

Animal	Liver	Spleen	Kidney	Medulla	Brain	Lung	Heart	Placenta	Ovary
1	-	-	-	-	-	-	-	**-**	**-**
1(C1*)	-	-	+	-	**-**	-	**-**	**-**	**-**
2	-	-	-	-	**-**	-	**-**	**-**	**-**
3	-	-	-	-	**+**	-	**-**	**-**	**+**
4	+	+	+	-	**-**	-	**-**	**-**	**-**
4 (C1*)	-	-	-	-	+	-	+	**-**	**-**
4 (C2*)	-	-	+	-	-	-	-	**-**	**-**
5	-	-	-	-	-	-	-	-	-
5 (C1*)	**-**	**-**	**-**	**-**	**-**	-	+	-	-
5(C2*)	-	-	-	-	**-**	**-**	**-**	**-**	**-**
Breeder	-	+	+	-	**-**	**-**	**-**	**-**	**-**

* Viable newborns

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Materials and methods: This section should give sufficient information to permit repetition of the study by others. Methods and apparatus used should be indicated, but specific brand names and models need to be mentioned only if significant. The source, e.g., city and state (if in the U.S.A.), both spelled in full, of special equipment or chemicals should also be given. If the source is outside the U.S.A., then the city and country should be given. Previously published or standard techniques are to be referenced, but not detailed. Generic descriptions should be given for unusual compounds used.
The primary heading for this section should be typed in all bold capital letters, starting at the left-hand margin of the page. The heading is unnumbered and ends without punctuation. Second-level headings in bold type should be on a separate line beginning at the left-hand margin. The initial letter of the first word is the only capital letter except as needed for proper nouns. These headings are unnumbered and end without punctuation. Third-level headings are indented for a paragraph, italicized, and end with a colon, also italicized. The initial letter of the first word is the only capital letter, except capitals needed for proper nouns. Text runs in immediately following this heading. Further subdivision should not be needed. If the materials and methods section is short, it should not be subdivided; it is unnecessary to provide headings, beyond the primary head, for a series of subsections comprising single paragraphs.
Results: This section should contain a concise account of the new information. Tables and figures are to be used as appropriate, but information presented in them should not be repeated in the text. Avoid detailing methods and interpreting results in this section. The results section may be subdivided and headed
as for the materials and methods section.
Discussion: An interpretation and explanation of the relationship of the results to existing knowledge should appear in the discussion section. Emphasis should be placed on the important new findings, and new hypotheses should be identified clearly. Conclusions must be supported by fact or data. The headings and subdivisions, if needed, in this section are as described for the materials and methods section. *Acknowledgments:* These should be concise. Ethics require that colleagues be consulted before being acknowledged for their assistance in the study. The heading for this section is as for the primary head described for the materials and methods section. Subdivisions are not used in this section.
Literature cited: Citations are arranged alphabetically. All references cited in the text must appear in the literature-cited section, and all items in this section must be cited in the text. Citation of unpublished studies or reports is not permitted, i.e., a volume and page number must be available for serials and a publisher, city, state, and full pagination for books. Abstracts not subjected to peer review may not be cited in the text or in the literature cited sections. Work may be cited as ‗‗in press'' only when the paper has been accepted for publication. If absolutely necessary, a statement may be documented in the text of the paper by ‗‗pers. comm.''. The citation is indicated in the style: (X. Y. Smith, pers. comm.).
Personal communications do not appear in the literature-cited section.
Style in the text:
Allen (1989)
(Allen, 1989)
(Allen and Smith, 1989)
(Allen et al., 1989)
(Jones, 1987; Allen, 1989)—chronological
(Jones, 1987; Allen, 1989; Smith, 1989)—chronological and alphabetical within year
(Jones, 1987, 1988a, 1988b, 1989)
Multiple authors with the same year of publication should be (Smith, Jones et al., 1988; Smith, Walker, and Jones, 1988), **not** (Smith et al., 1988a, 1988b)
Style in the literature cited section (note that indentations are no longer required):
Journal article, 1 author
Nollen, P. M. 1990. Chemosensitivity of *Philophthalmus megalurus* (Trematoda) miracidia. Journal of Parasitology **76:** 439–440.
Journal article, 2 authors
Edwards, D. D., and A. O. Bush. 1989. Helminth communities in avocets: Importance of the compound community. Journal of Parasitology **75:** 225–238.
Book
Schmidt, G. D., and L. S. Roberts. 1989. Foundations of parasitology, 4th ed. Times Mirror/Mosby College Publishing Company, St. Louis, Missouri, 750 p.

