Faculdade de Medicina de São José do Rio Preto
Programa de Pós-Graduação em Ciências da Saúde

Cinara de Cássia Brandão de Mattos

Toxoplasma gondii: prevalência de infecção, diagnóstico laboratorial e genótipos

São José do Rio Preto
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde, da Faculdade de Medicina de São José do Rio Preto, para obtenção do Título de Doutor em Ciências da Saúde, Eixo Temático: Medicina e Ciências Correlatas.

Orientador: Prof. Dr. Luiz Carlos de Mattos

Co-Orientadora: Profa. Dra. Vera Lúcia Pereira Chioccola

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CINARA DE CÁSSIA BRANDÃO DE MATTOS

Toxoplasma gondii: prevalência de infecção, diagnóstico laboratorial e genótipos

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Sumário

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Joana Greco Brandão e Nestor Brandão,

e às minhas filhas Bianca Maria Brandão de Mattos

e Giulia Luiza Brandão de Mattos.

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“Existe, durante a nossa vida, sempre dois caminhos a seguir:
aquele que todo mundo segue, e aquele que a nossa imaginação nos leva a seguir.
O primeiro pode ser o mais seguro, o mais confiável, o menos crítico, o que você encontrará mais amigos...
Mas, você será apenas mais um a caminhar.
O segundo, com certeza vai ser mais difícil, mais solitário, o que você terá maiores críticas...
Mas também, o mais criativo, o mais original possível.
Não importa o que você seja, quem você seja, ou que deseja na vida, a ousadia em ser diferente reflete na sua personalidade, no seu caráter, naquilo que você é.
E é assim que as pessoas lembrarão de você um dia."

(Ayrton Senna)

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Lista de Abreviaturas e Símbolos

µg	Micrograma
µl	Microlitro
µm	Micrômetro
AIDS	Síndrome da Imunodeficiência Adquirida
cnPCR	PCR convencional
DNA	Ácido desoxirribonucléico
dNTP	Desoxinucleotídeo Trifosfatado
DO	Densidade Óptica
EDTA	Ácido Etilenodiamino Tetra Acético
ELISA	Ensaio Imunoenzimático
FAMERP	Faculdade de Medicina de São José do Rio Preto
FUNFARME	Fundação Faculdade Regional de Medicina de São José do Rio Preto
HB	Hospital de Base
IFI	Imunofluorescência Indireta
IgA	Imunoglobulina de classe A
IgE	Imunoglobulina de classe E
IgG	Imunoglobulina de classe G
IgM	Imunoglobulina de classe M
KCl	Cloreto de Potássio
M	Molar
$MgCl_2$	Cloreto de Magnésio
mL	Mililitro
mM	Milimol
NaCl	Cloreto de Sódio
nM	Nanômetro
ODO	Outras Doenças Oculares
PB	Pares de Bases
PBS	Salina Tamponada com Fosfato
PCR	Reação em Cadeia da Polimerase

PCR-RFLP	Polimorfismo do Tamanho do Fragmento de Restrição
pM	Picomolar
qPCR	PCR em Tempo Real
RT	Retinocoroidite Toxoplásmica
SNC	Sistema Nervoso Central
T. gondii	*Toxoplasma gondii*
Tris-HCl	Tris Hidrocloreto
UV	Ultravioleta

Resumo

Toxoplasma gondii: **prevalência de infecção, diagnóstico laboratorial e genótipo**

Introdução: *T. gondii* é um parasito intracelular obrigatório e cosmopolita, cuja infecção de natureza congênita ou adquirida, resulta nas diferentes formas de toxoplasmose. A manifestação clínica desta doença é inespecífica e variável. Seu diagnóstico, essencialmente laboratorial, é direcionado a gestantes, neonatos, portadores de imunodeficiências, portadores de lesão ocular e mesmo indivíduos normais. Métodos sorológicos são frequentemente utilizados na caracterização de anticorpos IgM e IgG anti-*T. gondii*, porém aqueles de natureza molecular favorecem a caracterização gênica das cepas em isolados de amostras biológicas. **Objetivos**: O objetivo geral desta tese foi investigar a infecção por *T. gondii* na região Noroeste Paulista. Seus objetivos específicos foram: **1.** caracterizar a infecção em gestantes, neonatos e indivíduos com doenças oculares; **2.** avaliar a aplicabilidade do método de PCR em amostra de sangue periférico em pacientes com doenças oculares; **3.** identificar as cepas a partir de amostras de sangue periférico. **Casuísitca e Métodos:** dados epidemiológicos e amostras de sangue periférico de pacientes atendidos em Ambulatório de Gestação de Alto Risco e Retinopatia foram coletadas e analisadas quanto à infecção por *T. gondii* por métodos sorológicos (ELISA) e moleculares (cnPCR e qPCR). **Resultados:** entre 574 gestantes com média de idade igual a 27,2±6,5, 62% (345/556) mostraram-se reagentes para IgG e 3,4% (n=19/556), para IgM. Em 87 pares mãe-bebê, 64,4% (n=58) foram

reagentes para anti-*T. gondii* e 2,3% (n=2), para IgM; 92,9% (52/56) apresentaram IgG com avidez maior ou igual a 30%. Dentre 184 pacientes com diferentes doenças oculares, 26% (n=49) apresentaram toxoplasmose ocular, todos reagentes para IgG. O método PCR (cnPCR e qPCR) aplicado à análise do sangue periférico apresentou sensibilidade e especificidade iguais a 40,8% e 100%, respectivamente. Cinco pacientes com toxoplasmose ocular mostraram-se infectados pela cepa toxoDB#65. **Conclusão:** Os resultados demonstram que a prevalência de infecção por *T. gondii* em gestantes, neonatos e pacientes com doenças oculares é elevada na região Noroeste paulista, e permitem estimar os índices de infecção congênita na região. Além de descrever pela primeira vez a toxoplasmose ocular na região, este estudo reforça a importância dos métodos cnPCR e qPCR na caracterização laboratorial da infecção e da cepas toxoDB #65 a partir de amostras de sangue periférico de pacientes com infecção crônica.

Palavras-chave: *Toxoplasma gondii*, toxoplasmose congênita, toxoplasmose ocular, reação em cadeia da polimerase, genotipagem

Abstract

Toxoplasma gondii: prevalence of infection, laboratory diagnosis and genotype

Introduction: *T. gondii* is an obligate intracellular parasite and cosmopolitan, whose infection congenital or acquired, results in different forms of toxoplasmosis. The clinical manifestations of this disease are nonspecific and variable. Its diagnosis essentially laboratory is aimed at pregnant women, neonates, patients with immunodeficiencies, patients with eye injury and even normal individuals. Serological methods are often used in the characterization of IgM and IgG anti-*T.gondii,* but those of nature of molecular favoring genetic characterization of the strains isolated from biological samples. **Objectives:** The overall objective of this thesis was to investigate the infection by *T.gondii* in the northwestern region of São Paulo state. Its specific objectives were: 1. to characterize infection in pregnant women, newborns and people with eye diseases 2. to evaluate the applicability of the method of PCR inperipheral blood sample in patients with eye diseases 3. to identify the strains from peripheral blood samples. **Casuisitc and Methods:** epidemiological data and samples of peripheral blood from patients treated in Outpatient Clinic of High Risk Pregnancy and Retinopathy were collected and analyzed for infection by *T. gondii* using serological methods (ELISA) and molecular (cnPCR and qPCR). **Results:** among 574 women with mean age equal to 27.2 ± 6.5, 62% (345/556) showed positive for IgG and 3.4% (n = 19/556) for IgM. In 87 mother-newborn pairs, 64.4% (n = 58) were reactive for anti-*T. gondii* and 2.3% (n = 2),

IgM, 92.9% (52/56) had IgG avidity greater than or equal to 30%. Among 184 patients with different eye diseases, 26% (n = 49) had ocular toxoplasmosis, all reagents for IgG. The PCR (qPCR and cnPCR) applied to the analysis of peripheral blood showed sensitivity and specificity equal to 40.8% and 100%, respectively. Five patients with ocular toxoplasmosis were shown to be infected by the strain toxoDB # 65. **Conclusion**: The results show that the prevalence of infection with *T. gondii* in pregnant women, neonates and patients with eye diseases is high in the northwertern region of São Paulo state, and to estimate the rates of congenital infection in the region. In addition to describing the first time the ocular toxoplasmosis in the region, this study reinforces the importance of cnPCR and qPCR methods for the characterization of infection and laboratory strains toxoDB # 65 from peripheral blood samples of patients with chronic infection.

Keywords: *Toxoplasma gondii*, congenital toxoplasmosis, ocular toxoplasmosis, polymerase chain reaction, genotyping.

Introdução

Histórico

O gênero *Toxoplasma* foi descrito pela primeira vez no corpo do roedor *Ctnenodactyls gundii* por Nicolle e Manceaux, em 1908, na Tunísia.[1-3] Neste mesmo ano, Alfonso Splendore, identificou a presença deste este parasito em tecidos de coelhos, no Brasil.[3-5] Desde então o binômio *Toxoplasma gondii*, proposto por Nicolle e Manceaux, é usado para descrever este protozoário com morfologia em arco.

A infecção por *T. gondii* adquiriu interesse econômico e veterinário a partir da observação de casos de abortos sucessivos em rebanhos de ovinos na Nova Zelândia[6] e no Reino Unido.[7] Os primeiros casos de toxoplasmose em animais domésticos foram relatados em cães, na Itália,[8] e em Cuba, França, Alemanha, Índia, Iraque, Tunísia, União Soviética e Estados Unidos.[9] Curiosamente, a relação entre *T. gondii* e gatos foi descrita pela primeira vez em Nova York[10] e a elucidação de seu ciclo biológico se deu na década de 70.[5]

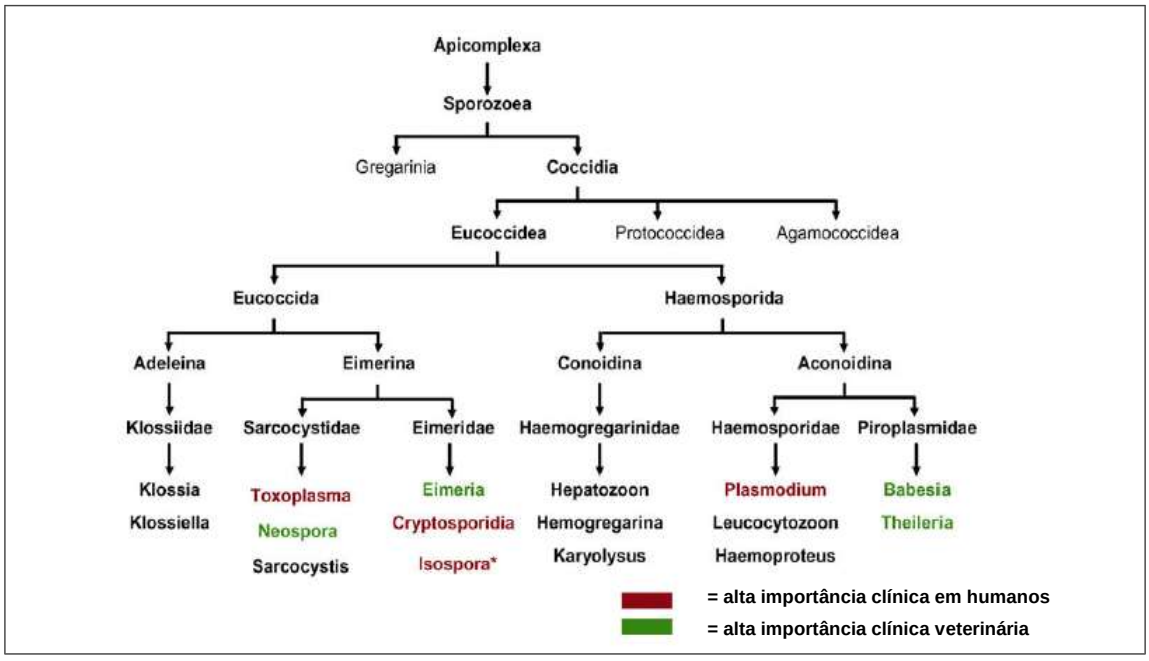
A descrição dos primeiros casos de lesões oculares relacionadas à infecção por *T. gondii* na, então, Tchekoslováquia[11,12] e no Brasil,[12] seguida de relatos de encefalomielite com evidências de transmissão congênita em humanos,[13,14] colocou a toxoplasmose em posição de destaque entre as doenças parasitárias. Além disso, o subseqüente desenvolvimento do primeiro método sorológico para o diagnóstico da infecção por *T. gondii* em humanos e

animais[15] favoreceu a sua investigação. Os incontáveis relatos apresentados nas décadas seguintes confirmaram a ampla distribuição da infecção por este parasito em todas as populações humanas ao redor do mundo e consolidou a toxoplasmose como doença de grande interesse médico, epidemiológico e veterinário.[16-18]

O Parasito

T. gondii é um parasito intracelular obrigatório que pertence ao Filo *Apicomplexa* (caracterizado pela presença de um pólo apical), Classe *Sporozoa*, Sub-classe *Coccidea*, Ordem *Eucicccidea*, Família *Sarcocystidae*, Sub-família *Toxoplasmatinae*, Gênero *Toxoplasma*, Espécie *Toxoplasma gondii,*[19,20] como ilustrado no quadro 1.

Quadro 1: Classificação Sistemática do *Toxoplasma gondii.* (Adapatado de Beck et al, 2009)[20]



Em função de sua classificação biológica, *T. gondii* apresenta parentesco evolutivo com outros microrganismos de grande interesse médico e epidemiológico como *Criptosporidium sp*, *Plasmodium sp* e *Babesia sp.*[20,21] A morfologia de *T. gondii* é múltipla e depende do habitat e do estado evolutivo em que o parasito se encontra. Possui três estágios infectantes denominados taquizoíto, bradizoíto e oocisto.[22]

O taquizoíto, também chamado de trofozoíto, se origina a partir do bradizoíto, tem forma de meia lua e infecta qualquer célula nucleada. É a única forma que se prolifera exponencialmente por um processo complexo denominado endodiogenia, onde cada taquizoíto cresce e gera duas células filhas, até formarem rosetas (figura 1).[3,22,23]

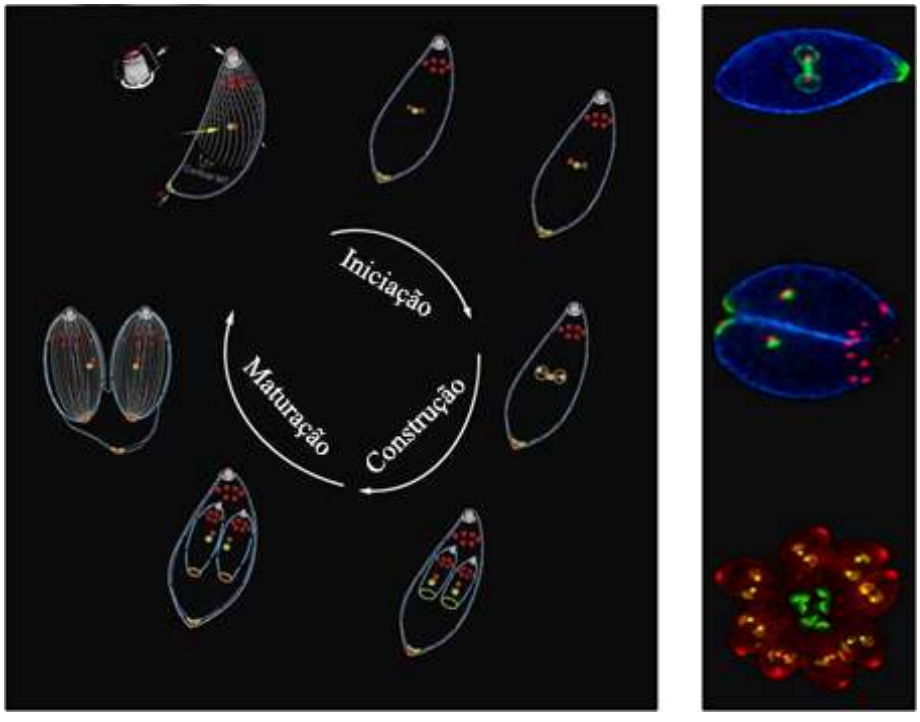


Figura 1. Representação esquemática da reprodução por endodiogenia realizada pelo taquizoíto do *T. gondii*. (Adaptada de Ke Hu, 2008).[24]

O bradizoíto, também chamado cisto tecidual, caracteriza o estágio de encistamento sendo altamente resistente à digestão por suco gástrico e ação dos quimioterápicos.[5,25,26] Esta forma desempenha importante papel no ciclo evolutivo do *T. gondii* pois se mantém no alimento preferencial dos hospedeiros carnívoros.[27]

Os oocistos contendo esporozoítos constituem a forma mais resistente do *T. gondii.* Quando presentes no intestino de felídeos são eliminados imaturos junto às fezes. Os oocistos esporulados contêm dois esporocistos que abrigam em seu interior quatro esporozoítos. Estas formas, ao entrarem em contato com os hospedeiros se diferenciam em bradizoítos.[22,28] As formas de bradizoíto e oocisto estão ilustradas na figura 2.