Chapter in edited book
Nesheim, M. C. 1989. Ascariasis and human nutrition. *In* Ascariasis and its prevention and control, D. W. T. Crompton, M. C. Nesbemi, and Z. S. Pawlowski (eds.). Taylor and Francis, London, U.K., p. 87–100.
Thesis or dissertation
Monks, W. S. 1987. Relationship between the density of *Moniliformis moniliformis* and distribution within the definitive host population. M.S. Thesis. University of Nebraska-Lincoln, Lincoln, Nebraska, 64 p.
Number of authors
If there are more than 10 authors, then include names of the 10, followed by et al.
Note that abbreviations are not used for titles or serial publications and that spaces appear between initials.
The literature cited section has a primary heading as described for materials and methods.
Footnotes: Footnotes are used only for the title page of regular articles to indicate authors' addresses and to whom correspondence should be sent. Those for tables are typed directly under the table to which they pertain. Footnotes appear at the end of the manuscript directly after the figure legends (see example at end of guidelines).
Tables: Tables are used only to present data that cannot be incorporated conveniently into the text. Ordinarily, values from statistical tests are not published as tables; tests employed and probability accepted for significance can be stated in the materials and methods section with significant differences indicated in tables by footnotes or in the text by a statement.
Tables must be designed to fit in 1 or 2 columns. Only rarely may they be designed to fit the height of a printed page. Generally, if the width does not fit the height of a typed page, the table is too wide. Tables may be continued on following pages to accommodate length, but pages cannot be photoreduced, single-spaced, oversized, or otherwise modified to contain additional material.
Tables are numbered with Roman numerals in a continuous series and so referenced, in sequence, in the text. Captions are typed above the data on the same page. Species names are spelled out in full (and italicized) the first time used in each caption. All columns in a table must have headings, with the first letter of the first word and proper nouns capitalized, e.g., Number sampled, % Recaptured.
Horizontal lines should be avoided in the body of the table; vertical lines are not permitted. If symbols are necessary, the table must be prepared as a line drawing and treated as a figure. Use of letters and numbers as superscripts or subscripts is not permitted. Table footnotes must be used in the sequence that follows: *, †, ‡, §, ||, #, ¶, **, ††.
Figures: All figure captions are to appear consecutively after the literature cited section. Do not place figure captions on the same page as the figures. Each figure or plate of figures must have a caption. The caption is written in paragraph style, beginning with the word ‗FIGURE.'' Captions are typed in roman, except when italic type is required, e.g., a genus and species. For plates, a summary statement should precede the specific explanation of each figure. Avoid repeating information for each figure that can be placed in the summary statement. Genus and species names are spelled out in full the first time used in each caption. The caption must contain an explanation of all abbreviations used on the figures and indicate the value of lines or scale bars used to show size. Moreover, size should not be indicated by magnification in the caption because the figure might not be printed at the size calculated.
Figures & Plates
Plates should be submitted composed of one or more figures ready for final publication. **Please note** that all figures should be submitted as separate files and **not** as part of the text. Figure legends should not be placed on the plate, they should be at the end of the manuscript file directly after the literature cited section. With the initial submission for review, each plate must conform to *Journal* specifications for publication. Plates must be sized to 1 column (88 mm) or 2 column (182 mm) width, and must be no longer than 220 mm in height to leave sufficient room beneath for a figure legend (in case of very long figure legends, plates must be adjusted to allow for the legend). When composing plates, sizing of individual figures should be proportional to information content of the individual figure. Plates are to be arranged fully rectangular, with a 1.4mm white space between all abutted images in plates creating a full rectangle. Plates must be submitted in any one of the following styles: black and white line art, grayscale, or color. Plates submitted as black and white line art, including line drawings, black and white graphs, and charts, must have a minimum resolution of 1200 dpi and must be submitted as TIFF, GIF, or EPS (postscript); bitmapped images will be returned to authors. Plates submitted as grayscale (halftone) photographs, charts, or graphs must have a minimum resolution of 300 dpi and must be submitted as TIFF, GIF, JPEG (quality level >9), or EPS. Plates submitted as color photographs, charts, or graphs, must have a minimum resolution of 450 dpi and must be submitted in CMYK. If color figures are submitted, please note that the cost of printing a color plate is $500, and is $75 for a version online. This is the responsibility of the authors. **Plates not intended for printing in color must be submitted in grayscale.**
All letters and numbers appearing on a plate must be in a sans-serif font, e.g., Helvetica, Ariel, Geneva, Gills-Sans, Lucida, or Verdana. All species binomena, such as those on phylogenetic trees, must

be italicized. Non-scientific names, e.g., —sp.‖ locality, strain, or reference label, must not be italicized. All figures in a plate are to be identified with a figure number or capital letter in the upper or lower left-hand corner, applied directly within the figure and without an added background, shadowing, outline, circle, period, or parentheses. At final print size, font height for numbers/letters identifying figures must be at least 4 mm and not more than 8 mm high. The font size used for labeling structures therein, must be at most two-thirds of the size of the numbers/letters used for identifying the figure and must be at least 2 mm high.

If possible, scale bars should be situated at the lower right-hand corner of the figure. Scale bars should be no thicker than 4 pts (or 1.4 mm at printed size) and normally would be at least 10 mm long and no longer than one-half the width of the figure being scaled. Scale bars (including those for substitutions on phylogenetic trees) may be labeled directly on the figure with their dimensions, bearing in mind that scale bars as generated by microscopic devices typically do not meet *Journal* standards for resolution or font specifications; alternatively, they may be defined in the figure legends. Separate figures in a plate may not share a single scale bar, except in the special circumstance in which all figures in the plate share the same scale.

Prior to review, the Graphics Associate Editor will examine the submitted paper to be certain that all figures conform to *Journal* specifications as described above. The Graphics Associate Editor will also evaluate the level of professional quality relative to what is expected for final publication. Manuscripts with figures that do not conform to *Journal* standards will be returned promptly to the author(s) for revision. **PLEASE NOTE:** Resampling poor resolution images to a larger size does not improve resolution. Resubmitted manuscripts that have merely re-sampled images to a larger size will be returned to the author again.

Taxonomic (Systematics) papers

Taxonomic papers have a distinct style that must be adhered to in preparing a manuscript. Single taxon papers will be considered only if presented in a comprehensive manner. A taxonomic paper should follow instructions given for a regular article, i.e., the title page, abstract, introduction, and materials and methods. In taxonomic papers, however, the results section is e replaced by a section headed **DESCRIPTION** (or **REDESCRIPTION**, as the case may be), beginning at the left-hand margin and the DESCRIPTION is followed in the next line by the italicized scientific name in bold type; it begins at the left-hand margin. Synonyms and reference to figures follow, each as a separate line at the left-hand margin (these are not in bold font). The text of the Description follows as a new paragraph beginning with *Diagnosis*. The DESCRIPTION section must be written using a telegraphic style, i.e., do not employ prepositions, start sentences with the subject, and refrain from using verbs except as absolutely necessary. This DESCRIPTION is followed by a **Taxonomic summary** section, headed as described for second-level headings in the instructions for the materials and methods section. The Taxonomic summary section comprises a listing of the type host, other hosts, site, locality, and specimens deposited. Each of these topics is italicized, but not indented. The *Host* subsection must include the full scientific name of the host, the authority‘s name, and an indication if *Symbiotype* specimens were deposited in a vertebrate museum along with accession numbers. The *Locality* should include map coordinates (e.g., 95°5'11"N, 48°3'15"W) as well as the name of the locality, e.g., ocean, river, etc., and the geopolitical region. *Prevalence and density* data are included when known. Museum accession numbers for appropriate type material (new taxa) and for voucher specimens (surveys) are required; the accession number of the museum must be preceded by the acronym of the appropriate museum. It is recommended that authors consider depositing one, or more, type or voucher specimens in the U.S. National Parasite Collection, Beltsville, Maryland, although this not a requirement for publication in the *Journal.* Appropriate photographic material should be deposited for descriptions of coccidia. Frozen tissues must also include accession numbers if deposited in a museum. In the case of phylogenetic studies involving, or based upon, molecular sequence data, novel nucleotide, or protein sequence data must be deposited in the GenBank Museum, and GenBank numbers must be obtained and added to the manuscript no later than the proof stage**.** The Taxonomic summary is followed by a **Remarks** section, headed as described for second level headings in the instructions for the materials and methods section. The Remarks section must include comparisons to all similar taxa so as to definitively distinguish new taxa from existing taxa; the section heading is typed in bold font and begins at the left margin. . The final section of a taxonomic paper should be the **DISCUSSION**, which includes a thoughtful dialogue regarding important considerations of phylogenentic position, molecular survey information, etc. It should be a synthesis section, placing the new findings in a phylogenetic and/or ecological context as appropriate.