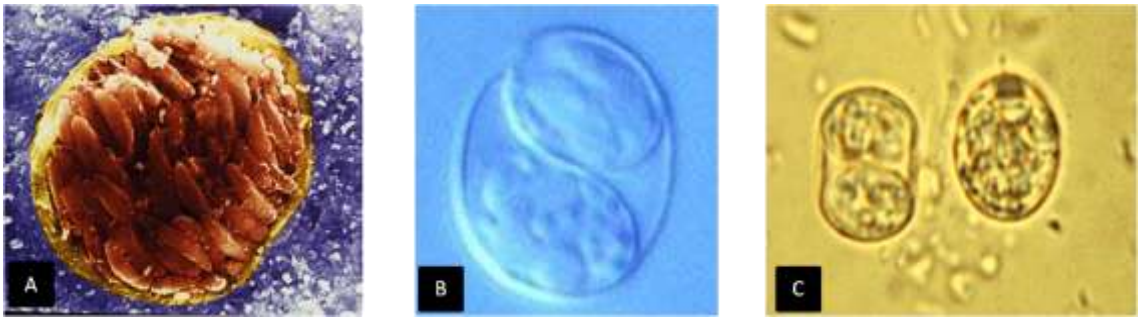


Figura 2. Em *A* cisto em cérebro de rato contendo bradizoítos; em *B* e *C* esporozoíto contendo oocisto esporulado e não esporulado em intestino de felino. *Fonte: A: foto de David Fegurson - http://sibleylab.wustl.edu/images/Bradyzoites.jpg.*[29] *B: foto de Steve J. Upton - http://www.k-state.edu/parasitology/625tutorials/Oocysts01.html*[30] *C: sem autoria - http://cal.vet.upenn.edu/projects/paralab/labs/lab10.htm#10*[31]

O ciclo evolutivo de *T. gondii* é heteroxeno, com os felinos atuando como hospedeiro definitivo, uma vez que nestes animais ocorrem as fases de reprodução sexuada e assexuada. Dentre os hospedeiros intermediários, nos quais ocorre apenas a reprodução assexuada, estão os animais de sangue quente como diversos mamíferos, aves, roedores e o homem.[32-34]

Infecção

A infecção por *T. gondii* pode ser congênita ou adquirida. A adquirida se dá, em geral, pela ingestão de alimentos ou água contaminados com oocistos e na congênita, o taquizoíto transpõe a barreira placentária e infecta o feto. Na maioria das vezes a infecção por este parasito é assintomática em indivíduos imunocompetentes.[26,35,36] Por outro lado, pode ser grave em pacientes imunodeprimidos, transplantados e neonatos.[26,37] A figura 4 ilustra as rotas de infecção por *T. gondii.*

Quando a infecção primária ocorre durante a gestação o feto, ao ser atingido, pode desenvolver malformações neonatais e complicações oculares. As conseqüências da infecção materno-fetal, portanto congênita, dependerão do grau de exposição do feto aos parasitas, da virulência da cepa infectante e do período gestacional em que ocorrer.[38,39] Os sinais clássicos da toxoplasmose congênita são hidrocefalia, retinocoroidite, calcificação cerebral e retardo mental.[5,40-43]

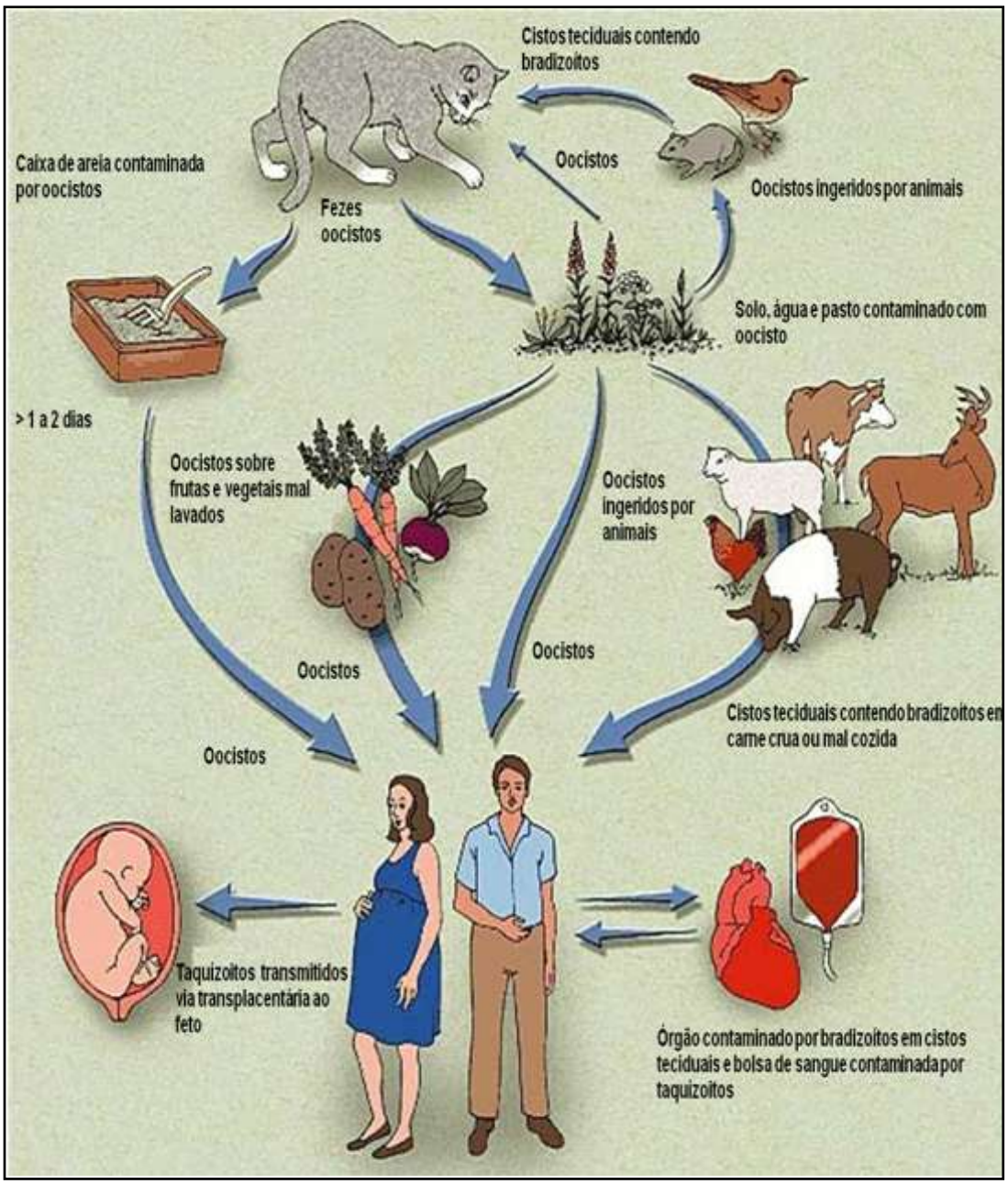


Figura 3. Vias de transmissão de *Toxoplasma gondii*. (Adaptada de Lynfield e Guerina, 1997)[44]

O contato com o solo, com carne industrializada, bem como os hábitos de higiene também contribuem para a aquisição da infecção por *T. gondii.*[45-50] Recentes relatos de surto de toxoplasmose na Índia e no Brasil reforçaram o papel da água contaminada como fonte de disseminação e infecção por este parasito.[51,52]

As recentes observações de que esquizofrenia, depressão, tentativas de suicídio e distúrbio bipolar estão associadas à infecção por *T. gondii*,[53-58] além de reforçar a importância deste parasito, oferecem novas oportunidades para a investigação de seu papel na patogênese de doenças neurológicas sem aparente relação com microrganismos.

A doença

A manifestação clínica da toxoplasmose é inespecífica e variável e as três formas comuns são a cerebral, a congênita e a ocular.

Em pacientes infectados com o HIV a mais freqüente manifestação da toxoplasmose é a encefalite toxoplásmica, a qual representa a principal causa de morbidade e mortalidade em doentes de AIDS; apresenta variabilidade de sinais como cefaléia, demência, ataxia, letargia, anomalias sensoriais e convulsões, necroses em ambos os hemisférios cerebrais, podendo se tornar sistêmica.[37,59-62] Estas alterações são semelhantes àquelas observadas em outras infecções oportunistas que acometem pacientes imunocomprometidos.[63]

A toxoplasmose congênita é caracterizada pela transmissão transplacentária ao feto, quando a infecção ocorre durante a gestação.[41,43,64] Há relatos de que 70% dos recém-nascidos infectados durante a gestação não apresentam sintomas ao nascimento[65] e 30% não demonstram evidências sorológicas da infecção congênita.[66,67]

As conseqüências da infecção materno-fetal dependerão do grau de exposição do feto aos parasitas, da virulência da cepa e do período gestacional em que houve a infecção causando malformações neonatais e complicações oculares. Os sinais clássicos da toxoplasmose congênita são similares a alguns dos sinais da toxoplasmose ocular e da toxoplasmose cerebral. Dentre eles estão a hidrocefalia, a coriorretinite, as calcificações cerebrais, a macrocefalia, a hidrocefalia, a cicatriz ocular, a perda de visão, a perda de audição e o retardamento mental.[40,63,66,68]

A toxoplasmose ocular é uma das mais importantes causas de uveíte posterior[37,69,70] e pode ocorrer tanto na fase fetal bem como após o nascimento (infância ou fase adulta).[71] Em todos os casos as manifestações clínicas podem ser precoces ou tardias com diferentes graus de comprometimento ocular.[72,73] O *T. gondii* forma cistos latentes diretamente na retina, que ao serem reativados originam a retinocoroidite. Embora muitos episódios de retinocoroidite possam ser assintomáticos, tem se observado que os mesmos podem resultar em perda visual, dor e fotofobia, isoladamente ou em conjunto.[74]

As lesões retinianas próprias da toxoplasmose ocular são focais, brancas e de pequena dimensão, mas com consistente reação inflamatória que se assemelha a uma imagem “nublada”. Resultam tanto do dano causado pelo parasito bem como da resposta imune do hospedeiro contra este protozoário. (64, 75)

Epidemiologia da toxoplasmose

A prevalência de infecção por *T. gondii* varia de acordo com o país e depende de fatores climáticos,[47] hábitos alimentares, higiene e idade dos indivíduos afetados.[34,46,76] A figura 4 ilustra os índices globais de infecção gestacional e congênita e a figura 5 ilustra os índices da toxoplasmose congênita no Brasil.[77] Os baixos índices observados em algumas áreas podem ser resultantes da escassez de investigação e/ou pela ausência de programas públicos de triagem materno-fetal e neonatal.[77-80]

Estudos epidemiológicos realizados em diferentes estados brasileiros nas últimas décadas revelaram grande variabilidade nos índices de infecção. Os estudos realizados em populações urbanas dos estados do Ceará e da Bahia verificaram que a prevalência de toxoplasmose varia de 22,8% a 71,5%.[81-83] Os índices de prevalência desta infecção em gestantes das cidades de São Paulo e Porto Alegre variaram entre 54,3% e 67,3 %, respectivamente.[84,85] Nas cidades de Brasília e Recife, os índices foram de 7,3% e 77,5% respectivamente.[86,87]

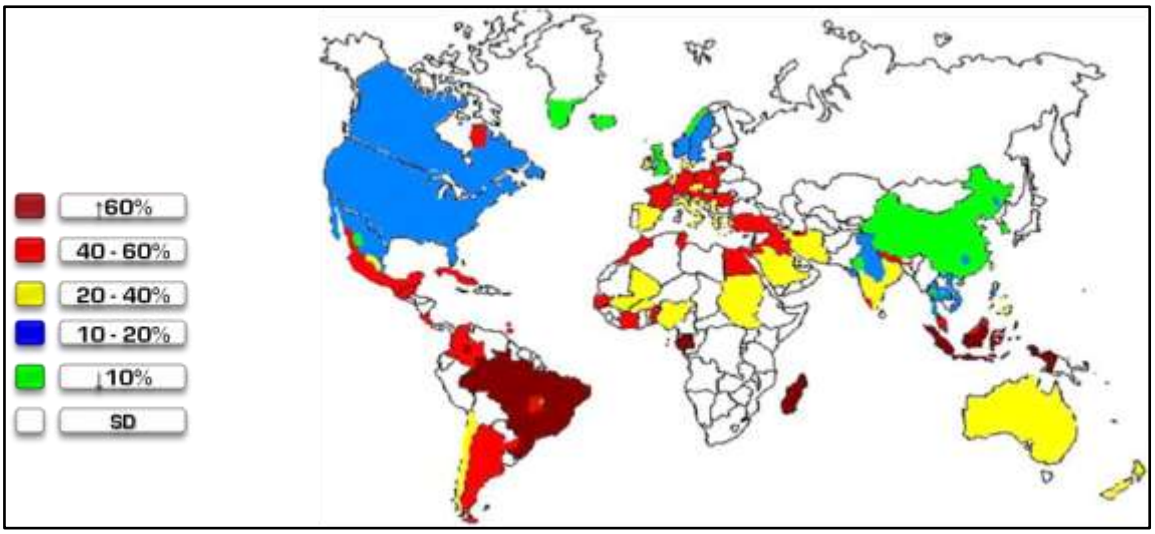


Figura 4. Prevalência mundial de infecção gestacional e congênita de *T. gondii*. (Adaptada de Pappas et al, 2009)[18] (SD = sem dados)

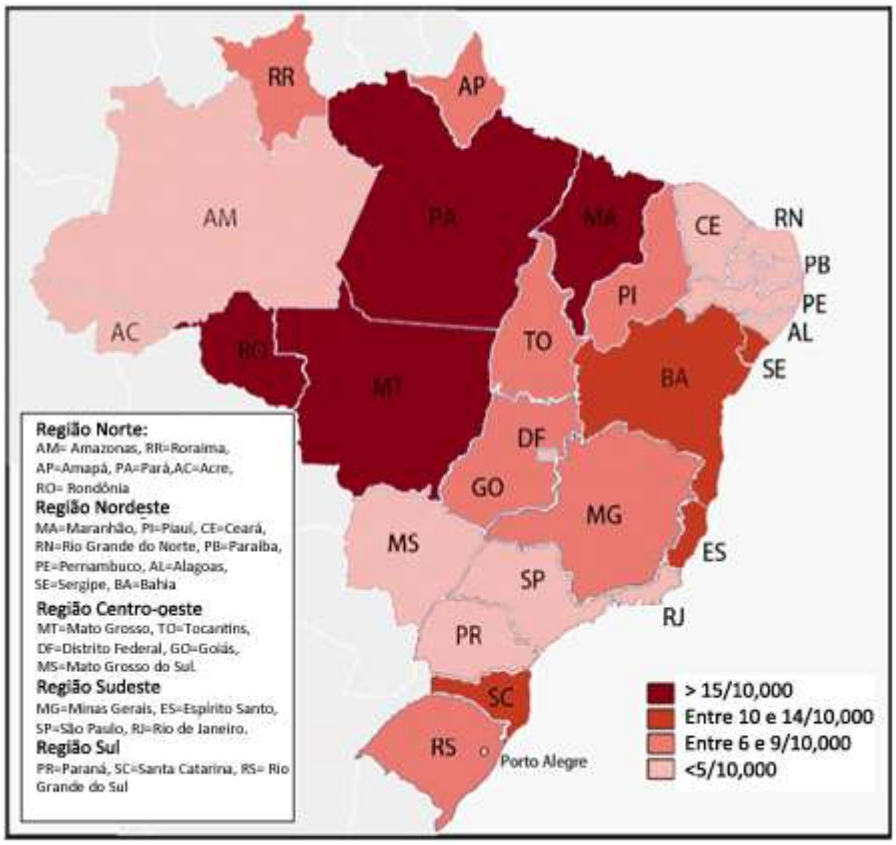


Figura 5. Distribuição nos Estados Brasileiros do número de casos de toxoplasmose congênita para cada 10.000 nascidos vivos, submetidos à triagem neonatal em papel filtro pelo método Luminex – 1995-2004. (Adaptada de Camargo Neto et al, 2010)[77]

A prevalência de retinocoroidite toxoplásmica é bastante elevada em adolescentes e adultos oriundos de diferentes regiões brasileiras e variam de 2% na região sudeste a 25% na região sul.[88-91] Glasner e colaboradores (1992) observaram no Rio Grande do Sul, prevalência de toxoplasmose ocular igual a 21,3 % em indivíduos maiores de treze anos de idade.[88] Silveira e *cols* constataram que um quinto dos indivíduos inicialmente soronegativos, se tornaram soropositivos após sete anos e 10% deles desenvolveram toxoplasmose ocular.[90]

No Estado de Pernambuco, foi observado que 56,2% dos casos de uveite posterior eram devidas à presença do *T. gondii*[92] e estudos conduzidos em outras regiões brasileiras relataram prevalência de toxoplasmose ocular menor que 4%.[93,94] Estes dados indicam que a toxoplasmose ocular é comum no Brasil e, portanto, justificam a investigação dos diferentes aspectos desta doença em nossa região. A tabela 1 ilustra os dados de estudos realizados no Brasil.

Tabela 1. Índices de infecção por *T. gondii* em diferentes populações brasileiras.

Cidade	Estado	Índices	N	População	Ref
Fortaleza	CE	71,5%	997	Diversos	Rey & Ramalho, 1999[83]
Londrina	PR	67%	1164	Gestante	Reiche et al, 2000[99]
Cuiabá	MT	70,7%	205	Puérpera	Leão et al, 2004[95]
Goiânia	GO	65,8%	2563	Mulheres	Avelino et al, 2004[100]
Alto Uruguai	RS	74,5%	1583	Gestante	Spalding et al, 2005[46]
Belo Horizonte	MG	61,2%	420	Puérpera	Carellos et al, 2008[98]
Recife	PE	77,5%	503	Gestante	Porto et al, 2008[87]
Porto Alegre	RS	67,3%	2513	Gestante	Lago et al, 2009[85]
São Luís	MA	66,4%	3037	Gestante	Costa et al, 2010[96]
Salvador	BA	56,4%	2229	Gestante	Rebouças et al, 2011[97]
S José do Rio Preto	SP	64,1%	1006	Gestante	Rodrigues et al, 2011[101]
Araraquara	SP	58%	233	Gestante	Isabel et al, 2007[102]
Botucatu	SP	60,0%	913	Gestante	Olbrich Neto et al, 2004[103]

Diagnóstico laboratorial

A investigação laboratorial da infecção por *T. gondii* em humanos tem sido direcionada a grupos de risco tais como neonatos, portadores de imunodeficiências, pacientes transplantados, portadores de lesão ocular e mesmo indivíduos normais. Devido à ausência e a diversidade de sinais clínicos o diagnóstico da toxoplasmose é essencialmente laboratorial.[26]

A maioria dos métodos disponíveis são sorológicos, produzidos *in-house* ou disponibilizados em *kits* comerciais, embora métodos moleculares tenham sido introduzidos nos últimos anos.

Métodos sorológicos como imunofluorescência indireta (IFI), ensaio imunoenzimático (ELISA), quimioluminescência, fluorescência (ELFA) e

imunoblot identificam anticorpos das classes IgG e IgM específicos para *T. gondii*, bem como permitem analisar a avidez daqueles de classe IgG.[104-108] Além disso, são úteis na caracterização de anticorpos de classe IgA os quais auxiliam o diagnóstico neonatal.[109,110]

Além destes, o teste para verificar a avidez de anticorpos IgG anti-*T. gondii* também é muito utilizado para "datar" a época de infecção. A identificação de anticorpos IgA anti-*T. gondii* também auxilia na caracterização da infecção em pacientes com AIDS, neonatos e portadores de toxoplasmose ocular.[111-117]

A resposta imune ao *T. gondii*, mediada por anticorpos da classe IgE é de curta duração e por isso, a presença desses anticorpos pode ser considerada como mais um indicador de infecção recente.[107,108,118] Contudo, avaliações na concentração de IgE não são comumente utilizadas.[119,120]

Os avanços tecnológicos que levaram à identificação e seqüenciamento de partes do genoma de *T. gondii* possibilitaram a introdução de métodos moleculares no diagnóstico de infecção bem como na toxoplasmose de importância clínica como as formas cerebral e congênita.[121-126]

O diagnóstico molecular se dá pela identificação de seqüências gênicas do *T. gondii* com o uso da reação em cadeia da polimerase convencional (cnPCR) ou em tempo real (qPCR). Estes métodos oferecem maior rapidez, sensibilidade e especificidade na identificação deste parasito em diversos materiais biológicos

ou amostras clínicas tais como sangue, urina, líquido amniótico, líquido cefalorraquiano, humor aquoso, humor vítreo e alimentos.[127-134] O gene *B1* de *T. gondii* tem sido amplamente utilizado como marcador, embora outros genes também serem amplificados para efeito de diagnóstico molecular da infecção por *T. gondii.*[131,135,136]

O uso dos métodos moleculares vem auxiliando na caracterização clínica da infecção especialmente em pacientes imunocomprometidos, submetidos à transplante de órgãos, portadores do vírus HIV, gestantes de alto risco e neonatos.[106,137-139]

Diversidade gênica do *Toxoplasma gondii*

A caracterização gênica da população de *T. gondii* tem como papel principal a compreensão dos padrões epidemiológicos, as diferenças nas manifestações clínicas da doença em sí e também, no suporte para o desenvolvimento de novas estratégias para vacinação, tratamento e diagnóstico.[140]

As primeiras análises genéticas de populações permitiram o agrupamento das cepas de *T. gondii* em três linhagens designadas tipo I, II e III.[122,141,142,] Estes estudos foram realizados em animais experimentais e concluíram que a virulência e a evolução da infecção variam de acordo com cepa de *T. gondii* utilizada.

Alguns estudos propuseram a correlação entre a variabilidade de cepas com a gravidade da doença humana[143,144] e com a velocidade e capacidade de migração tecidual e de transposição de membranas biológicas.[145,146]

Gradativamente foram realizados estudos em cepas provenientes de diferentes regiões do mundo, como Estados Unidos, Europa, Ásia e África. O uso de diferentes marcadores moleculares mostraram que a maioria das cepas apresentavam características gênicas do tipo II.[141,147-150] Aqueles realizados com cepas isoladas na América do Sul mostraram que as mesmas são mais virulentas e com predominância dos tipos I ou III.[144,151,152]

No Brasil, a maioria dos estudos de caracterização gênica do *T. gondii* foi realizado em animais.[153-157] Outros estudos analisaram isolados provenientes de amostras biológicas humanas na tentativa de se esclarecer a relação entre as diferentes cepas e a infecção humana.[156,158,159]

Estudos recentes demonstram a diversidade gênica das cepas da América Latina em comparação às da América do Norte e da Europa, assim como as da América do Sul e da América do Norte.[153,160,161] Novos modelos de segregação foram propostos. As figuras 6 e 7 ilustram a estrutura da linhagem populacional do *T. gondii* e a hipótese sobre a origem das linhagens I, II e III.