Research notes

Manuscripts are to be organized in the following format and sequence with all pages, numbered consecutively. *Title page:* Research Notes do not have a running head, but should be identified as such on the title page, i.e., RH: RESEARCH NOTE. On first page, give the title of the note in bold type and capitalize the first letter of all principal words. On a separate line, give the names of the authors, also in bold type, and capitalize only the authors‘ initials and first letter of the last name. The addresses currently are run in after

the last author‘s name, with a comma in bold type separating the names and beginning address. Follow with addresses of the authors in roman type, joined by semicolons, matched to authors other than the first one by symbols like those used for regular articles. Begin all lines at the left-hand margin. Identify the corresponding author with an acceptable symbol; this is the person with whom the editor will correspond and to whom page proofs will be sent.

Abstract: An abstract is to be provided as described for articles.

Text: The text of a research note is written without sections and without extra spacing between paragraphs. Acknowledgments may be given, without heading, as the last paragraph. Literature is cited in the text as described for articles.

Literature cited, tables, figure captions, and figures: These items are in the form and sequence described for articles.

Review articles

Invited review articles should be submitted using the format described for articles, except that other section names may be used in place of the materials and methods, results, and discussion sections. Headings should be restricted to major headings and no more than 2 sublevels. Use of tabular data or figures from the work of others must be consistent with copyright law, and it is the responsibility of the author to supply appropriate permissions when the manuscript is submitted.

Critical comments

Manuscripts are to be organized in the following format and sequence with all pages, beginning with the title page, numbered consecutively.

CRITICAL COMMENT…

Title: Starting at the left-hand margin, give the title of the article in bold type and capitalized. Immediately following title, give the name of the author(s) in bold type.

Names and addresses of authors: These follow the title. The style is as described for research notes.

Text: The text is written without subdivision. Literature citations are made as for articles. Acknowledgments may be included as an un-headed final paragraph.

Literature cited: If citations have been used in the text, the literature cited section is as described for articles.

Tables, figure captions, and figures: When present, these are as described for articles.

Book reviews Manuscripts are organized in the following format and sequence with all pages, beginning with that for the title, numbered consecutively.

Title: Give the title of the book being reviewed, and other critical information, in the following style:

Toxoplasmosis of Animals and Man, by J. P. Dubey and C. P. Beattie. CRC Press, Boca Raton, Florida. 1988. 220 p. ISBN Number. Hardcover $124.95.

Note that, unlike in the literature cited section, the first letters of principal words are capitalized. The words ‗‗edited by‘‘ are substituted for ‗‗by‘‘ when appropriate. The book title, etc., should begin at the left-hand margin.

Text: Begin as a new paragraph immediately following the book title. The text usually is not subdivided. If literature must be cited, a headed literature cited section follows the text in the style described for articles. Figures and tables should not be used.

Name and address of author: This information follows the text or, if present, the literature cited section. The reviewer‘s name should be in bold type; the address should follow, but not in bold type.

General points of style

Scientific names: The full binomen is written out at the first use of a species name. At subsequent use, the genus is abbreviated by use of the first letter, except at the beginning of a sentence where it is written out. Genera and species should be italicized, not underlined, throughout the manuscript. Author and date citations for scientific names need not be used in non-systematic papers. In systematic papers, author and date citations are used the first time a taxon is mentioned in the abstract and the text, but not subsequently except as described for tables and figures. Use must be according to the International Code of Zoological Nomenclature and should be consistent for all parasite and host species mentioned. Author and date citations used only as authorities for scientific names do not appear in the literature cited section.

Authors are reminded that names of taxa are not names of organisms, e.g., *Fasciola* is the name of a genus (a group of related species) and as such it does not lay eggs, ingest cells, possess a sucker, etc. These are properties of organisms.

Ecological terms: The terms prevalence, incidence, intensity, mean intensity, density, relative density, abundance, infrapopulation, suprapopulation, site, niche, and habitat are to be used as recommended by the ASP Ad Hoc Committee on the Use of Ecological Terms in Parasitology (1982, *Journal of Parasitology* **68:** 131–133); also see Bush et al. (1997, *Journal of Parasitology* **83:** 575–583) for an expanded and updated treatment of ecological terminology.

Mathematical and chemical notations: Authors should attempt to write mathematical equations so

that they can be set in 1 line of type. When 1 unit appears in a denominator, use the solidus, e.g., g/m^2; for 2 or more units in a denominator, use negative exponents, e.g., g·m^{-2}·day^{-1}. Manuscripts submitted to the *Journal of Parasitology* should conform to the same conventions as those used for chemical and biochemical/molecular nomenclature.

All chemical structures not accommodated by a single line of type must be drafted and reproduced as figures.

Use of numbers: In the text, numbers should be Arabic numerals except when beginning a sentence. Naked decimals are not permitted in the text, tables, legends, or on figures, i.e., 0.1, **not** .1. Numbers greater than 999 must have commas. Metric units are to be used in all articles. The 24-hour system is used to indicate time, e.g., 1500 hr.

Acronyms and abbreviations: At first use, acronyms are placed in parentheses following the name written out in full. At subsequent use, the acronym alone is used. An acronym may begin a sentence. Sentences may not begin with an abbreviation, and abbreviations are as recommended in the Council of Science Editors (CSE) style manual. The *Journal* uses all International System of Measurement (SI) metric unit abbreviations. Common CSE and SI abbreviations include the following (the same abbreviation is used for plural form):

wk (week)
hr (hour; use 0–2400 hr for time)
sec (second)
min (minute)
mo (month)
day (not abbreviated)
n. sp. (new species)
n. gen. (new genus)
L (liter; but ml for milliliter)
RH (relative humidity)
p.o. (per os)
s.c. (subcutaneous)
i.pl. (intrapleural)
i.p. (intraperitoneal)
PI (post-inoculation, or post-infection)
p. (page)
ad lib. (ad libitum) U.S.A. (as a noun)
U.S. (as an adjective)
sp. gr. (specific gravity)
t-test
U-test
P (probability)
(arithmetic mean) x
r (correlation coefficient)
n (sample size)
SD (standard deviation of the mean)
SE (standard error of the mean)
df (degrees of freedom)
NS (not significant)

Basic SI units

Meter m
Kilogram kg
Second sec
Ampere A
Volt V
Mole mol

Prefixes for SI units

Factor	Prefix	Symbol
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	µ
10^{-9}	nano	n
10^{-12}	pico	p
10^{1}	deca	da
10^{2}	hecto	h
10^{3}	kilo	k
10^{6}	mega	M
10^{9}	giga	G
10^{12}	tera	T

Miscellaneous: Unless stated otherwise, U.S.A. is understood for locations, including addresses of authors, and is not stated.

Words and abbreviations in Latin and other non-English languages, except for genus and species names, are not italicized. American spelling supersedes English spelling.

No and none are treated as singular, e.g., no worm was found. If this form is not satisfactory, avoid use of the words.

The suffix -like is hyphenated only in combination with a name in italic type or to avoid a triple l.

Studies involving sacrifice of animals are outside the scope of the *Journal*; however, many appropriate studies involve killing of animals.

Because manuscripts are accepted only with the understanding that the work was conducted in compliance with all relevant laws and within the ASP policy on animal care and use, a separate statement regarding animal care and use is not printed as a part of each paper. Likewise, use of the word euthanasia is redundant.

Papers are not dedicated to individuals. Dedication is only at the direction of the ASP Council and it is in the form of an entire issue.

Revising manuscripts

When manuscripts are returned for revision, a cover letter from the editor provides directions that must be followed carefully. A point-by-point statement of what has been revised and a brief rebuttal of those criticisms not addressed should be provided. All suggestions of the reviewers and the associate editor and editor must be addressed individually. Reviewers are usually kept anonymous and assigned numbers to retain anonymity. The revised manuscript and the author‘s comments may be reviewed again (by the same referee[s]), subject to the discretion of the editor.

Retain a complete and exact copy of the manuscript, tables, and figures for reference.