Diante da diversidade típica das cepas comuns na América do Sul bem como de seu difícil isolamento, a aplicabilidade de *kits* comerciais produzidos na

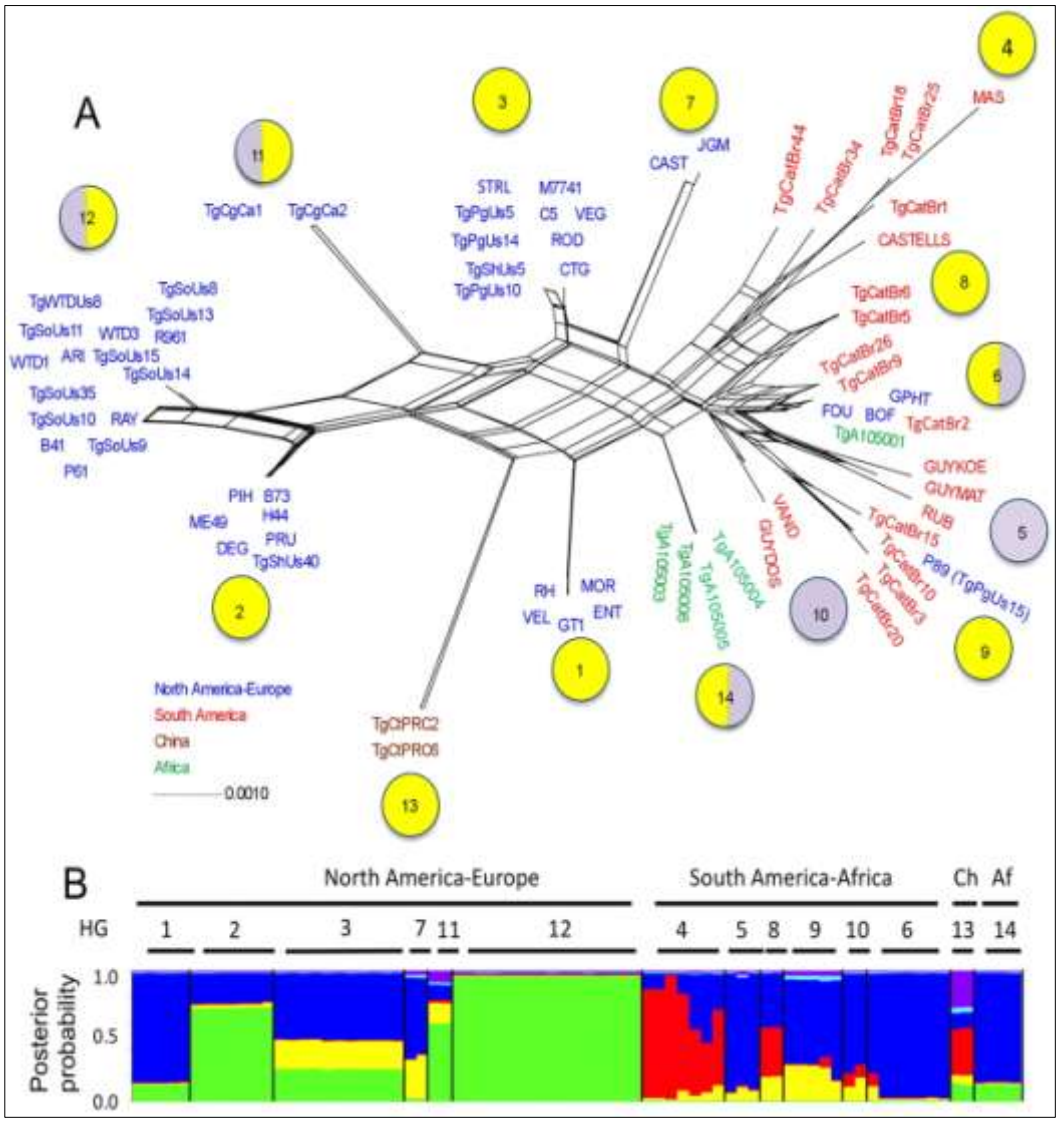


Figura 6. Análise da estrutura populacional de *T. gondii*. Em *A* rede desenvolvida a partir da análise da sequência do íntron 5 de 74 cepas de *T. gondii*, evidenciando a separação geográfica entre as cepas Norte Americanas-Européias (em azul) e Sul Americanas (vermelho), os círculos indicam o cromossomo Chrla monomórfico (amarelo) e divergentes (azuis). Em *B* análise de ancestralidade da população de *T. gondii* a partir de seqüenciamento dos 74 isolados. (Haplogrupos = HG, China =Ch, África = Af). (Adaptada de Khan et al, 2011)[153]

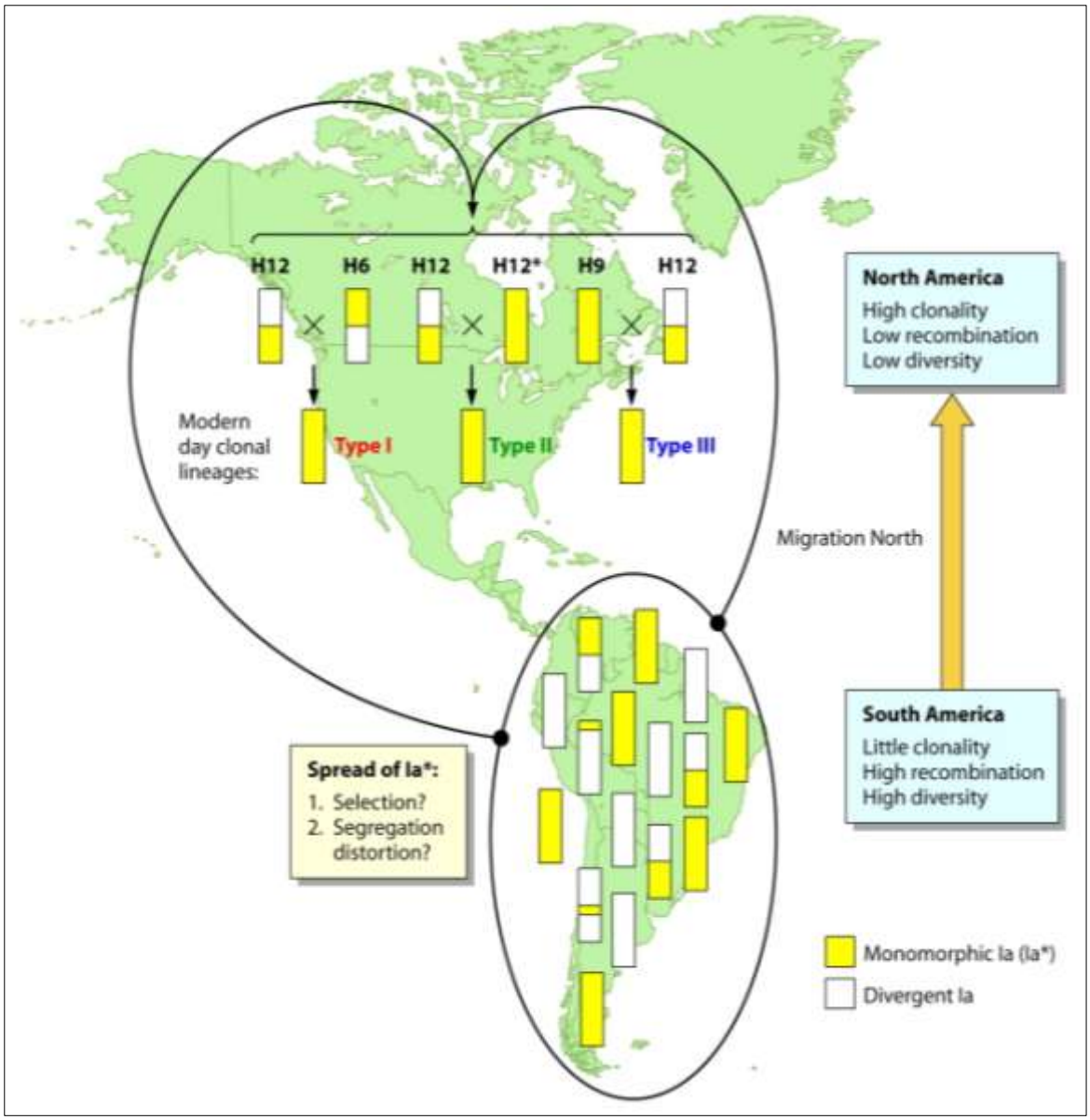


Figura 7. Modelo proposto por Walzer & Boyle, sobre a possível migração das cepas monomórficas ChrIa da América do Sul para a América do Norte, sugerindo que essas poderiam ter originado as cepas das linhagens clonais I, II e III norte-americancas e européias, considerando os fluxos de migração aviária e humana. (Adaptada de Walzer & Boile, 2012)[161]

Europa e na América do Norte na caracterização da infecção por *T. gondii* em pacientes brasileiros, pode ser questionada. É possível que antígenos presentes nos *kits* comerciais e extraídos de cepas distintas daquelas comuns no Brasil, não reajam de forma adequada com anticorpos específicos para aquelas predominantes em nosso país. Estas particularidades inerentes à diversidade parasitária e imune devem ser consideradas e discutidas no diagnóstico sorológico da infecção por *T. gondii*. Estes aspectos foram considerados nos artigos que compõe essa tese.

O panorama atual da infecção por *T. gondii* no Brasil demonstra que persistem mais perguntas que respostas, especialmente em uma região em que são escassos estudos sobre as diferentes formas de manifestação da toxoplasmose e de seu agente etiológico. O interesse em contribuir para a construção de conhecimentos que possam auxiliar na compreensão de vários aspectos da epidemiologia, do diagnóstico e da caracterização do *T. gondii* justifica o investimento na realização desta tese.

Objetivos

Este estudo foi realizado pelo Laboratório de Imunogenética do Departamento de Biologia Molecular da Faculdade de Medicina de São José do Rio Preto em cooperação com o Ambulatório de Gestação de Alto Risco e Medicina Fetal e o Ambulatório de Retinopatia do Hospital de Base da Fundação Faculdade Regional de Medicina de São José do Rio Preto, e com o Laboratório de Biologia Molecular de Parasitos do Instituto Adolfo Lutz e teve como objetivos:

1. Caracterizar a infecção por *T. gondii* em gestantes, neonatos e indivíduos com doenças oculares;
2. Avaliar a aplicabilidade do método de PCR em amostra de sangue periférico em pacientes com doenças oculares;
3. Caracterizar cepas de *T. gondii* a partir de amostras de sangue periférico.

Resultados

Os resultados serão apresentados em formato de artigos publicados em revistas indexadas, formatados conforme as normas da revista selecionada, bem como em resumos apresentados em congressos ou reuniões científicas.

Os artigos I, II e III estão relacionados ao objetivo 1. O artigo III expressa o objetivo 2 e o artigo IV é relacionado ao objetivo 3.

Os resumos I e II estão relacionados aos 3 objetivos. Ambos, artigos e resumos que compõe essa tese, estão listados abaixo e foram organizados em sequência lógica para apresentação do assunto da tese em si, independente da data de publicação.

Artigos:

I. Márcia Aparecida dos Santos Gonçalves; **Cinara de Cássia Brandão de Mattos**; Lígia Cosentino Junqueira Franco Spegiorin; Denise Cristina Mós Vaz Oliani; Antonio Hélio Oliani; Luiz Carlos de Mattos. Seropositivity rates for toxoplasmosis, rubella, syphilis, cytomegalovirus, hepatitis and HIV among pregnant women receiving care at a public health service, São Paulo state, Brazil. Brazilian Journal of Infectious Disease 2010;14(6):601-605.

doi: 10.1590/S1413-86702010000600009 – JCR 2010: 0.811

Co-orientação de Iniciação Científica de Bolsista BIC-FAMERP – Curso Graduação em Enfermagem FAMERP e Trabalho de Conclusão de Curso de Enfermagem FAMERP.

Este artigo foi recomendado como leitura pelo Europrise Science Update.

II. **Cinara de Cássia Brandão de Mattos;** Lígia Cosentino Junqueira Franco Spegiorin; Cristina da Silva Meira; Thaís da Costa Silva; Ana Iara da Costa Ferreira; Fabiana Nakashima; Vera Lúcia Pereira-Chioccola; Luiz Carlos de Mattos. Anti-*Toxoplasma gondii* antibodies in pregnant women and their newborn infants in the region of São José do Rio Preto, São Paulo, Brazil. *São Paulo Medical Journal/Evidence for Health Care 2011;129(4):261-266*

doi: 10.1590/S1516-31802011000400010 – JCR 2010: 0.577

III. **Cinara de Cássia Brandão de Mattos**; Cristina da Silva Meira; Ana Iara da Costa Ferreira; Fábio Batista Frederico; Roberto Mitsuyoshi Hiramoto; Gildásio Castello de Almeida Jr; Luiz Carlos de Mattos; Vera Lúcia Pereira-Chioccola. Contribution of laboratory methods in diagnosing clinically suspected ocular toxoplasmosis in Brazilian patients. *Diagnostic Microbiology and Infectious Disease 2011;70(3):362-366*

doi: 10.1016/j.diagmicrobio.2011.02.002 – JCR 2010: 2.426

Este artigo foi recomendado como leitura pelo Programa de Educação Continuada da Sociedade Brasileira de Infectologia

IV. Isabelle Martins Ribeiro Ferreira; José Ernesto Vidal; **Cinara de Cássia Brandão de Mattos**; Luiz Carlos de Mattos; Daofeng Qu; Chunlei Su; Vera Lúcia Pereira-Chioccola. *Toxoplasma gondii* isolates: Multilocus RFLP–PCR genotyping from human patients in Sao Paulo State, Brazil identified distinct genotypes. *Experimental Parasitology 2011; 129(2):190–195*

doi:10.1016/j.exppara.2011.06.002 – JCR 2010: 1.869

Resumos:

I. **Cinara de Cássia Brandão de Mattos**; Lílian Beani; Lígia Cosentino Junqueira Franco Spegiorin; Sabrina Baring; Isabelle Martins Ribeiro Ferreira; Vera Lúcia Pereira-Chioccola; Luiz Carlos de Mattos. Brazilian case report: Postnatal diagnosis of gestational and congenital toxoplasmosis of a newborn and his mother due to an atypical *Toxoplasma gondii* strain. *In:* ICOPA XII - International Congress of Parasitology, 2010, Melbourne, Austrália. ICOPA XII Conference Abstracts, 2010. p.836-836.

II. **Cinara de Cássia Brandão de Mattos**; Fábio Batista Frederico; Ana Iara da Costa Ferreira; Cássia Rúbia Bernardo; Isabelle Martins Ribeiro Ferreira; Vera Lúcia Pereira-Chioccola; Luiz Carlos de Mattos. Epidemilogical data from 5 patients with toxoplasmic retinochoroiditis and infected by toxodb#65 strain – case report. *In:* 11th Toxomeeting International Congress on Toxoplasmosis, 2011, Ottawa, Canadá. 11th International Congress on Toxoplasmosis - Abstract Book, 2011. p.A13

BRIEF COMMUNICATION

Seropositivity rates for toxoplasmosis, rubella, syphilis, cytomegalovirus, hepatitis and HIV among pregnant women receiving care at a Public Health Service, São Paulo State, Brazil

ABSTRACT

Infectious and parasitic diseases affecting women during their reproductive age may result in vertical transmission. The aim of this study was to determine the seroprevalence for TORSCH among pregnant women receiving care at a university hospital. Records of 574 pregnant women who received medical attention from January 2006 to December 2007 were assessed. The mean age was 27.2 ± 6.5 years ranging from 13 to 44. The results of the immunodiagnostic tests were: 62.0% (345/556) for IgG and 3.4% (19/556) for IgM anti-*T. gondii*; 93.1% (433/465) for IgG and 0.6% (3/465) for IgM anti-rubella; 0.9% (5/561) for VDRL; 1.8% (10/554) for HBsAg; 0.7% (4/545) for anti-HCV and 2.1% (11/531) for HIV. In conclusion, the results of immunodiagnostic tests for the TORSCH panel among pregnant women attending a perinatal service of a university hospital are in agreement with those reported by previous studies and by governmental sources.

Keywords: toxoplasmosis; rubella; infectious disease transmission; vertical; pregnancy complications; infectious; hepatitis.

[Braz J Infect Dis 2010;14(6):601-605]

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We declare no conflict of interest.

INTRODUCTION

Infectious and parasitic diseases affecting pregnant women are a potential public health problem, as they may cause congenital abnormalities or be vertically transmitted.[1,2] The prevalence rates of these diseases are frequently associated with the pregnancy period. The risks are associated with their higher rates in less well educated populations.[3] Knowledge of the prevalence and incidence of these infectious-parasitic diseases in pregnant women is of great importance in the planning of maternal-fetal health programs strategies, screening and treatment of these diseases.[4]

Serological tests to diagnose infections of the TORSCH (Toxoplasmosis, Rubella, Syphilis, Cytomegalovirus, Hepatitis B and C, and HIV) are mandatory, as they pose a potential life threatening risk for the pregnant woman and their fetus, in addition to the newborn baby after delivery.[3,4] With the exception of infections by cytomegalovirus, the other diseases are routinely investigated in all pregnant women attending the high-risk pregnancy and fetal medicine unit of Hospital de Base (FUNFARME) in São José do Rio Preto, a reference center for the northwest region of São Paulo State.

Few studies have assessed the prevalence of the TORSCH infections among pregnant women in Brazil. In the north of Paraná State, rates of 67.0% (IgG-toxo), 1.8% (IgM-toxo), 89.0% (IgG anti-rubella), 1.2% (IgM anti-rubella), 1.6% (syphilis), 0.8% (hepatitis B), 0.8% (hepatitis C) and 0.6% (HIV) were reported in one study.[3] In the state of Mato Grosso do Sul, the percentages were 0.4% for IgM-toxo, 0.03% for IgM anti-rubella, 0.8% for syphilis, 0.3% for hepatitis B, 0.1% for hepatitis C and 0.2% for HIV.[4]

Recent studies with pregnant women in the northwest region of São Paulo State reported IgG-toxo antibodies in 64.1%[5] and 1.03% of HIV seropositivity,[6] but did not detail the results of other TORSCH infections. The aim of this study was to assess the prevalence of infections of the TORSCH group among pregnant women attending a university hospital.

MATERIAL AND METHOD

Ethical considerations

This study was approved by the Research Ethics Commit- tee of the São José do Rio Preto Medical School (FAMERP – protocol 168/2007). The need for a written consent of patients was waivered as all the data were retrospectively collected from the patients' hospital records.

Selection of patients' records

A total of 624 hospital records of pregnant women who had attended the high-risk pregnancy and fetal medicine unit of Hospital de Base (FUNFARME) in São José do Rio Preto from January 2006 to December 2007, were included. Of these, 574 who had records with the necessary data to be retrieved for the proposal of this work were selected.

Data collection

Data were collected from records through an epidemiological form and later transferred to a Excel spreadsheet (version 2003).

Statistical analysis

The GraphPad Instat software version 3.06 was employed to calculate the rates of seropositivity for the tested infections. The chi-squared test was used to compare proportions; a level of significance of 0.05 was considered statistically significant.

RESULTS

The mean age selected pregnant women was 27.2 ± 6.5 years ranging from 13 to 44 years.

Table 1 shows the seropositivity rates for the TORSCH International protocol, with the exception of cytomegalovirus (CMV). As this test is not mandated by the Brazilian Health Ministry it is not performed at our institution.