Correcting proof and ordering reprints

Authors are responsible for the accuracy of their proofs and, therefore, what ultimately is printed in the *Journal.* **Corrected proofs must be returned to the editor promptly**, ideally on the same day as received. Proofs are sent by e-mail and corrections can be sent by e-mail to Dr. Gerald W. Esch at: esch@wfu.edu. Proofs are to be corrected, not revised. Additions usually are disallowed except to correct errors made in typesetting and by the editor. Correction of errors made by the author may be billed to the author at the rate of $5.00 each. Queries on the proof are to be answered by ‗‗yes‘‘ or ‗‗no‘‘; and if there is no change to be made you should write ―ok as set‖; otherwise do not use ‗‗ok‘‘ or ‗‗stet.‘‘ Reprints can now be ordered online; a link is provided. Only the author designated to receive correspondence receives proof and reprint order forms. It is the responsibility of this author to clear the proof with other authors and to provide the opportunity for them to order reprints. Reprint orders are to be sent to Allen Press using the standardized form provided.

SCHEDULE FOR PRINTING INSTRUCTIONS

These instructions can be found online at: http://asp.unl.edu

ACKNOWLEDGMENTS

These instructions are a revision of policies and practices formulated by previous editors. The staff at Allen Press, especially Annielaurie Seifert, Mary Reilly, and Valerie Pierce, contributed ideas and advice for the revision.

Gerald W. Esch, Department of Biology, Wake Forest University, P.O. Box 7629, Winston-Salem, North Carolina 27109.

Name /PARA/2008_guide 04/14/2008 03:51PM Plate # 0-Composite pg 8 # 8 **EXAMPLE FOR FULL-SIZED ARTICLE**

The following presents a sample layout of the way in which your manuscript should appear for a full-sized article.

RH: NADLER ET AL.- GENETICS OF GEOGRAPHIC VARIATION IN *A. SUUM* (not to exceed 60 characters and spaces)

GENETIC STRUCTURE OF MIDWESTERN *ASCARIS SUUM* POPULATIONS: A COMPARISON OF ISOZYME AND RAPD MARKERS

Steven A. Nadler, Rachel L. Lindquist*, and Thomas J. Near

Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115. *e-mail: snadler@ucdavis.edu* (the e-mail address is of the corresponding author ONLY)

ABSTRACT: Isozyme and random amplified polymorphic DNA (RAPD) markers were used to characterize the genetics of geographic variation among population samples of *Ascaris suum.* . . .

Molecular markers are of great potential utility for revealing intraspecific variation among parasite populations. (**Note:** This was the first sentence of the Introduction, that it is not preceded by a heading.) By studying the genotypes or haplotypes of individuals. . . .

MATERIALS AND METHODS

Ascaris suum adults were collected from the intestines of pigs at a local meat processing plant. . . .

RESULTS

Isozyme data

Six of the 13 loci surveyed showed evidence of polymorphism; however, only 3 of the 6 variable loci showed sufficient enzymatic activity
and resolution on gels to score. . . .

RAPD data and comparative analysis of markers

Of the 29 RAPD primers surveyed, 9 yielded amplification products that met both subjective. . . .

DISCUSSION

Isozyme data

Geographic variation: Relatively few studies have focused on the genetics of geographic variation among populations of parasitic helminths. In part, this is due to. . . .

Polymorphisms: A paradigm of parasite population structure is that parasitic organisms are characterized by small populations with high levels of inbreeding. . . .

Genetic drift

Genetic drift among *A. suum* infrapopulations may be promoted by their small effective population size and founder effects. The overall sex ratio. . . .

ACKNOWLEDGMENTS

We are grateful to Mr. Don Temperly for assistance at FDL. . . .

LITERATURE CITED

ANDERSON, R. M., AND R. M. MAY. 1978. Regulation and stability of host-parasite population interactions. I. Regulatory process. Journal of Animal Ecology **47:** 219–247.

ANDERSON, T. J. C., M. E. ROMERO-ABAL, AND J. JAENIKE. 1993. Genetic structure and epidemiology of *Ascaris* populations: Patterns and host affiliation in Guatemala. Parasitology **107:** 319–334.

_____, _____, AND J. JAMES. 1995. Botulism. Journal of Parasitology **81:** 1–10.

MONKS, W. S. 1987. Relationship between the mean density of *Moniliformis moniliformis* and distribution within the definitive host population. M.S. Thesis. University of Nebraska-Lincoln, Lincoln, Nebraska, 64 p.