Table 1. Seropositivity rates of toxoplasmosis IgG and IgM, rubella IgG and IgM, syphilis, hepatitis B, hepatitis C and HIV in pregnant women attending the Gynecol- ogy and Obstetrics Outpatients Clinic of Hospital de Base (FUNFARME) in São José do Rio Preto, São Paulo State, Brazil from January 2006 to December 2007

Test	n	Seroreagent	%
Toxoplasmosis			
IgG	556	345	62.0
IgM	556	19	3.4
Rubella			
IgG	465	433	93.1
IgM	465	3	0.6
Syphilis	561	5	0.9
Hepatitis B (HBsAg)	554	10	1.8
Hepatitis C (anti-HCV)	545	4	0.7
HIV1/2	531	11	2.1

Detailed information of the serological test results for toxoplasmosis was available for 556 pregnant women. The mean age of the pregnant women with risk of congenital transmission (n = 230; non-reagent [IgG and IgM] and reagent serology for IgG + IgM) was 26.3 ± 6.6 years and for those without risk (n = 326; reagent serology for IgG) it was 27.8 ± 6.4 years (p = 0.005).

Out of 465 women with available anti-rubella serological results, the mean age of pregnant women with risk of congenital transmission (n = 35; non-reactive serology [IgG and IgM] and reactive serology for IgG + IgM) was 28.9 ± 7.4 years and 27.1 ± 6.6 years for those without risk (n = 430; serology reactive for IgG; p = 0.11).

For syphilis, the mean age of the seropositive pregnant women was 19 ± 4.2 years and 27.2 ± 6.5 years for the seronegative women (p = 0.004).

The mean ages of pregnant women with and without HBsAg antigen were 25.3 ± 8.1 and 27.1 ± 6.5 years, respec tively (p = 0.37). In respect to hepatitis C, the mean ages were 31.7 ± 4.2 for seropositive women and 27.1 ± 6.5 years for seronegative women (p = 0.15).

In relation to serology for HIV, the mean age of seropositive pregnant women was 30.7 ± 5.4 years and 27.1 ± 6.6 years of seronegative women (p = 0.07).

DISCUSSION

The aim of this study was to determine the prevalence of seropositive immunodiagnostic tests of the TORSCH panel group in 574 pregnant women attending a university hospital in the northwest region of São Paulo State. Results of this cross-section study are representative of the population from the northwest region of São Paulo State. The observed mean age of the pregnant women was in line with a previous study reporting results of women from the same region.[6]

In the present study more than 60% of the pregnant women tested positive for IgG anti-*T. gondii* antibodies, which is in agreement with other studies carried out in Brazil reporting seropositivity rates ranging from 22.8% to 71.5%.[7-12] Two recent studies performed in the northwest region of São Paulo State found rates similar to those reported in the current study.[5,13]

Pregnant women at risk of congenital transmission of toxoplasmosis were younger than those without risk. Most humans tend to produce high-avidity anti-*T. gondii* IgG antibodies following infection with *T. gondii* as a result of the development of immune memory.[14,15] Additionally, older individuals seem to be exposed to a greater number of stimuli including re-infections by this parasite, without necessarily manifesting the clinical form of the disease.[15] Thus, we can presume that the majority of seropositive pregnant women are probably immune and with no risk of congenital transmission of *T. gondii*.

On the other hand, seronegative pregnant women or those seropositive for IgG and IgM are at risk of transmission and adequate precautions need to be taken.

Serology for rubella was performed in fewer cases, although more than 93% of the pregnant women presented with evidence of immunization with reactivity for IgG.

In several countries, including Brazil, vaccination campaigns against rubella in women have contributed to the reduction of acute cases of the disease thereby protecting susceptible patients and their fetuses.[16,17] This strategy of increasing the immunization rates is translated in the high rates of IgG seropositivity seen in this study.

The percentage of pregnant women at risk for congenital transmission of rubella was lower than 1% and the mean age of these individuals was not significantly different from those without risk. The last vaccination campaign against rubella carried out in Brazil was aimed at women in reproductive age, i.e., between 12 and 39 years old.[18] This may explain, at least in part, the lack of differences in the mean ages of pregnant women with and without risk for congenital transmission.

The positivity rate for VDRL in this study (0.9%) was lower than that reported in other publications.[19,20] Adolescence is a phase of life in which the risk of acquiring sexually transmitted diseases is higher. In a study performed in the state of Pará, it was observed that 15% of the mothers who gave birth to a baby with syphilis were younger than 20 years old.[21,22] These data support the results of the current study that also found a lower mean age among pregnant women with positive serology for VDRL. Congenital syphilis is preventable; control measures adopted in Brazil aim at reducing the number of cases to 1 in 1,000 newborns.[20]

The age of pregnant women with and without reactive tests for hepatitis B was similar and the rate of HBsAg positivity (the surface antigen of the hepatitis B virus) was within the range for persistent infection by HBV reported in Brazil. Studies performed in several states have demonstrated frequencies ranging between 0.3% and 13%, including among pregnant women.[3, 23-25]

Previous knowledge of the serological test results for hepatitis B in pregnant women is of extreme importance as the risks of congenital transmission increase with maternal viral load. This disease should receive constant attention in pregnant women, as infected newborn babies present a high risk of developing chronic forms of the disease due to their immunological immaturity.[24, 26, 27]

The frequency of anti-HCV antibodies found in this study is lower than that reported in the general population,[28,29] but is close to that observed in pregnant women.[30,31] Moreover, pregnant women, with and without seropositivity had similar mean ages.

The hepatitis C virus is responsible for more than 90% of the hepatitis previously classified as non-A and non-B,[32] with the great majority of infected individuals remaining asymptomatic.[33] This is of great importance, as pregnant women, even when asymptomatic, can transmit the virus to their fetuses and newborn babies.

The HIV-infection rate found in this study is elevated and may be related to the High-Risk Gestation Outpatient Clinic being a regional reference center and that São José do Rio Preto is a reference center for the treatment of sexually transmitted diseases including AIDS.[34] The mean ages of the pregnant women with and without anti-HIV antibodies were similar.

In Brazil, the National HIV/AIDS Program has involved city and state governments, as well as non-governmental organizations, in the fight against HIV/AIDS for more than two decades. By means of an epidemiological surveillance the National Program keeps track of temporal and spatial trends in the occurrence of AIDS and HIV infection, aiming at guiding control actions of the epidemic at every level of public healthcare (SUS).[35] Heterosexual transmission of HIV increased the number of infected women throughout the world, including in Brazil, thus raising the number of cases of children with AIDS due to perinatal and transplacental transmission.[23, 36, 37]

The rate of vertical transmission of HIV, with no intervention, is about 25%. However, several studies have demonstrated a drop in transmission to 0% to 2% and a significant reduction in the incidence of cases of AIDS in children, as a result of preventive measures such as the use of combined anti-retroviral agents, elective C-sections, the use of chemoprophylaxis with AZT in the perinatal period and restricting breastfeeding in the newborn.[38-40]

The results of this study underscore the importance of diagnosing congenitally and perinatally transmitted infectious-parasitic diseases for the health of pregnant women and their newborn babies, in agreement with prior publications.[3,20,41] Early diagnosis allows preventive measures and treatment to be implemented in the antenatal and postnatal periods.[3,4,41,42]

CONCLUSIONS

In conclusion, the prevalence of the TORSCH group infections in pregnant women attending a teaching hospital are in agreement with those previously reported by epidemiological studies and by government sources.

ETHICAL APPROVAL

This study was approved by the Research Ethics Committee of the Medicine School in São José do Rio Preto (FAMERP – protocol 168/2007).

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Anti-*Toxoplasma gondii* antibodies in pregnant women and their newborn infants in the region of São José do Rio Preto, São Paulo, Brazil

Anticorpos anti-*Toxoplasma gondii* em gestantes e seus neonatos na região de São José do Rio Preto, São Paulo, Brazil

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KEY WORDS:
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Prenatal diagnosis.
Neonatal screening.

PALAVRAS-CHAVE:
Toxoplasma gondii.
Testes sorológicos.
Gravidez de alto risco.
Diagnóstico pré-natal.
Triagem neonatal.

ABSTRACT

CONTEXT AND OBJECTIVE: Toxoplasmosis transmission during pregnancy can cause severe sequelae in fetuses and newborns. Maternal antibodies may be indicators of risk or immunity. The aim here was to evaluate seropositivity for anti-*Toxoplasma gondii* (anti-*T. gondii)* immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies and IgG avidity in pregnant women and their newborn infants.

DESIGN AND SETTING: Cross-sectional study in a high-risk pregnancy outpatient clinic.

METHODS: Serum samples from pregnant women (n = 87) and their respective newborns (n = 87) were evaluated for anti-*T. gondii* antibodies using indirect immunofluorescence (IIF) (IgM and IgG), enzyme- linked immunosorbent assay (ELISA) (IgG) and an avidity test.

RESULTS: Anti-*T. gondii* antibodies were identified in 64.4% of the serum samples from the mothers and their infants (56/87). Except for two maternal serum samples (2.3%), all others were negative for anti-*T. gondii* IgM antibodies, using IIF. The results showed that 92.9% of the pregnant women had high IgG avid- ity indexes (> 30%) and four samples had avidity indexes between 16 and 30%. Two women in the third trimester of pregnancy were positive for anti-*T. gondii* IgM antibodies; their babies had avidity indexes between 16 and 30%. The avidity indexes of serum from the other 83 newborns were similar to the results from their mothers.

CONCLUSIONS: The results showed that 2% of the pregnant women were at risk of *T. gondii* transmission during the gestational period. These data seem to reflect the real situation of gestational toxoplasmosis in the northwestern region of the state of São Paulo.

RESUMO

CONTEXTO E OBJETIVOS: A toxoplasmose, quando transmitida durante a gestação, pode causar graves sequelas em fetos e neonatos. Anticorpos maternos podem ser indicadores de risco ou de imunidade. O objetivo foi avaliar a positividade dos anticorpos das classes imunoglobulina M (IgM) e imunoglobulina G (IgG) anti-*Toxoplasma gondii* (anti-*T. gondii*), bem como a avidez de IgG em gestantes e seus neonatos. TIPO DE ESTUDO E LOCAL: Estudo transversal em ambulatório de gestação de alto risco.

MÉTODOS: Anticorpos *anti-T. gondii* foram avaliados em amostras de soro de gestantes (n = 87) e seus respectivos neonatos (n = 87) com o uso dos métodos imunofluorescência indireta (IFI) (IgM e IgG), ensaio imunoenzimático (ELISA) (IgG) e avidez.

RESULTADOS: Anticorpos anti-*T. gondii* foram identificados em 64,4% das amostras de soro das mães e seus bebês (56/87). Com exceção de duas amostras de soro materno (2,3%), todas as demais foram nega- tivas anticorpos IgM anti-*T. gondii* determinado pela IFI. Os resultados mostraram que 92,9% das gestantes tinham índices elevados de avidez de IgG (> 30%) e 4 amostras apresentaram índices de avidez entre 16-
30%. Duas gestantes no terceiro trimestre da gravidez eram positivas IgM anti-*T. gondii*; seus bebês apre- sentaram índices de avidez entre 16 e 30%. Os índices de avidez dos soros dos outros 83 recém-nascidos foram semelhantes àqueles encontrados nas amostras maternas.

CONCLUSÕES: Os resultados mostraram que 2% das gestantes estavam sob risco de transmissão de *T. gondii* durante o período gestacional. Estes dados parecem refletir a real situação da toxoplasmose gesta- cional na região noroeste do Estado de São Paulo.

INTRODUCTION

Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite within the apicomplexa classification that can infect many different species of mammals and birds. Humans can be infected by consumption of raw or undercooked red meat or vegetables, unpasteurized milk or contaminated water, or after contact with cat feces.[1] Additionally, fetuses can be infected by transplacental transmission, a condition that may cause significant sequelae in babies. The life cycle of toxoplasmosis is shown in Figure 1.

During acute infections, the parasites differentiate within tissue cysts in the muscles and brain. Most primary infections are asymptomatic and, in fact, only 10-20% of all patients infected by *T. gondii* are symptomatic. In these cases, toxoplasmosis can be a serious public health problem.[2,3]

Fetuses of women acutely infected during pregnancy may present with severe damage, which also constitutes an important public health problem due to the resulting high morbidity and mortality rates. Most congenitally infected newborn babies have no clinical signs but are at risk of developing retinochoroiditis during childhood or adolescence.[4]

The risk of fetal contamination and the severity of sequelae depend on the stage of pregnancy at which the mother becomes infected.[4,5] Early in pregnancy, infections are less likely to cross the placental barrier, but when this does occur the consequences are more serious. In general, when infection occurs late in preg- nancy, babies have mild symptoms or are asymptomatic at birth.[6-8] High parasite counts in the amniotic fluid are associated with severe outcomes.[8] However, time of infection during preg- nancy is not the only factor that contributes towards the different outcomes, since parasitic virulence is also important with regard to the severity of the disease.[9]

Different studies have reported the seroprevalence of toxoplasmosis in pregnant women and newborns in different regions of the world, including South America.[10] Additionally, Brazilian studies have demonstrated that infection rates among pregnant women vary according to the geographical region.[10-16] However, there are only a few studies evaluating anti-*T. gondii* antibodies and their avidity in mothers and their newborns in the state of São Paulo. Further studies that analyze anti-*T. gondii* antibodies in paired mother-baby serum samples may contribute towards better understanding of congenital toxoplasmosis in specific regions.[15,17]

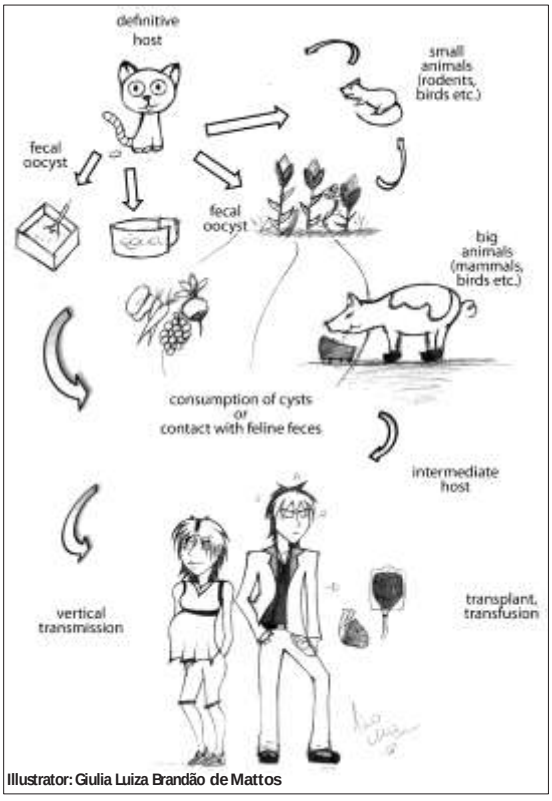

Figure 1. *Toxoplasma gondii* life cycle and human infection.

OBJECTIVES

The aim of this study was to evaluate the seropositivity of pregnant women and their newborn infants for anti-*T. gondii* immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies. The pregnant women were attended at a reference outpatient clinic for high-risk pregnancies in São José do Rio Preto. This region, located in the northwest of the state of São Paulo, is composed of 96 municipalities with a population of around 1.5 million (Figure 2).[18]

METHODS

Clinical samples

This cross-sectional descriptive study analyzed the positivity of 174 serum samples for anti-*T. gondii* IgM and IgG antibodies and the avidity of IgG antibodies. From May 2005 to June 2007, 87 serum samples were collected from consecutive pregnant women at different gestational ages as follows: (i) 13 women in the first trimester; (ii) 36 women in the second trimester; and (iii) 38 women in the third trimester. Another 87 samples were collected from the babies' umbilical cords at birth. All the pregnant women selected for this study were considered as having "high-risk pregnancy", as determined by the Ministry of Health's policy.[19] They were attended and gave birth at the high-risk gestational outpatient clinic of the teaching hospital (Hospital de Base) of Fundação Faculdade Regional de Medicina (Funfarme),

São José do Rio Preto, state of São Paulo, Brazil. This is a tertiary-care regional reference center. The number of samples evaluated represented 23.7% of the total number (n = 367) of high-risk pregnant women attended during the period of this study, as reported in our previous paper.[20]

After blood collection (around 5 ml) from mothers and from the umbilical cords, the samples were immediately sent to the Immunogenetics Laboratory of the Department of Molecular Biology, Faculdade de Medicina de São José do Rio Preto (Famerp). The serum samples were stored at -20 ºC until use. All samples were assayed by means of indirect immunofluorescence (IIF) (for IgM and IgG), enzyme-linked immunosorbent assay (ELISA) (for IgG) and an avidity test. All the pregnant women gave their written consent for the procedures and the institution's Ethics Committee approved this study (case number 295/2008).

Toxoplasma gondii and antigens

T. gondii RH strain tachyzoites were grown and maintained in the ascites of Swiss mice by means of intraperitoneal inoculation. At three to four-day intervals after infection, peritoneal fluid from each mouse was collected in phosphate-buffered saline (PBS) solution at pH 7.2. The mixture was centrifuged at 1,000 g for 10 minutes. The sediment containing the parasites was washed twice in PBS, the parasites were counted and the concentration was determined in order to prepare the antigens. For IIF antigens, the centrifuge pellets were suspended in PBS at a concentration of 2 x 10^7 cells/ml. The tachyzoites were incubated in 2% buffered formalin for 30 minutes at 37 °C, washed twice in PBS, centri- fuged at 1,000 g for 10 minutes and finally fixed on glass slides. For ELISA, the crude extract of tachyzoites was obtained as pre- viously described.[21] The parasites were sonicated (10 cycles of 1.0
A/minute for five minutes with two-minute intervals). Subsequently, the aliquots were dissolved in 0.3 M NaCl (sodium chloride) and the protein concentration was determined in a Nanodrop ND1000 spectrophotomer.

Serological reactions

IIF was carried out as previously described,[22] in order to determine whether anti-*T. gondii* IgG and IgM antibodies were present or absent. The samples were used in serial dilutions and assayed in duplicate. The dilutions went from 1:4 to 1:4096, and the cutoff point was determined as 1:16. For ELISA and the *Toxoplasma*-specific IgG avidity assay, the samples were assayed in duplicate at a dilution of 1:500. The optical density (OD) cutoff for ELISA at a wavelength of 492 nm was 0.190. The *Toxoplasma*-specific IgG avidity assay was performed as previously described.[22] The basic ELISA test was used except that: (i) each serum sample was analyzed in two fourfold titration rows at a dilution of 1:500; (ii) after one hour of incubation at 37 °C, the first row was washed three times with 250 ml of 6 M urea in PBS containing 0.05%

Figure 2. Map of Brazil indicating location of state of São Paulo and map of São José do Rio Preto region including 96 municipalities.[18]

Tween 20, in order to remove low-avidity antibodies from their binding sites. The control row was washed three times using the buffer without urea. The formula to calculate the IgG avidity index was: OD values under dissociative conditions/OD values of control without urea x 100.

A low avidity index (up to 15%) was indicative of an infection within the previous five months; an avidity index between 16 and
30% was indicative of an infection more than five months ago; and a high avidity index (over 30%) represented chronic infection. For ELISA, the absorbance values were subtracted from the background, and the arithmetic mean was calculated. The cut-off was calculated for each reaction using a serum panel from 20 healthy individuals (data not shown).

Statistical analysis

Fisher's exact test was used to evaluate associations in the serological analysis, between maternal and newborn samples.

RESULTS

Among the 87 pregnant women evaluated, 43.7% (n = 38) were Caucasians, 44.8% (n = 39) were of mixed race, 10.3 (n = 9) were blacks and 1.2% (n = 1) were Amerindians. The mean age and gestational age were 27.5 years (± 6.9) and 25.5 weeks (± 8.4), respectively.

Anti-*T. gondii* IgG antibodies, as determined by ELISA and IIF, were identified in 64.4% (56/87) of both the maternal and the umbilical cord serum samples. The samples from the other 31 pregnant women and their babies (35.6%) were negative for toxoplasmosis. All the maternal serum samples except for two (2.3%) were negative for anti-*T. gondii* IgM antibodies, as determined by IIF. In both of these cases, the antibodies were detected in the pregnant women during the third trimester of gestation. However, IgM antibodies were not isolated in the serum of the newborns, since fetuses are unable to produce IgM antibodies.[14] These two pregnant women did not give their consent for amniotic fluid to be collected and therefore the fetal infection could not be confirmed by means of the polymerase chain reaction (PCR).

The results showed that 92.9% (52/56) of the pregnant women infected with *T. gondii* had high avidity indexes for IgG antibodies (≥ 30%). Samples with avidity of less than 15% were not found. However, four serum samples had avidity indexes between 16 and
30%. Of these, two samples were from pregnant women in the third trimester of pregnancy who were positive for anti-*T. gondii* IgM antibodies, and their babies had avidity indexes between 16 and 30%. The other two women were in their second trimester of gestation and their babies presented avidity indexes of up to 30%. The avidity indexes of the other serum samples from the umbilical cords were similar to those found in their mothers. These results are shown in detail in Table 1. The results from the maternal and newborn serological analyses were not statistically significant (IIF/ELISA IgG: P = 1.000; IgG avidity: P = 0.6788).

DISCUSSION

Since toxoplasmosis is highly prevalent in Brazil and causes seri- ous problems during pregnancy,[10] we decided to investigate the serum status of a group of high-risk pregnant women with regard to anti-*T. gondii* IgM and IgG antibodies. These patients were attended and their babies were born at a high-risk pregnancy out- patient clinic in São José do Rio Preto. Our results showed that
64.4% of the women with high-risk pregnancies had toxoplasmosis. These data suggest that the rate of positive findings of *T. gon- dii* infection was high in this group and thus corroborate other studies from the same region of the state of São Paulo[20,23] and from other Brazilian states.[15-17,24-28] The similarities between this and other studies carried out in some Brazilian states[15-17,28] may reflect homogeneity regarding the laboratory diagnostic strate- gies used.

The avidity index helps to identify the acute phase of infections by this parasite.[29] In this study, the majority of the infected women (92.9%) were in the chronic phase of infection (avidity indexes higher than 30%). Only anti-*T. gondii* IgG antibodies with high avidity were detected in serum samples from their babies. Since the avidity indexes were identical to those of the maternal serum and the methods used in this study were unable to differentiate IgG antibodies from mothers and babies, it can be assumed that the antibodies presented by the newborns originated from the mothers. Therefore, the majority of the pregnant women evaluated in this study seemed to present a protective level of humoral immunity against *T. gondii*, without a risk of congenital transmission. Anti-*T. gondii* IgM antibodies were identified in 2.3% of the pregnant women. These antibodies were detected in the third trimester of gestation. Simultaneously, anti-*T. gondii* IgG had avidity indexes between 16 and 30%. The data suggest that these women probably became infected around five months prior to testing; in other words, within the first trimester. When primary maternal infection occurs in this period, around 15% of the fetuses can become infected.[13,14,16] The fetuses of these two pregnant women were probably not infected during gestation, although this condition is not conclusive. There have been reports that 70% of newborns infected during gestation do not present symptoms at birth.[30] Additionally, around 30% of newborns do not demonstrate serological evidence of congenital infection at birth, even when the mothers present with IgM antibodies.[4,14]

Since these data demonstrate that 2.3% of the pregnant women became infected during gestation, it can be assumed that this is the level of risk of congenital transmission of *T. gondii* in the northwestern region of the state of São Paulo. This figure corroborates our additional observations.[23] These observations highlight the importance of early diagnosis and good-quality methodology for evaluating pregnant women and newborn babies in healthcare services. This, together with the risks implicit in congenital transmission, emphasizes the need for continuous educational programs and constant monitoring of pregnant women from regions where the prevalence of infection by this parasite is high.

Despite the small number of serum samples evaluated, the results from this study shed some light on the clinical importance of combined mother-newborn evaluation using serological methods to detect not only IgM and IgG anti-*T. gondii* antibodies but also IgG avidity. Furthermore, these results draw attention to the need to investigate patient samples consisting of larger

Table 1. Determination of anti-*Toxoplasma gondii* antibodies in maternal and umbilical cord serum samples using indirect immunofluorescence (IIF), enzyme-linked immunosorbent assay (ELISA) and an immunoglobulin G (IgG) avidity test, São José do Rio Preto, state of São Paulo, Brazil

	IIF/ELISA IgG* (n = 174)		IIF IgM (n = 87)		IgG avidity† (n = 174)		
	Negative	Positive	Negative	Positive	≤ 15%	16-30%	≥ 30%
Newborn	31	56	Not determined	Not determined	0	2	54
Maternal	31	56	85	2	0	4	52
1st trimester	4	9	13	0	0	0	9
2nd trimester	14	22	36	0	0	2	20
3rd trimester	13	25	36	2	0	2	23

*P = 1.000; †P = 0.6788 (calculated by means of Fisher's exact test)
IIF = indirect immunofluorescence; ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G; IgM = immunoglobulin M.

numbers of mother-newborn pairs, given the epidemiological importance of toxoplasmosis.

CONCLUSIONS

This study demonstrated that 64.4% of the pregnant women in the northwestern region of the state of Sao Paulo became infected with *T. gondii* before pregnancy and that most of them had immune protection with high avidity indexes. Nonetheless, the study suggests that an epidemiologically significant proportion of the fetuses may be at risk of congenital transmission of *T. gondii*.

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Contribution of laboratory methods in diagnosing clinically suspected ocular toxoplasmosis in Brazilian patients☆

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Abstract

This prospective study evaluated the value of laboratorial diagnosis in ocular toxoplasmosis analyzing peripheral blood samples from a group of Brazilian patients by immunologic and molecular methods. We analyzed blood samples from 184 immunocompetent patients with ocular disorders divided into 2 groups: Group I, composed of samples from 49 patients with ocular toxoplasmosis diagnosed by clinical features; Group II, samples from 135 patients with other ocular diseases. Samples were assayed by conventional polymerase chain reaction (cnPCR), real-time PCR (qPCR) for Toxoplasma gondii, indirect immunofluorescence reaction (IF), avidity test (crude tachyzoite lysate as antigen), and excreted-secreted tachyzoite proteins as antigen (ESA-ELISA). cnPCR and qPCR profiles were concordant in all samples. Positive PCR was shown in 40.8% of group I patients. The majority of the positive blood samples (75%) were taken from patients with toxoplasmic retinochoroiditis scars, and the others (25%), from patients with retinal exudative lesions. Despite that 86 of the 135 patients from Group II had asymptomatic toxoplasmosis, all DNA blood samples had negative PCR. Concordant results were shown in the data obtained by serologic methods. Around 24% of the patients with ocular toxoplasmosis had high antibody titers determined by ESA-ELISA and IF. Anti-ESA antibodies are shown principally in patients with active infection. Collectively, these data demonstrate the presence of tachyzoites in the blood of patients with chronic infection, supporting the idea of recurrent disease. Circulating parasites in blood of immunocompetent individuals may be associated with the reactivation of the ocular disease.

Keywords: Ocular toxoplasmosis; ESA-ELISA; Molecular and serologic diagnosis; PCR

1. Introduction

Toxoplasmic retinochoroiditis is the most common lesion caused by *Toxoplasma gondii* infection and may occur either immediately or long after the initial infection or in reactivation (Montoya and Remington 1996; Vallochi et al., 2008). Rupture of dormant cysts in the retina can release viable parasites that induce necrosis and inflammation. Alternatively, retinochoroiditis might result from a hypersensitivity reaction triggered by unknown causes (Commodaro et al., 2009), resulting in irreversible damage to the retina involved, whose consequences may be severe visual morbidity (Remington et al., 2001). In addition, toxoplasmic retinochoroiditis has been reported as more severe in Brazil than in Europe (Gilbert et al., 2008).

Despite the diagnosis of ocular toxoplasmosis is typically based on clinical analysis, the laboratorial tests normally can help the definitive diagnosis. The presence of anti-*T. gondii* IgG antibodies does not confirm the toxoplasmic etiology, but such antibodies can often persist at high titers for years after the acute infection, and a negative result generally discards the diagnosis (Ongkosuwito et al., 1999). *T. gondii*

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DNA has been identified in ocular tissue sections and vitreous fluid (Grigg et al., 2001; Montoya and Remington, 1996; Rothova et al., 2008).

To evaluate the value of the laboratorial diagnosis in ocular toxoplasmosis, this study analyzed peripheral blood samples from a group of Brazilian patients with ocular toxoplasmosis by immunologic and molecular methods.

2. Materials and methods

2.1. Patients and clinical samples

This prospective study was conducted for 12 months (July 2009 to June 2010). We analyzed blood samples from 184 patients with ocular alterations admitted and treated at the Ophthalmology Outpatients Clinics from Fundação Faculdade Regional de Medicina-Hospital de Base, São José do Rio Preto, São Paulo, Brazil. All patients were immunocompetent and were divided into 2 groups according to clinical data. Group I consisted of 49 patients with ocular toxoplasmosis including 38 with toxoplasmic retinochoroiditis scars and 11 with retinal exudative lesions. Group II consisted of 135 patients with other diseases including cataracts; vitreous or retinal detachment due to other diseases; type II diabetes; vascular occlusions; age-related macular degeneration; glaucoma; toxocariasis; cytomegalovirus infection; cornea transplants; non-toxoplasmic uveitis; hypertensive retinopathy macular alterations, no light perception, and melanoma choroid. From each patient, before specific treatment, 5 mL of peripheral blood with EDTA was collected for DNA extraction and 5 mL of blood in dried tubes for serologic diagnosis. All patients provided written informed consent and the institutional review boards of the ethics committees of all institutions approved this study.

2.2. Molecular and serologic diagnosis

The molecular diagnosis included conventional polymerase chain reaction (cnPCR) and real-time quantitative polymerase chain reaction (qPCR). The serologic diagnosis was composed of indirect immunofluorescence (IF), avidity test, and enzyme-linked immunosorbent assay (ELISA) using excreted-secreted tachyzoite proteins (ESA) as antigen (ESA-ELISA).

2.3. *T. gondii* source

T. gondii (RH strain) was grown and maintained in Swiss mice by intraperitoneal inoculation. Tachyzoites were harvested from the peritoneal cavities in phosphate buffered saline (PBS), pooled, then centrifuged, washed twice at 2000 × g for 10 min, and used to infect tissue cultures. Tachyzoites were maintained in VERO cells, at 37 °C 5% CO_2, in Eagle's medium containing 292 mg/L L-glutamine, 110 mg/L sodium pyruvate, 1 g/L glucose, 2.2 g/L sodium bicarbonate, 100,000 U/L penicillin, 133 mg/L streptomycin, and 10% fetal bovine serum (FBS). DNA control and antigens for immunofluorescence reaction (IF) and ELISA-avidity test were made using tissue culture tachyzoites. The supernatants of culture infected cells were used in ESA recovery (ESA-ELISA).

2.4. Antigens

For IF, culture tachyzoites were washed twice, counted, and suspended in PBS at a concentration of 2×10^7 cells/mL. Parasites were incubated in 2% buffered formalin for 30 min at 37 °C, washed twice in PBS at 1000 × g for 10 min, and fixed on glass slides. For avidity test, the crude tachyzoite antigen was obtained by sonication of tachyzoites (10 cycles of 1.0 A/min, for 5 min with 2-min intervals). The crude antigen was dissolved in 0.3 mol/L NaCl and the protein concentration was determined (Colombo et al., 2005). For ESA-ELISA, the proteins were recovered from infected culture supernatant with tachyzoites. Infected VERO cells were previously washed 3 times and the medium was replaced by another aliquot without FBS. After 48 h postinfection, culture supernatants were harvested, filtered through a 0.22-μm-pore-size filter, and 10 μg/mL of a cocktail of protease inhibitors was added, containing (per milliliter) 20 μm AEBSF, 10 μm EDTA, 1.3 μm bestatin, 0.14 μm E-64, 10 nm leupeptin, and 3 nm aprotinin. The supernatants, referred to as ESA, were concentrated in a Speed Vac (Jouan S.A., evaporator centrifuge RC 10.09; Saint-Herblain, Cedex, France) and dialyzed against PBS (Meira et al., 2008).

2.5. Serologic reactions

IF was carried out as previously described (Colombo et al., 2005). The reactions were made in order to determine the presence or absence of anti-*T. gondii* IgG and IgM antibodies. Samples were used in serial dilutions and assayed in duplicate. Sera were diluted from 1:4 to 1:4.096, and the cutoff was considered to be 1:16. Low titers were considered to be between 1:16 and 1:64; intermediate, 1:256 and 1:512; and high titers, above 1:1024. *T. gondii*-specific IgG avidity assay was performed exactly as described before (Colombo et al., 2005; Korhonen et al., 1999). ELISA plates were incubated overnight at 4 °C with the "crude tachyzoite antigen" dissolved in 0.1 mL of 0.1 mol/L $NaHCO_3$, pH 8.5. After removal by washing plates with PBS, pH 7.2, containing 0.05% Tween 20, the free binding sites were blocked by 5% skim milk-PBS for 30 min. Each serum sample, diluted 1:500 in 5% skim milk-PBS, was analyzed in two 4-fold titration rows. After 1 h of incubation at 37 °C, the first row was washed 3 times with 250 μL of 6 mol/L urea in 0.05% PBS-Tween 20 to remove low-avidity antibodies from their binding sites. The control row was washed 3 times with the same buffer without urea. The wells were incubated for an additional 60 min at 37 °C with a horseradish peroxidase-conjugated goat anti-human IgG. After a new wash cycle with 0.05% PBS-Tween 20, substrate solution (0.1 mol/L citric acid, 0.2 mol/L Na_2HPO_4, 0.05% o-phenylenediamine, 0.1% H_2O_2) was added to each well.

The plates were left to stand at room temperature in the dark for 30 min. Color development was stopped by adding 50 µL of 4N H_2SO_4. Absorbance values were measured in an ELISA reader (Labsystems Multiskan, MS Plate Reader, Minneapolis, MN) with a 492-nm filter. The optical density (OD) cutoff was 0.143 at 492-nm wavelength and was calculated in each reaction using a sera panel of 20 healthy individuals with negative serology for toxoplasmosis. The absorbance values were subtracted from the background, and the arithmetic mean was calculated. The IgG avidity index was calculated as previously described (Korhonen et al., 1999). The ratio was calculated as: (OD values under dissociative conditions)/(OD values of untreated controls without urea) − 100. A low avidity index (15%) represented the predictive value for an infection of less than 5 months, and an index between 15% and 30% represented the predictive value for an infection of more than 5 months. A high avidity index (over 30%) determined a chronic infection. The procedure used in ESA-ELISA was similar to the one described above. The differences were as follows: i) ELISA plates were incubated with ESA as antigen; ii) each serum sample was assayed in duplicate; iii) OD results were transformed in ELISA-relative values that represent the ratio of the absorbance of each serum sample at an optical density of 492 nm to the cutoff value (serum OD/cutoff OD). Values greater than 1.0 were considered reactive. Low ELISA-relative values were considered to be between 1 and 5; and high, above 6 (Meira et al., 2008).

2.6. DNA Purification

DNA of blood samples was extracted using PureLink Genomic DNA Kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions (Mesquita et al., 2010). Before performing the DNA extraction, blood samples were centrifuged and washed with PBS at 2500 × g for 10 min. The supernatants with plasma were discarded. In order to lyse the erythrocytes, the packed cells were mixed with 3 times the volume of a buffer containing 150 mmol/L ammonium chlorate, 1 mmol/L potassium bicarbonate, 0.1 mmol/L EDTA (pH 7.3), incubated for 15 min at room temperature under mild shaking and centrifuged for 10 min at 3000 × g. The blood pellets, containing only nuclei cells, were digested with proteinase K (20 µg/mL) in 50 mmol/L Tris-HCl, 25 mmol/L EDTA (pH 8.0), 2% sodium dodecyl sulfate, and incubated for 30 min at 56 °C. DNA pellets were resuspended in ultrapure water. DNA extracted from the tachyzoite pellets using the PureLink kit was used as a positive control in the PCR reactions. DNA purity was determined by the OD ratio at 260 and 280 nm in a NanoDrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.7. Primer selection

B22/B23 primer pair was used to amplify a 115-bp sequence from a specific repetitive region of the B1 gene in cnPCR. To control the course of DNA extraction and check for PCR inhibitors, all samples were assayed using β1/β2, which amplified a 140-bp fragment of the human β-globulin gene. For qPCR, the primer set amplified another region of B1 gene and the description is B1Tg-F (forward) 5′-CAAGCAGCGTATTGTCGAGTAGAT-3′; B1Tg-R (reverse) 5′-GCGTCTCTTTCATTCCCACA-TTTT-3′; and B1Tg-TM (TaqMan probe, FAM dye-labeled) FAM 5′-CAGAAAGGAACTGCATCCGTT-3′. TaqMan probe had NFQ as reporter quencher (Mesquita et al., 2010).

2.8. cnPCR and qPCR

cnPCR was carried out in a LongGene Thermal Cycler (LongGene) in a final volume of 25 µL. DNA from samples (5 µL) or controls was mixed with and 25 pmol of each primer and additional components from a kit (Go Taq Green Master Mix, Madison, WI) purchased from Promega. The PCR mix (12.5 µL) was composed of 1 U of Taq DNA polymerase, 10 mmol/L Tris-HCl, pH 8.5; 50 mmol/L KCl; 1.5 mmol/L $MgCl_2$; and 200 mmol/L of each dNTP. Each amplification run contained 2 negative controls (ultrapure water and a DNA negative for toxoplasmosis) and 1 positive control (DNA extracted from RH strain). The thermal cycle conditions were made by one initial denaturation cycle for 5 min at 95 °C, 35 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min. The procedure was completed by a final cycle extension for 5 min. PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. DNA fragments were visualized under UV illumination. The images were analyzed by a Mini Bis Gel Imager and Documentation (DNR Bio-Imaging Systems, Jerusalem, Israel). The size of fragments was based on comparison with a 100-bp ladder. qPCR was performed with an Applied Biosystems 7500 Real Time PCR System in a final volume of 20 µL. The clinical samples or control DNA (3 µL) was added to a reaction mixture containing 10 µL of 2× TaqMan Universal PCR Master Mix and 1 µL of the ″assay mix″ that included i) the forward primer, 18 µmol/L; ii) the reverse primer, 18 µmol/L; and iii) the TaqMan MGB probe FAM dye-labeled, 5 µmol/L. Amplification runs contained 2 negative controls and 1 positive control, as described in the cnPCR. The amplification conditions included a 2-min, 50 °C step for optimal AmpliErase UNG activity and 95 °C for 10 min. Next, 40 cycles were performed at 95 °C for 15 s and 60 °C for 1 min.

2.9. Data analysis

All discordant results were repeated at least twice. Clinical diagnoses were used to establish cnPCR and qPCR sensitivities and specificities and were calculated as: i) percent of sensitivity = ratio of true positives/true positives + false negatives × 100; and ii) percent of specificity = ratio of true negatives/true negatives + false positives × 100. The cutoff of ESA-ELISA and avidity test corresponded to the mean plus 2 times the standard deviation of optical density of

492 nm (OD). In ESA-ELISA, the OD results were transformed into ELISA-relative values as previously described (Meira et al., 2008).

3. Results

This study evaluated the laboratorial diagnosis of ocular toxoplasmosis by analyzing peripheral blood samples from 184 Brazilian patients with ocular alterations. The tests included cnPCR, qPCR, IF, avidity test, and ESA-ELISA.