NESHEIM, M. C. 1989. Ascariasis and human nutrition. *In* Ascariasis and its prevention and control, D. W. T Crompton, M. C. Mescheim, and Z. S. Pawlowski (eds.). Taylor and Francis, London, U.K., p. 87–100.

SCHMIDT, G. D., AND L. S. ROBERTS. 1989. Foundations of parasitology, 4th ed. Times Mirror/Mosby Publishing Company, St. Louis, Missouri, 750 p.

FIGURE 1. Random amplified polymorphic DNA fingerprints for 11 *Ascaris suum* individuals. . . .

FIGURES 2–4. Isozymes and infrapopulations. (**2**) Gels showing the genetic make-up of. . . . (**3**) Comparison of 4 infrapopulations. . . . (**4**) Isozymes from *Ascaris suum.*. . . .

*Department of Biology, Southeast Missouri State University, Cape Girardeau, Missouri 63701. **EXAMPLE FOR RESEARCH NOTE**

The following represents a sample layout of the manner in which the file on your diskette should appear for a RESEARCH NOTE.

RH: RESEARCH NOTE

HEPATIC SARCOCYSTOSIS IN A HORSE

C. R. Davis, B. C. Barr*, J. R. Pascoe†, H. J. Olander‡, and J. P. Dubey§, Department of Anatomy, Physiology and Cell Biology, University of California, Davis, California 95616; *University of California, California Veterinary Diagnostic Laboratory, Davis, California 95616; †Department of Surgical and Radiological Sciences, University of California, Davis, California 95616; ‡Department of Pathology, Microbiology and Immunology, University of California, Davis, California 95616; and §Parasite Biology and Epidemiology Laboratory, Livestock and Poultry Sciences Institute, Agriculture Research Service, United States Department of Agriculture, Beltsville, Maryland 20705-2350. *e-mail: jdubey@anri.barc.usda.gov* (the e-mail address is of the corresponding author ONLY)

ABSTRACT: This report examines the phylogenetic relationships of *Tetrabothrium* spp. . . .

The various species of *Tetrabothrium* are examined within an evolutionary context (this constitutes the first sentence of the text).

If you wish to acknowledge, add without a title as an indented sentence immediately following the last paragraph of the

RESEARCH NOTE.

LITERATURE CITED

(See **EXAMPLE** for full-sized article.)

Figure legends will follow.

ANEXO A

Licença da Comissão de Ética no Uso Animal (CEUA/UFRPE)

USO EXCLUSIVO DA CEUA-UFRPE

LICENÇA Nº- 007/2010

DATA DE PROTOCOLO

DATA DE APROVAÇÃO

DATA DE VENCIMENTO DA LICENÇA

PRPPG

COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO

SOLICITAÇÃO DE LICENÇA PARA USO DE ANIMAIS

1. IDENTIFICAÇÃO DO SOLICITANTE

NOME	RINALDO APARECIDO MOTA
INSTITUIÇÃO DE ORIGEM	UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
CARGO/FUNÇÃO	PROFESSOR ASSOCIADO II
DEPARTAMENTO/UNIDADE ACADÊMICA	DEPARTAMENTO MEDICINA VETERINARIA
ENDEREÇO ELETRÔNICO E TELEFONE	rinaldo.mota@hotmail.com

2. DADOS DA EQUIPE

	NOME	FORMAÇÃO/QUALIFICAÇÃO	FUNÇÃO
RESPONSÁVEL (IS)	**Prof. Dr. Rinaldo Aparecido Mota**	PhD, Pós Doutor em Parasitologia Veterinária, professor associado II de Bacterioses dos Animais Domésticos e Doenças Infecciosas da Reprodução de Ruminantes, Departamento de Medicina Veterinária,Universidade Federal Rural de Pernambuco (UFRPE).	RESPONSÁVEL
COLABORADOR (ES)	**Msc. Flaviana Santos Wanderley**	Médica veterinária, Especialista em Parasitologia, Mestre em Desenvolvimento Sustentável e Doutoranda no Programa de Biociência Animal da Universidade Federal Rural de Pernambuco	COLABORADORA
	Prof. Dr. Wagnner José Nascimento Porto	Médico veterinário, Doutor em Ciência Veterinária, mestre em Ciência Veterinária, prof. Adjunto da Universidade Federal de Alagoas,	COLABORADOR

Informar titulação e/ou cursos realizados.

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