The results of the immunologic and molecular tests are shown in Table 1. The results of molecular diagnosis (cnPCR and qPCR) were consistent in all samples. By the analysis of peripheral blood from the patients with ocular toxoplasmosis, both methods presented 40.8% (20) sensitivity and 100.0% (135) specificity, respectively.

Anti-*T. gondii* IgG antibodies were demonstrated in sera from 135 of 184 studied patients, including all patients from Group I (49 patients) and 86 patients of Group II. All of them had chronic toxoplasmosis infection as determined by negative anti-*T. gondii* IgM antibodies (IF) and positive anti-*T. gondii* IgG antibodies with high avidity (ELISA-avidity test) in sera. The remaining 49 patients of Group II were sera-negative for toxoplasmosis, as they presented all reactions as negative.

The majority (77.6%) of the sera collected from Group I patients (with ocular toxoplasmosis) had low (19 patients) and intermediary (19 patients) IF titers. However, high IF titers (1:1048 to 1:4096) were shown in 22.4% of them (11 patients).

ESA of anti-*T. gondii* IgG antibodies could determine active infection (Meira et al., 2008). High antibody titers determined by ESA-ELISA were shown in sera from 12 patients (24.5%). The other sera from 37 (75.5%) patients had no (4 patients) or low (33 patients) anti-ESA antibodies.

The majority of the sera (91.8%) from the 86 Group II patients with toxoplasmosis had low (41 patients) and intermediary (38 patients) IF titers. Only 8.1% (7 patients) of them had high IF titers (1:1048 to 1:4096). Low anti-ESA antibodies were shown in 80 sera of this group of patients. Therefore, active infection by *T. gondii* detected by high ESA-ELISA titers or PCR was not found in any Group II patient.

4. Discussion

The evaluation of molecular diagnosis in ocular disease is usually performed in intraocular fluid samples, but the methods used for sample collection are invasive. On the other hand, PCR in peripheral blood samples has been successfully used for cerebral toxoplasmosis diagnosis (Colombo et al., 2005; Mesquita et al., 2010; 2010a). The great difference is that patients with cerebral toxoplasmosis generally are immunosuppressed and the parasites are relatively easy to determine by PCR in blood.

Table 1
Laboratory diagnosis of toxoplasmosis (PCR, IF, and ESA-ELISA) of 184 Brazilian patients with ocular disorders, from northwestern São Paulo state

	Ocular toxoplasmosis (group I)		Other ocular diseases (group II)	
	No. of patients	%[a]	No. of patients	%[a]
PCR				
Positive	20	40.8	0	0
Negative	29	59.2	135	100
Total	49		135	
IF				
Negative	0	0	49	37.0
Titers[b]				
Low	19	38.8	41	30.0
Intermediary	19	38.8	38	28.0
High	11	22.4	7	5.0
Total	49		135	
ESA-ELISA				
Negative	04	8.1	55	40.7
RV[c]				
Low	33	67.4	80	59.3
High	12	24.5	0	0
Total	49		135	

[a] Sensitivity and specificity, expressed in percent for PCR (both reactions), were determined considering the clinical diagnosis described in the Materials and methods section.

[b] Titer values: low, 16-32; intermediary, 256-512; and high, 1024-4096.

[c] ELISA-relative values (RV): serum OD/cutoff OD. Values greater than 1.0 were considered reactive. Low RV (1-5), chronic infection; and high RV (N6), active disease.

In this study, we applied cnPCR and qPCR protocols routinely used for molecular diagnosis in our laboratory (Colombo et al., 2005; Mesquita et al., 2010; 2010a; Vidal et al., 2004). To obtain high-quality reactions, certain laboratorial controls must be included as follows: i) use of primer sets amplifying regions of the gene B1, as they showed to be highly specific and sensitive in Brazilian samples; ii) clinical samples should be processed rapidly within 48 h of collection to prevent Taq polymerase inhibition; iii) as the amount of human chromosomal DNA and the DNA pathogen can vary from sample to sample and interfere in the results, all samples must be quantified after extraction; iv) DNA extraction and PCR inhibitors must be evaluated by a β1-β2 marker that amplifies a PCR fragment of human β-globulin gene; v) the clinical center must be informed to collect blood samples of the suspected patients before the specific therapy. According to these procedures, positive amplifications showed that no substance present in DNA samples inhibited the reaction. The results of cnPCR and qPCR were consistent in 100% of samples and, as expected, the specificity was 100%. Despite that, 86 of the 135 patients from group II (with other ocular diseases) had asymptomatic toxoplasmosis, but all DNA blood samples had negative molecular diagnosis.

Positive PCR was shown in 40.8% of Group I patients (with ocular toxoplasmosis). The majority of the positive blood samples (75%) were taken from patients with

toxoplasmic retinochoroiditis scars (15); and 25% of blood samples were taken from patients with retinal exudative lesions (5).

Concordant results were shown in the data obtained by serologic methods. Despite the fact that the majority of sera from these patients had low anti-*T. gondii* antibody titers, around 24% of them had high titers (IF and ESA-ELISA). Previous studies showed at least 2 important points about active infection. One is that anti-*T. gondii* antibodies determined in IF are statistically higher in patients with active infection than in asymptomatic ones (Colombo et al., 2005; Hellerbrand et al., 1996). The other is that specific antibodies induced changes in the reactivity of serum toward *T. gondii* ESA. When ESA was used as antigen in ELISA, sera from symptomatic and asymptomatic individuals presented different reactivity. This provides clear evidence that anti-ESA antibodies are present principally in patients with active infection (Meira et al., 2008; Pereira-Chioccola et al., 2009).

Collectively, these data demonstrate the presence of tachyzoites in the blood of patients with chronic infection, supporting the idea of recurrent disease, as reported previously (Silveira et al., 2010). Circulating parasites in blood of immunocompetent individuals may be associated with the reactivation of the ocular disease.

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Toxoplasma gondii isolates: Multilocus RFLP–PCR genotyping from human patients in Sao Paulo State, Brazil identified distinct genotypes

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abstract

This study investigated the genetic characteristics of Toxoplasma gondii samples collected from 62 patients with toxoplasmosis in Sao Paulo State, Brazil. DNA samples were isolated from blood, cerebrospinal fluid and amniotic fluids of 25 patients with cerebral toxoplasmosis and AIDS, two patients with acute toxoplasmosis, 12 patients with ocular toxoplasmosis, six newborns with congenital toxoplasmosis and 17 pregnant women with acute infection. Diagnosis of toxoplasmosis was based in clinical, radiological and laboratory features. Genotyping was performed using multilocus PCR–RFLP genetic markers including SAG1, SAG2, 5′- and 3′-SAG2, alt.SAG2, SAG3, BTUB, GRA6, C22-8, c29-2, L358, PK1 and Apico. Among the 62 clinical samples, 20 (32%) were successfully genotyped at eight or more genetic loci and were grouped to three distinct genotypes. Eighteen samples belonged to ToxoDB Genotype #65 and the other two samples were identified as ToxoDB Genotypes #6 and #71, respectively (http://toxodb.org/toxo/). Patients presenting Genotypes #6 and #71 had severe and atypical cerebral toxoplasmosis, characterized by diffuse encephalitis without extensive brain lesions. These results indicate that T. gondii Genotype #65 may have a high frequency in causing human toxoplasmosis in Sao Paulo State, Brazil. This unusual finding highlights the need to investigate the possible association of parasite genotypes with human toxoplasmosis.

1. Introduction

Toxoplasma gondii is a widespread protozoan parasite that infects one third of the global human population (Dubey, 2008). Genetic diversity of T. gondii strains has been an interesting and important subject of research. In the past decades, the development of highly sensitive and simple molecular methods facilitated the detection, diagnosis and genotyping of this important parasitic pathogen. With these molecular methods, it is possible to study the potential correlation between parasite genotype and disease patterns in infected patients, epidemiology, as well as population biology of T. gondii (Dubey, 2008; Pereira-Chioccola et al., 2009).

High resolution of genotyping can be achieved using multilocus RFLP–PCR, microsatellite and multilocus sequencing typing methods (Ajzenberg et al., 2004; Khan et al., 2007; Su et al., 2010). In North America, Europe, and Africa, most isolates belong to the clonal Type I, II, and III lineages, and there is no host boundary for different genotypes (Khan et al., 2007; Dardé et al., 1987; Sibley and Boothroyd, 1992; Lehmann et al., 2004). In these regions, Type II strains were predominant and commonly isolated from clinical cases of toxoplasmosis. Among the three lineages the difference at DNA sequence level is less than 1% on average, and it was suggested that these three lineages were expanded globally in the past 10,000 years (Su et al., 2003). Studies conducted in Brazil and French Guiana challenged the idea that T. gondii was clonal with very small genetic variability. Genotyping results from these regions showed a higher genetic variability with distinct genotypes not identified in North America, Europe and Africa (Ajzenberg et al., 2004; Fazaeli et al., 2000; Khan et al., 2006; Lindstrom et al., 2006; Su et al., 2006; Demar et al., 2007; Dubey et al., 2008; Ferreira et al., 2008; Pena et al., 2008; Vaudaux et al., 2010). Analysis of 125 isolates from domestic animals in Brazil re-

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vealed 48 genotypes and four of them had multiple isolates from different hosts and locations, and they were considered to be the common clonal lineages in Brazil (Pena et al., 2008).

Genetic analysis of T. gondii infection in human is of importance to understand the epidemiology, transmission patterns and mechanisms of the disease. However, the difficulty of strain isolation in human cases limits the data regarding T. gondii strain types. Despite that, genotyping studies were previously applied in human congenital infection (Ajzenberg et al., 2002; Nowakowska et al., 2006), human ocular infections (Vallochi et al., 2005), and AIDS patients (Ferreira et al., 2008; Fuentes et al., 2001; Gallego et al., 2006), which provided preliminary information for further studies. However, these early studies were limited to identify distinct isolates, since fewer (Pereira-Chioccola et al., 2009; Ajzenberg et al., 2004; Khan et al., 2007) genetic markers were used.

It is known the high prevalence of toxoplasmosis in the Brazilian population with an elevated morbidity and mortality in ocular and cerebral toxoplasmosis. Parasites had been detected in blood and cerebrospinal fluid (CSF) of these patients (Vidal et al., 2004; Vidal et al., 2005; Colombo et al., 2005). Early studies using limited number of genetic markers were inappropriate to distinguish the most of the polymorphic Brazilian strains (Ferreira et al., 2008). With the advent of multilocus PCR–RFLP such limitation can be overcomed. This study was aimed to investigate the genetic characteristics of the T. gondii strains isolated from patients with toxoplasmosis in Sao Paulo State by using recently developed typing method.

2. Materials and methods

2.1. Patients and clinical samples

This study was conducted using clinical samples from HIV-infected patients, newborns, pregnant women and immunocompetent patients admitted and treated at different public hospitals or clinics located Sao Paulo State, Brazil. All samples received by Laboratório de Parasitologia (Instituto Adolfo Lutz) and suspected of toxoplasmosis were tested during routine diagnosis. Diagnosis of toxoplasmosis was based in clinical, radiological and laboratory features. The laboratorial methodologies included: (i) serological exams as IgG and/or IgM antibodies to T. gondii detected by indirect immunofluorescence reaction (IF) (Colombo et al., 2005); (ii) conventional PCR (cnPCR) and real-time PCR (qrtPCR) (Colombo et al., 2005; Mesquita et al., 2010a). For patient with some immunosuppression, $CD4^+$ counts and serology for HIV were also investigated. During the period of January 2007–2010, positive results for toxoplasmosis were shown in DNA samples extracted from blood, CSF or amniotic fluids of 25 patients with cerebral toxoplasmosis and AIDS, two patients with acute toxoplasmosis, 12 patients with ocular toxoplasmosis, six newborns with congenital toxoplasmosis and 17 pregnant women with acute toxoplasmosis. The institutional review boards of Ethics Committees of all involved Institutions approved this study.

2.2. T. gondii reference strains

The reference strains GTI, RH, PTG, ME49, CTG, VEG, TgCgCa1 (COUGAR), MAS, TgCatBr5 and TgCatBr64 were used as positive control and as genotype indication in each genetic marker. Parasites were grown and maintained in VERO tissue cells. Tachyzoites were harvested from culture supernatants, centrifuged and washed twice at 2,000g for 10 min in phosphate-buffered saline, pH 7.2 (PBS). The parasite pellets were used for DNA extraction. RH strain was used also for antigen preparation in IF. Tachyzoites were incubated in 2% buffered formalin for 30 min at 37 oC, washed twice in PBS at 1,000g for 10 min, and fixed on glass slides (Colombo et al., 2005).

2.3. Indirect immunofluorescence reaction

The reactions were made in order to determine the presence or absence of anti-T. gondii IgG and IgM antibodies and carried out as previously described (Colombo et al., 2005). Samples were used in serial dilutions and assayed in duplicate. The sera were diluted from 1:4 to 1:4.096, and the cutoff was considered to be 1:16. Each reaction contained two negative controls (PBS and one negative serum) and one positive control (positive serum).

2.4. DNA extraction

DNA molecules were extracted from blood, amniotic fluid and tachyzoites using PureLink Genomic DNA Kits (Invitrogen) according to the manufacturer's instructions and described before (Mesquita et al., 2010a). Blood samples were centrifuged and washed with PBS at 2,500g for 10 min. The supernatants with plasma were discarded. In order to lyse the erythrocytes, the packed cells were mixed with three times the volume of a buffer containing 150 mM ammonium chlorate, 1 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.3, incubated for 15 min at room temperature under mild shaking and centrifuged for 10 min at 3,000 g. The blood pellets, containing only nucleated cells were digested with proteinase K (20 g/ml) in 50 mM Tris–HCl, 25 mM EDTA, pH 8.0, 2% sodium dodecyl sulfate and incubated for 30 min at 56 oC. DNA pellets were dissolved in ultra pure water. DNA from CSF samples was extracted as described before (Vidal et al., 2004). Samples were centrifuged for 10 min at 3,000g. After washing the packed cells twice in PBS, whole cells were lysed by incubation at 100 oC for 5 min in 50 μl of ultrapure water. DNA purity was determined by the ratio of O. D. at 260 and 280 nm in a NanoDrop ND1000 (Thermo Scientific).

2.5. Conventional PCR (cnPCR)

The reactions were carried out as described before (Mesquita et al., 2010b). The DNA samples (or controls) and 25 pmol of each primer were added to a kit purchased from Promega (Go Taq Green Master Mix). The PCR mix (12.5μl) was composed of 1 U of Taq DNA polymerase, 10 mM Tris–HCl, pH 8.5; 50 mM KCl; 1.5 mM $MgCl_2$; and 200 mM of each of each dNTP. Each amplification run contained two negative controls (ultrapure water and a negative DNA for toxoplasmosis) and one positive control (DNA extracted from RH strain). The primer pair used was B22/B23, which amplified a 115-bp sequence from a specific repetitive region of the B1 gene as target (Colombo et al., 2005; Burg et al., 1989). To control the course of extraction and check for PCR inhibitors, all samples were assayed using the primer pair b1/b2, which amplified a 140 bp fragment of the human b-globulin gene. After thermal cycles, PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide and visualized under UV illumination.

2.6. Real time PCR (qrtPCR)

The amplifications were performed with an Applied Biosystems 7500 Real Time PCR System as described before (Mesquita et al., 2010a). The clinical samples (or controls) were added to a reaction mixture containing 10 μl of 2× TaqMan Universal PCR Master Mix and 1 μl of the ''Assay Mix'' that included: (i) the forward primer, 18 μM; (ii) the reverse primer, 18 μM; and (iii) the TaqMan MGB probe FAM dye-labeled, 5 μM. The primer set was B1Tg (TaqMan probe, FAM dye-labeled and NFQ as quencher) that amplified an-

other region of B1 gene (Mesquita et al., 2010a). Amplification runs contained two negative controls and one positive control, as used in cnPCR.

2.7. PCR–RFLP for genotyping

The genotypes of T. gondii were performed using multilocus PCR–RFLP, as previously described (Su et al., 2010, 2006). The genetic markers were SAG1, SAG2 (5′-and 3′-SAG2, alt.SAG2) SAG3, BTUB, GRA6, C22-8, c29-2, L358, PK1 and Apico. The set of reaction included: (i) multiplex PCR; (ii) nested PCR; and (iii) amplified product treatment with restriction enzymes. Each reaction set included two negative controls (ultrapure water and a negative DNA for toxoplasmosis); and at least five positive controls (reference strains).

2.8. Data analysis

Clinical and laboratory diagnosis were used to establish patient status. The genotypes were compared, identified and matched to those listed in ToxoDB at http://toxodb.org/toxo/.

3. Results

All patients analyzed in this study had clinical and/or laboratory diagnosis of toxoplasmosis. They had, at least, one positive PCR (cnPCR or qrtPCR); and IgG or IgM antibodies anti-T. gondii, detected by IF (except the patient Ct262 that had negative serology) (Table 2).

DNA samples were isolated from blood, CSF and amniotic fluids of 25 patients with cerebral toxoplasmosis and AIDS, two patients with acute toxoplasmosis, 12 patients with ocular toxoplasmosis, six newborns with congenital toxoplasmosis and 17 pregnant women with acute toxoplasmosis. From these 62 clinical samples, 20 (32%) were successfully genotyped at eight or more genetic loci (Fig. 1 and Table 1). Three genotypes were identified and matched to those listed in ToxoDB. Eighteen of the 20 samples belonged to ToxoDB Genotype #65 and the other two samples, Ct180 and Ct534 belonged to Genotype #71 and Genotype #6, respectively. Genotype #65 was previously identified from chicken and cat in Brazil (isolates IDs: TgCkBr89 and TgCatBr82, both strains were virulent to mice) (Dubey et al., 2008; Pena et al., 2008). Genotype #71 was previously identified from chickens in Brazil (isolates IDs: TgCkBr26 and TgCkBr69) (Dubey et al., 2008). Genotype #6 was identified in different animals and is a major genotype in Brazil (Type BrI). Strains belonging to this genotype are generally virulent to mice (Pena et al., 2008).

The clinical and laboratorial details of the 20 patients, for which T. gondii isolates were thoroughly genotyped, were shown in Table 2. Among the 18 patients infected with Genotype #65 isolates, acute toxoplasmosis was shown in three immunocompetent patients (At704, At358 and At176). One of whom was a woman that developed the acute infection during the pregnancy but without symptomatology (At176). One patient (CT262), negative for HIV and without IgG and IgM to T. gondii, had acute infection with focal cerebral toxoplasmosis. This patient had positive PCR and biopsy results for T. gondii. Focal cerebral toxoplasmosis characterized by multiple expansive brain lesions were observed in six patients (Ct331, Ct384, Ct441, Ct527, Ct581, and Ct244). They were treated with conventional anti-toxoplasmosis treatment (sulphadiazine, pyrimetamine and folinic acid) and five of them were discharged home. One patient (Ct581) had focal cerebral toxoplasmosis and cytomegalovirus encephalitis and eventually died. Toxoplasmic retinochoroiditis scars were shown in one patient with AIDS (Ot539) and six immunocompetents (Ot692, Ot571, Ot607, Ot616, Ot622, and Ot508). Congenital toxoplasmosis was shown in one infant with 6 months (Cot477).

Genotypes #71 and #6 were isolated from two patients with diffuse cerebral toxoplasmosis (Ct180) and (Ct534), respectively. Both patients had atypical cerebral toxoplasmosis, characterized by diffuse encephalitis without visible expansive brain lesions in images. Both cases had positive PCR for T. gondii in CSF and the patient (Ct180, Genotype #71) had brain histopathologic findings consistent with toxoplasmosis, including the identification of stage-specific antigens of T. gondii. These two patients eventually died, despite specific anti-toxoplasmosis therapy.

The 20 patients had active toxoplasmosis at different periods (Table 2) and localities of São Paulo State. Thus, these data demonstrated that there was no indication of any outbreak among these patients.

4. Discussion

Genotyping of T. gondii samples from clinical infections is challenging by the chronic nature of the infection, which is characterized by tissue cysts and an absence of circulating parasites. When in active infection, however, small quantities of T. gondii may enter blood stream and therefore detectable. Multiplex PCR–RFLP made it possible to genotype multiple loci using small among of DNA extracted from tissues such as CSF, amniotic fluid, or blood. As the sensitivity of this method is estimated to be 10-genome equivalent per PCR (Su et al., 2010), only a small portion of clinical samples can be genotyped. Among the 62 clinical DNA samples studied, 32% of them were typed.

The three genotypes determined in these clinical samples were previously identified from farm or pet animal infections in Brazil, indicating the importance of these animals serving as the reservoirs for human infection. Genotype #65 was previously identified in chicken (TgCkBr89) from Rio de Janeiro (14) and cat (TgCatBr82) from Sao Paulo State (Pena et al., 2008). This genotype differs at a single locus (SAG1) from the major genotype BrIV in Brazil (Pena et al., 2008). In our genotyping experiments, we did observed ambiguity of alleles (allele I vs. u-1) at SAG1 locus, but due to low DNA concentration, this problem could not be resolved with confidence. Therefore, there is possibility that this genotype is identical to type BrIV, one of the major lineages in Brazil.

The frequency of Genotype #65 is unexpected, given the highly diverse T. gondii population in Brazil. Dominance of one particular T. gondii genotype in human toxoplasmosis has been reported previously. Analysis of 88 T. gondii isolates associated with toxoplasmosis with immunocompromised patients in France showed that majority of these cases were caused by the clonal type II strains (Ajzenberg et al., 2009). This reflects the high prevalence of type II strains in Europe (Ajzenberg et al., 2002). Recent human toxoplasmosis outbreaks were also reported in South America (Demar et al., 2007; Vaudaux et al., 2010). In each of these cases, oocysts derived from a single T. gondii strain contaminating the environment are the likely source of infection. However, in current study, there was no indication of outbreak in the patients studied, since they had active toxoplasmosis at different periods and localities of São Paulo State.

There are several possible explanations for the dominance of Genotype #65 in these patients. First, PCR–RFLP has a biased sensitivity towards Genotype #65. Second, PCR contamination falsely amplified the same DNA sequence in a large number of samples. Third, Genotype #65 is responsible for considerable part of human infections in patients in Sao Paulo, Brazil. Fourth, Genotype #65 is highly virulent to human and when activated, it can produce larger amount of parasites than other genotypes, therefore becomes easier to be detected by PCR. Since the variation of DNA sequence

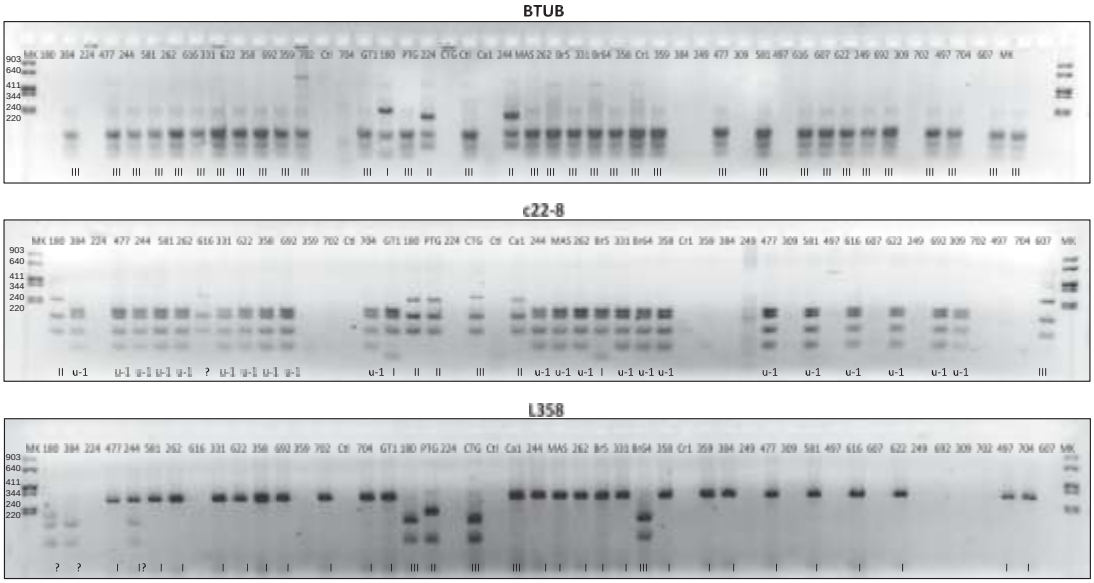

Fig. 1. Representative gel images of RFLP genotyping (markers BTUB, c22-8 and L358). Sample IDs are at the top of the gel images, genotype results are at the bottom. GT1, PTG, CTG, Ca1(TgCgCa1), MAS, Br5(TgCatBr5) and Br64(TgCatBr64) are reference strains (positive controls), Ctl is the negative control. MK is the DNA size marker with 903, 640, 411, 344, 240 and 220 base pair in fragment sizes, respectively.

Table 1
T. gondii genotypes determined in 20 human clinical samples.

Clinical sample code[a]	Markers											
	SAG1	5′ + 3′ SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Genotype
At704	I	I	II	III	III	III	u-1	I	I	nd	I	ToxoDB #65[b]
At358	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
At176	I	I	II	III	III	III	nd	nd	I	III	I	ToxoDB #65
Ct262	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ct384	I	I	II	III	III	III	u-1	I	I	nd	I	ToxoDB #65
Ct527	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ct581	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ct244	I	I	II	III	III	III	u-1	I	I	nd	I	ToxoDB #65
Ct331	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ct441	I	I	II	III	III	III	nd	I	I	nd	nd	ToxoDB #65
Ct180	I	III	III	III	III	III	II	I	III	III	I	ToxoDB #71[c]
Ct534	I	I	I	III	I	II	u-1	I	I	I	I	ToxoDB #6[d]
Ot692	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ot571	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ot622	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ot508	I	I	II	III	III	III	u-1	nd	I	nd	nd	ToxoDB #65
Ot539	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ot607	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Ot616	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65
Cot477	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65

Nd: not determined.
[a] At (acute toxoplasmosis); Ct (cerebral toxoplasmosis); Cot (congenital toxoplasmosis); Ot (ocular toxoplasmosis) and patient number code.
[b] The listed Genotypes were previously identified in chicken (TgCkBr89) and cat (TgCatBr82) isolates (14, 16).
[c] The listed Genotypes were previously identified in chickens (TgCkBr26 and TgCkBr69) (14).
[d] The listed Genotypes were previously identified in cat (TgCatBr2) and chicken (TgCkBr144) isolates. This genotype is also known as type Brl (16). All from Brazil and published in ToxoDB (http://toxodb.org/toxo/).

among different T. gondii strains is limited (less than 1%), it is not likely the first scenario will be true. Since direct typing of Toxoplasma in tissue samples is very sensitive to contamination, particularly true when DNA concentration of Toxoplasma is close to or below detection threshold. It is possible that the high frequency of Genotype #65 is the result of contamination. However, in our experiments, both positive and negative controls were included to monitor contamination (Fig. 1), and questionable results were re-tested to rule out contamination. To further minimize the influence of potential contamination, we excluded all samples that can't be successfully typed for more than eight out of the 11 loci, assuming these samples have low concentration and were not suitable

Table 2
Clinical and laboratory diagnostic results of the 20 patients with toxoplasmosis listed in Table 1.

Clinical Sample code/sample collection[a]	Age (years)	Diagnosis[b] (clinical, images and laboratory data)	cnPCR	qrtPCR[c] (CT)	IF-IgG[d]	IF-IgM[d]	$CD4^+$ lym counts[e]	HIV (serology)	Brain histo-pathology for T. gondii	In hospital outcome	Toxoplas-mosis treatment	Genotype
At704-Bl-11/09	33	Acute toxoplasmosis	Pos	22.7	1:256	1:16	nd	Neg	nd	Alive	Yes	ToxoDB #65
At358-Bl-08/08	4	Acute toxoplasmosis	Pos	31.9	1:1024	1:1024	nd	Neg	nd	Alive	Yes	ToxoDB #65
At176-Af*-04/07	19	Acute toxoplasmosis (pregnancy – asymptomatic)	Pos	nd	1:16	Neg	nd	Neg	nd	Alive	Yes	ToxoDB #65
Ct262-Bl-10/07	29	Acute and focal cerebral toxoplasmosis	Pos	nd	Neg	Neg	498	Neg	pos (biopsy)	Alive	Yes	ToxoDB #65
Ct384-Bl-09/08	39	Focal cerebral toxoplasmosis	Pos	36.3	1:4000	Neg	184	Pos	nd	Alive	Yes	ToxoDB #65
Ct527-Bl-05/09	36	Focal cerebral toxoplasmosis	Pos	32	1:4000	Neg	89	Pos	nd	Alive	Yes	ToxoDB #65
Ct581-Bl-08/09	10	Focal cerebral toxoplasmosis and cytomegalovirus encephalitis	Pos	22.6	1:4000	Neg	2	Pos	nd	Death**	Yes	ToxoDB #65
Ct244-Bl-08/07	39	Focal cerebral toxoplasmosis	Pos	nd	1:256	Neg	56	Pos	nd	Alive	Yes	ToxoDB #65
Ct331-Bl-06/08	42	Focal cerebral toxoplasmosis	Pos	27.3	1:256	Neg	9	Pos	nd	Alive	Yes	ToxoDB #65
Ct441-Bl-12/08	42	Focal cerebral toxoplasmosis	Pos	29.9	1:16	Neg	17	Pos	nd	Alive	Yes	ToxoDB #65
Ct180-CSF-05/07	40	Diffuse cerebral toxoplasmosis	Pos	31.0	1:4000	Neg	93	Pos	pos (necropsy)	Death	Yes	ToxoDB #71
Ct534-Bl/CSF-06/09	45	Diffuse cerebral toxoplasmosis	Pos	32.9	1:4000	Neg	8	Pos	nd	Death	Yes	ToxoDB #6
Ot692-Bl-11/09	27	Ocular toxoplasmosis	Pos	32	1:16	Neg	nd	Neg	nd	Alive	Yes	ToxoDB #65
Ot571-Bl-08/09	77	Ocular toxoplasmosis	Pos	36	1:16	Neg	nd	Neg	nd	Alive	Yes	ToxoDB #65
Ot622-Bl-11/09	54	Ocular toxoplasmosis	Pos	28.1	1:16	Neg	nd	Neg	nd	Alive	Yes	ToxoDB #65
Ot508-Bl-03/09	14	Ocular toxoplasmosis	Pos	35	1:4000	Neg	299	Neg	nd	Alive	Yes	ToxoDB #65
Ot539-Bl-06/09	56	Ocular toxoplasmosis	Pos	28	1:16	Neg	nd	Pos	nd	Alive	Yes	ToxoDB #65
Ot607-Bl-09/09	36	Ocular toxoplasmosis	Pos	36.4	1:1024	Neg	nd	Neg	nd	Alive	Yes	ToxoDB #65
Ot616-Bl-09/09	60	Ocular toxoplasmosis	Pos	25	1:16	Neg	nd	Neg	nd	Alive	Yes	ToxoDB #65
Cot477-Bl-01/09	0.6	Congenital toxoplasmosis	Pos	18	1:16	Neg	nd	Ng	nd	Alive	Yes	ToxoDB #65

[a] At (acute toxoplasmosis); Ct (cerebral toxoplasmosis); Cot (congenital toxoplasmosis); Ot (ocular toxoplasmosis); patient number and clinical sample: Bl, blood; Af, amniotic fluid and CSF, cerebrospinal fluid. Date (month/year) of the clinical sample collection.
[b] Diagnosis was defined by clinical, images and laboratory data as described in Section 2.
[c] qrtPCR results were shown in CT (cycle threshold). Values below 40 were considerate positive (Mesquita et al., 2010a,b).
[d] Reagent serum and CSF were considered up to 1:16 and 1:4, respectively.
[e] Number of $CD4^+$ T lymphocytes/μl of blood.
* IF were determined in serum sample.
** Cytomegalovirus encephalitis was concomitant to diagnosis of cerebral toxoplasmosis. nd, no determined; neg, negative; pos, positive.

for direct genotyping. Though contamination cannot be ruled out completely in these experiments, it is not likely the major explanation to the genotyping results. The third and fourth scenarios are of biological significance. However, direct genotyping of clinical samples cannot distinguish the two possibilities. Therefore, isolate T. gondii strains for further analysis is absolutely necessary.

The other genotypes identified were Genotype #71 which was previously identified in chicken (TgCkBr26 and TgCkBr69) from Rio de Janeiro (Dubey et al., 2008). The genotype #6 is the major virulent lineage type BrI previously reported in Brazil (Ferreira et al., 2008). It was also the cause of waterborne outbreak of toxoplasmosis in Santa Isabel in Brazil (Vaudaux et al., 2010). Both Genotype #71 and #6 were isolated from patients with severe and diffuse cerebral toxoplasmosis. Although scarce, there are some prior reports about disease presentation of human toxoplasmosis and parasite genotypes, particularly related to non-clonal genotypes from North America (Grigg et al., 2001), Europe (Bossi and Bricaire, 2004; Delhaes et al., 2010), and Brazil (Khan et al., 2006). In addition, a recent study showed that T. gondii causes more severe ocular disease in congenitally infected children in Brazil compared with Europe and suggest that marked differences may be due to infection with more virulent genotypes of the parasite that predominate in Brazil but are rarely found in Europe (Gilbert et al., 2008). However, the extent to which host genetics, immune status, parasitic burden, parasitic genotype, and exposure rate contribute to clinical disease is unknown.

This study showed that it is possible to directly genotype clinical T. gondii infection in human patients using multilocus PCR–RFLP method. The results of this study revealed an unusual high frequency of Genotype #65. This can be explained by two possibilities: (i) Genotype #65 is responsible for a large proportion of human infections in Sao Paulo, Brazil; or (ii) Genotype #65 is highly virulent to human and can lead to high parasite burden in tissues than other genotypes, therefore can be preferentially detected by PCR. This study highlighted the need to isolate T. gondii strains from patients for further analysis and to evaluate potential association of T. gondii Genotype #65 with toxoplasmosis in human patients.

Acknowledgments

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Resumo I

Abstract ID: ABSJF-FC3JX-MM7KL-BP33R

Brazilian case report: Postnatal diagnosis of gestational and congenital toxoplasmosis of a newborn and his mother due to an atypical *Toxoplasma gondii* strain

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Introduction: The diagnosis of toxoplasmosis in Brazil is a challenge for healthcare professionals due to the diversity of strains and the difficulty to access news technologies. Objective: The aim of this study is to report on a case of congenital toxoplasmosis characterized by an unusual discrepancy between serology and molecular tests. *Methods: History - Mother:* The pregnant was referred to the high-risk antenatal unit with a gestational age of 28 weeks, because of the echographic findings. By the Advia Centauro® automated method, she was IgM- and IgG- for anti-*Toxoplasma* antibodies in both prenatal and postnatal periods. She lived in a rural area and reported that she ate free-range chicken but did not drink unpasteurized milk. After the baby's birth, peripheral blood samples were submitted to PCR and ELISA tests. *Newborn:* Birth by normal delivery at 33 weeks (Capurro method) with weight 3360g,

Apgar 4/7, the infant presented with hepatosplenomegaly, petechia, thrombocytopenia, seizures and a clinical picture of congenital toxoplasmosis. By TORCH syndrome investigation, the infant's serology was IgM- and IgG- for toxoplasmosis using automated method; he was not treated at this time for toxoplasmosis. At 26 days, samples of CSF and peripheral blood were sent for analysis by cn-PCR and qrt-PCR. Markers used in both reactions amplified a segment of the *B1* gene of *T.gondii*. In parallel, the *T. gondii* isolate was genotyped; serum and CSF were tested by ELISA and IFI with antigens extracted from tachyzoites of an in-house RH strain. Results: Both serum and CSF were seronegative according to serological tests (ELISA and IFI), thereby confirming the results of the automated method. However the cn-PCR results were positive for blood and qrt-PCR results were positive for CSF. Genotyping showed that the mother and newborn hosted an atypical strain of *T. gondii*. Conclusions: although the serological tests showed negative results, molecular methods (cn-PCR and qrt-PCR) confirmed congenital infection by an atypical strain of *T. gondii* in agreement with the clinical diagnosis of the baby. This case report shows that serological tests alone, although widely used, are insufficient to elucidate cases of infection by atypical strains of *T. gondii*, and so molecular tests with higher specificity are needed.

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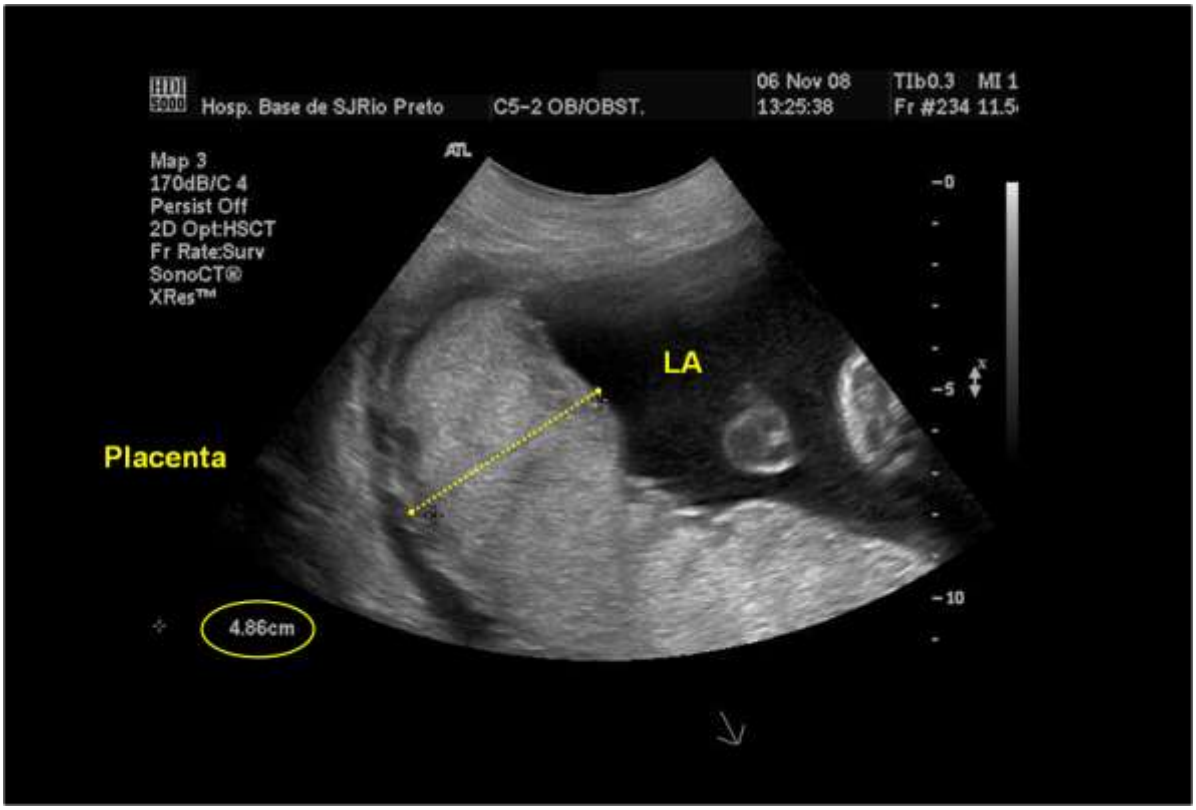

Figura 1: Imagem ultrasonográfica apresentando placenta com implantação posterior, com grau I de maturidade, espessura aumentada de 4,86cm, quantidade de líquido amniótico aumentado (ILA: 21,29), realizada em equipamento com sonda convexa multifrequencial. (Philips, HDI 5000)

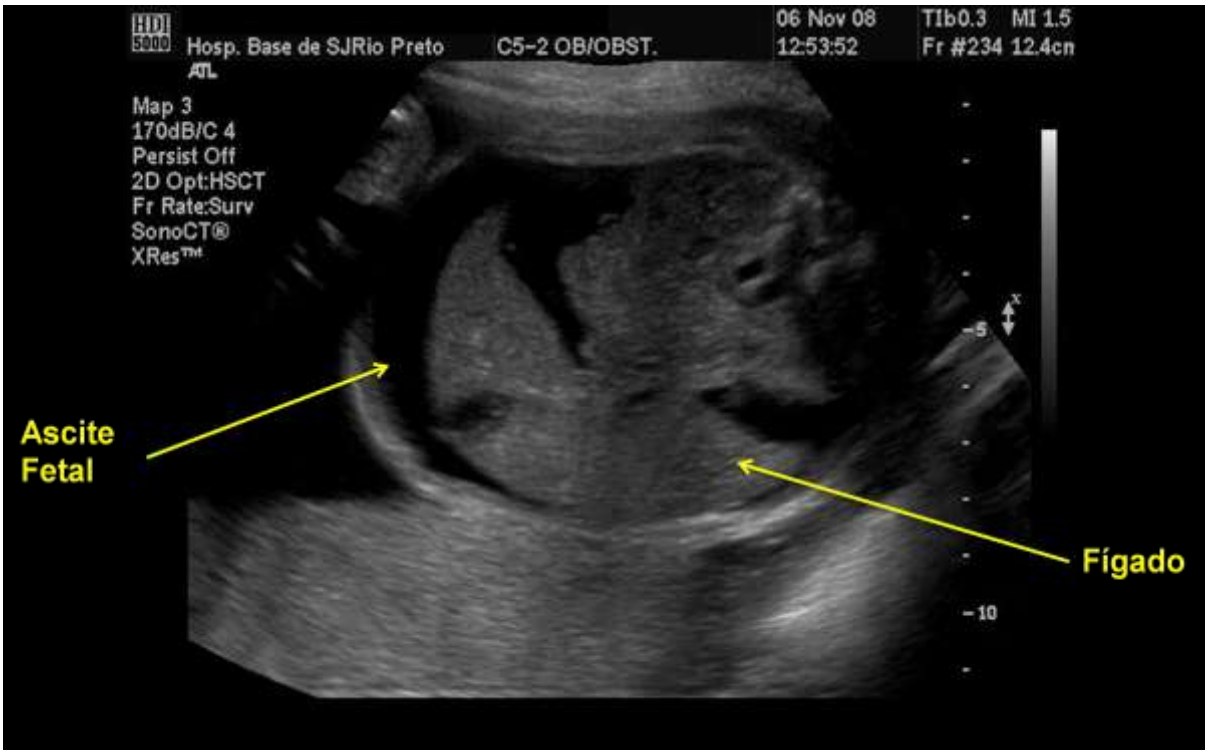

Figura 2: Imagem ultrasonográfica de abdome fetal, com idade de 28 semanas e 6 dias, apresentando ascite fetal, realizada em equipamento com sonda convexa multifrequencial. (Philips, HDI 5000)

A13. **Epidemiological data from 5 patients with toxoplasmic retinochoroiditis and infected by toxodb#65 strain**

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Toxoplasmic retinochoroiditis (TR) is the most common ocular lesion caused by *Toxoplasma gondii* and may occur after the congenital/acquired infection immediately or lately by reactivation. The lesions are often necrotic destroying the architecture of the neural retina and sometimes involving the choroid. It has been reported as more severe in Brazil than in Europe. Here we report epidemiological findings from 5 Brazilian patients (3 women and 2 men) with TR living in a country region of São Paulo State. Serological and molecular diagnosis were made. Next, multilocus PCR-RFLP was used to genotype the *T. gondii* DNA isolates, using the genetic markers SAG1, SAG2 (5´-and-3´) newSAG2, SAG3, BTUB, GRA6, C22-8, c29-2, L358, PK1 and Apico. Epidemiological data like age, education, eating habits, pets, among others were collected. All of them were positive for anti-*T. gondii* IgG antibodies, determined by ELISA and indirect immunofluorescence; and for PCR, using a specific regionfrom gene B1 as target. The isolates were characterized as toxodb#65 strain, a usual Brazilian *T. gondii* genotype. The patients lived in small cities nearby São José do Rio Oreto, São Paulo State, Brazil, in urban area with sewage and reported drinking tap water. Among them, 4 received less than 2 miminum wages and 1 was unemployed; in some moment of his lives, 2 receives blood transfusion, 4 had pets (cats and dogs), 2 usually barefoot, 3 drank crude milk, 2 ate undercooked or raw meat, 1 had higher education and 4 had less than 8 years of schooling. These results show that TR in the country region of São Paulo affecting mainly low income community and low school levels. Besides, it confirms that eating habits and pets are associated with the infection by *T. gondii.*

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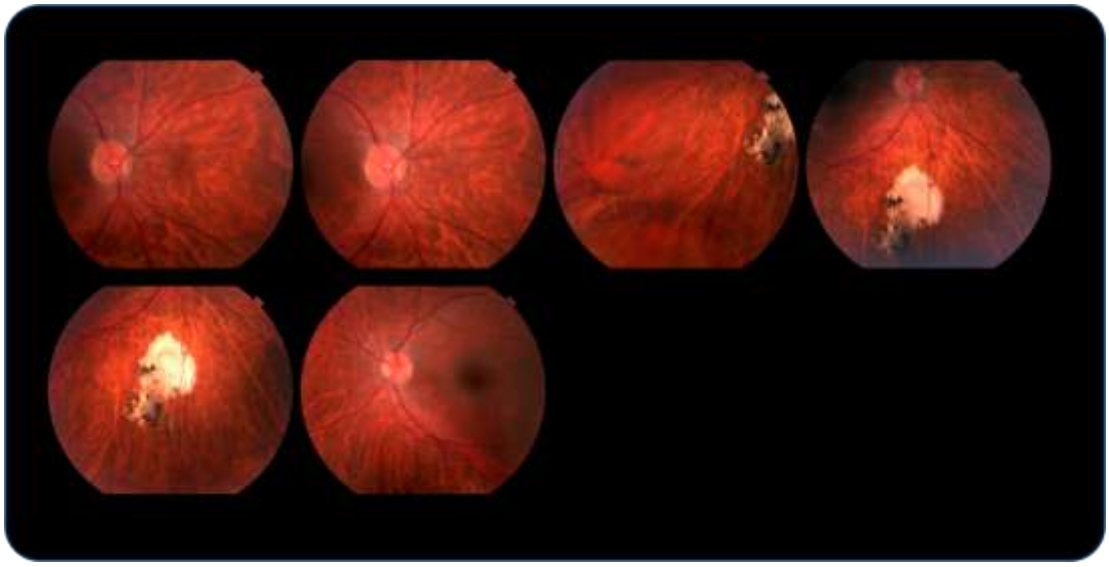
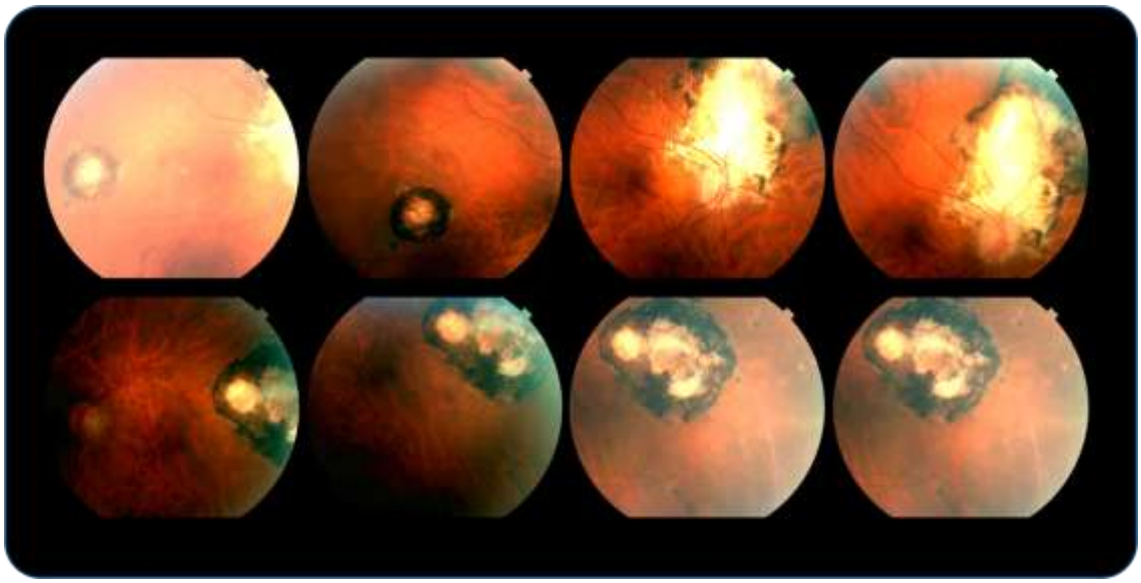


Case 1. Female, 27 years old, toxoplasmic retinochoroiditis scars in right eye, live in urban area of Jales city, with sewage and reported drink tap water, had pets (dog and cat), usually barefoot, eat undercooked or raw meat, higher education, recieves 1.5 minimum wages.



Case 2. Female, 60 years old, toxoplasmic retinochoroiditis scars in both eyes, live in urban area of Mirassol city, with sewage and reported drink tap water, had pets (dog and cat), no eat undercooked or raw meat, drink unpasteurized milk, primary school level, receives 2 minimum wages.

Comentários finais

O objetivo geral desta tese foi avaliar a infecção por *T. gondii* em gestantes, neonatos e portadores de doenças oculares, e analisar a aplicabilidade dos métodos PCR convencional e PCR em tempo real no diagnóstico de infecção em amostras de sangue periférico de portadores de doenças oculares, bem como caracterizar cepas deste parasito.

Três aspectos fundamentaram a decisão de conduzir o estudo na região noroeste do estado de São Paulo. Primeiro, esta região apresenta elevado índice de infecção por *T. gondii* e este fato atrai a atenção para investigações de natureza epidemiológica, diagnóstica e molecular que contribuem para o conhecimento e a caracterização de uma doença que, embora ainda não tenha status de negligenciada e não seja de notificação compulsória, representa um importante problema de saúde.

Segundo, esta região apresenta considerável desenvolvimento econômico, social e demográfico, bem como infra-estrutura de saneamento básico que contribuem para a prevenção e a redução de casos de doenças infecciosas e parasitárias que ainda comprometem a qualidade de vida em outras áreas do país, incluindo aquelas disseminadas por água e alimentos contaminados. Estas características foram ponderadas durante a elaboração do projeto original uma vez que deveriam estar contribuindo com a redução da infecção por *T. gondii*.

Terceiro, os Ambulatórios de Gestação de Alto Risco e Medicina Fetal e de Oftalmologia do complexo FAMERP/FUNFARME são referências em atendimento médico nessa região do estado de São Paulo e favorecem o acesso a um grande número de pacientes com condições clínicas bem definidas, assim como às amostras biológicas indispensáveis para as análises realizadas.

Os resultados apresentados nos artigos que compõe esta tese corroboram observações e conclusões anteriormente publicadas, mas também acrescentam novos conhecimentos sobre a importância do *T. gondii* no eixo materno-fetal, no diagnóstico molecular e na caracterização genotípica de cepas deste parasito que circulam em nossa região.

Poucos estudos epidemiológicos sobre a infecção por *T. gondii* e as doenças dela decorrente foram anteriormente conduzidos no noroeste paulista e não há relatos acessíveis sobre a variabilidade genética do *T. gondii* em isolados e nem em sangue periférico dos residentes na região. Portanto, parte do trabalho que compõe esta tese é pioneiro no que se refere à investigação da toxoplasmose no noroeste paulista.

Os resultados alcançados com o primeiro artigo permitem concluir que prevalência de infecção por *T. gondii* em gestantes de alto risco atendidas em um ambulatório especializado do noroeste paulista é elevada, em comparação a outras doenças infecciosas e parasitarias de importância gestacional. A

obtenção de dados desta natureza pode ser de grande valor na adoção de medidas de proteção a saúde da mulher.

Embora os resultados deste artigo sejam baseados em dados de prontuários médicos de gestantes atendidas entre 2006 e 2007 no Ambulatório de Gestação de Alto Risco, os índices encontrados mostram que quase dois terços delas apresentam anticorpos anti-*T. gondii* (62,0% IgG; 3,4% IgM). A presença destas IgG, quando de alta avidez, confere relativa proteção imune e reduz sensivelmente os riscos de transmissão congênita. Contudo, a presença de IgM e de IgG de baixa avidez, além de não excluir tal risco, representa um indicador de risco e desperta a atenção para a transmissão congênita da toxoplasmose.

O segundo artigo consistiu na análise pareada de amostras de soro de gestantes e seus respectivos neonatos e os resultados alcançados confirmaram as observações feitas no primeiro artigo. Além, de confirmar o elevado índice de infecção por este parasito, seus resultados demonstraram que a maioria das gestantes apresentou anticorpos anti-*T. gondii* de alta avidez (acima de 30%). Estes dados são importantes, pois reforçam as conclusões do primeiro artigo de que a maioria das gestantes apresenta relativa proteção imune a este parasito resultante do desenvolvimento de imunidade humoral.

Pequeno percentual (2,3%) das gestantes analisadas apresentaram anticorpos IgM anti-*T. gondii*. Como estes anticorpos constituem um importante indicador

de infecção/doença de fase aguda, fica evidente que a transmissão congênita do *T. gondii* está ocorrendo na região noroeste. Portanto, os aspectos econômicos, sociais e demográficos e a infra-estrutura de saneamento básico da região noroeste não estão isoladamente contribuindo para a redução da transmissão da toxoplasmose congênita. Este aspecto desperta a atenção para a necessidade de tomadas de medidas adicionais de educação da população da região para a prevenção da toxoplasmose.

O terceiro artigo traz importantes contribuições para o diagnóstico laboratorial da toxoplasmose ocular. O diagnóstico sorológico da infecção por *T. gondii* predominou durante as últimas décadas e foi reforçado com a introdução de kits comerciais. A demonstração deste parasito no sangue ficava restrita a casos de doença em fase aguda. Entretanto, a clonagem e o seqüenciamento de parte do genoma do *T. gondii* abriu novas oportunidades para o diagnóstico molecular, inclusive em indivíduos com perfil sorológico típico de infecção crônica.

O uso dos métodos PCR convencional e PCR em tempo real foi crucial na demonstração do material genômico de pacientes com cicatrizes retinocoroidianas e infecção crônica bem como naqueles com infecção aguda por *T. gondii*. A sensibilidade observada na análise molecular foi baixa em comparação a especificidade e é possível que esta diferença decorra da baixa parasitemia que os pacientes apresentam tanto na fase aguda como na fase

crônica da infecção. Estas observações corroboram relatos previamente publicados no Brasil.

Outra grande contribuição deste artigo é que o uso de antígenos extraídos e secretados (ESA) por *T. gondii* na investigação de anticorpos contra este parasito revela reatividade diferencial entre o soro de pacientes com infecção ativa e com infecção crônica.

Finalmente, o quarto artigo representa um avanço na investigação da toxoplasmose ocular na região noroeste do Estado de São Paulo. Em suas análises foram incluídas amostras de pacientes com outras formas de infecção por *T. gondii*, mas aquelas coletadas de pacientes com toxoplasmose ocular indicam que o genótipo ToxoDB#65 prevalece nesta região. Este genótipo, caracterizado com o uso do método PCR-Multiplex, foi previamente identificado em isolados de animais domesticados no Brasil.

Estudos adicionais voltados a identificar os genótipos de *T. gondii são* necessários para determinar se outros genótipos são encontrados na região uma vez que este parasito Apicomplexa apresenta considerável diversidade genética. Além disso, seria adequado avaliar se o genótipo ToxoDB#65 ou ainda outros presentes na região noroeste do Estado de São Paulo tem impacto clínico no desenvolvimento da toxoplasmose ocular bem como na reativação da infecção.

Em síntese, os resultados apresentados nesta tese estão alinhados a importância da investigação da toxoplasmose no Brasil e apresentam dados valorosos que poderão contribuir para a tomada de medidas educacionais da população, bem como de esclarecimento e de orientação para a toxoplasmose ocular na região noroeste do Estado de São Paulo.

Conclusão

1. Os artigos integrantes desta tese demonstram altos índices de infecção por *T. gondii* em gestantes e sugere os índices de infecção congênita para a região. Além disso, descreve pela primeira vez a toxoplasmose ocular na região;

2. Os métodos de cnPCR e qPCR auxiliam na caracterização laboratorial e clínica da toxoplasmose ocular, pode ser utilizado em amostras de sangue periférico de pacientes com infecção crônica e com suspeita de toxoplamose congênita;

3. É possível genotipar *T. gondii* a partir de amostra de sangue periférico de indivíduos acometidos

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Anexo I – Parecer Comitê de Ética em Pesquisa FAMERP

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
Autarquia Estadual - Lei n.° 8899 de 27/09/94
(Reconhecida pelo Decreto Federal n.° 74.179 de 14/06/74)

Parecer n.° 295/2008

COMITÊ DE ÉTICA EM PESQUISA

O Protocolo nº 4534/2008 sob a responsabilidade de Luiz Carlos de Mattos com o título "Diagnóstico laboratorial da toxoplasmose com enfoque nas infecções congênitas e cerebral" está de acordo com a resolução CNS 196/96 e foi aprovado por esse CEP.

Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa em Seres Humanos (CEP) deverá receber relatórios semestrais sobre o andamento do Estudo, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, para conhecimento deste Comitê. Salientamos ainda, a necessidade de relatório completo ao final do Estudo.

São José do Rio Preto, 11 de agosto de 2008.

Prof. Dr. Antonio Carlos Pires
Coordenador do CEP/FAMERP

Av. Brigadeiro Faria Lima, 5416 - 15090-000 - São José do Rio Preto - SP - Brasil
Tel. (17) 3201-5700 - Fax (17) 3229-1777 - www.famerp.br

Anexo II – Parecer Comitê de Ética em Pesquisa FAMERP – Extensão

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO

Autarquia Estadual - Lei n.° 8899 de 27/09/94
(Reconhecida pelo Decreto Federal n.° 74.179 de 14/06/74)

COMITÊ DE ÉTICA EM PESQUISA

O Comitê de Ética em Pesquisa em Seres Humanos da Faculdade de Medicina de São José do Rio Preto aprovou a extensão datada de 01 de dezembro de 2010, referente ao protocolo n.º **4534/2008** sob a responsabilidade de **Luiz Carlos de Mattos** com o título "Diagnóstico laboratorial da toxoplasmose com enfoque nas infecções congênitas e cerebral".

São José do Rio Preto, 07 de dezembro de 2010.

Prof. Dr. Fernando Batigália
Coordenador do CEP/FAMERP

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Anexo III – Parecer Comitê de Ética em Pesquisa FAMERP

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
Autarquia Estadual - Lei n.º 8899 de 27/09/94

Parecer n.º 319/2008

COMITÊ DE ÉTICA EM PESQUISA

O Protocolo nº 4533/2008 sob a responsabilidade de Vera Lucia Pereira Chioccola com o título "Genotipagem de cepas polimórficas de Toxoplasma gondii provenientes de pacientes com Toxoplasmose" está de acordo com a resolução CNS 196/96 e foi aprovado por esse CEP.

Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa em Seres Humanos (CEP) deverá receber relatórios semestrais sobre o andamento do Estudo, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, para conhecimento deste Comitê. Salientamos ainda, a necessidade de relatório completo ao final do Estudo.

São José do Rio Preto, 11 de agosto de 2008.

Prof. Dr. Antonio Carlos Pires
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