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CENTRO DE SAÚDE E TECNOLOGIA RURAL
CAMPUS DE PATOS – PB
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

Aspectos epidemiológicos da toxoplasmose em suínos e galinhas de criações domésticas no estado da Paraíba, Brasil: Soroprevalência e fatores de risco, isolamento e genotipagem de *Toxoplasma gondii*

THAIS FERREIRA FEITOSA

PATOS-PB
2017

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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal de Campina Grande como requisito parcial para a obtenção do título de Doutor em Medicina Veterinária.

THAIS FERREIRA FEITOSA

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RESUMO

A prevalência da infecção e as cepas de *T. gondii* em galinhas e suínos variam de acordo com a região estudada e, no estado da Paraíba pouco se sabe sobre a prevalência e genótipos de *T. gondii* que circulam em galinhas e suínos. Portanto, este trabalho teve como objetivo realizar a sorologia, isolamento e genotipagem de *T. gondii* proveniente de suínos e galinhas do estado da Paraíba, Brasil. Para isso, foram realizadas coletas de amostras sanguíneas de 483 galinhas e 120 suínos, nestas amostras sanguíneas foi realizado o teste de Reação de Imunofluorescência Indireta (RIFI) para anticorpos anti-*T. gondii*. Nos animais positivos foi realizado o bioensaio em camundongos para isolamento do protozoário. Nos isolados obtidos foi realizado a genotipagem por PCR-Polimorfismo no Comprimento do Fragmento de Restrição (PCR-RFLP) utilizando 12 marcadores genéticos (SAG1, SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L258, PK1, CS3 e Apico). Em galinhas, observou-se uma prevalência de 31.5% (152/483) para *T. gondii* e dos 71 animais submetidos ao bioensaio em camundongos, foram obtidos 33 (46.5%) isolados. Nos suínos, verificou-se 12,5% (15/120) de animais positivos na RIFI e foi possível isolar o parasito em 80% (12/15) dos bioensaios realizados. Conseguiu-se revelar os genótipos completos em 30 dos 33 isolados de galinhas submetidos a genotipagem. Foram identificados nove genótipos, destes, três foram identificados como novos em seis isolados. Já nos suínos foram utilizados os 12 isolados desta pesquisa e, adicionalmente, 13 isolados de estudos anteriores, obteve-se o genótipo completo de 19, e estes foram agrupados em 12 genótipos, três inéditos. O genótipo mais frequente tanto em suínos como em galinhas foi o #013. Não foi observado nenhum tipo clonal I, II e III. Observou-se os genótipos BrIII, #13, #116 e três inéditos presentes simultaneamente em suínos e galinhas. Conclui-se que a prevalência de *T. gondii* em galinhas e suínos é alta no estado da Paraíba, que é alta a diversidade genética do parasito e que existem genótipos que circulam frequentemente entre suínos e galinhas no estado da Paraíba.

Palavras-chave: Bioensaio, Marcadores genéticos, PCR-RFLP, RIFI.

Feitosa, Thais Ferreira. "Epidemiological aspects of toxoplasmosis in domesticated pigs and chickens in the state of Paraíba, Brazil: Seroprevalence and risk factors, isolation and genotyping of *Toxoplasma gondii*". Patos, PB: UFCG, 2017. 137p. (Thesis-Doctorate Degree in Veterinary Medicine)

ABSTRACT

The prevalence of infection and the strains of *T. gondii* in chickens and pigs vary according to the region studied. In the state of Paraíba a little is known about the prevalence and genotypes of *T. gondii* that circulate in these animals. Therefore, this study aimed to perform serology, isolation and genotyping of *T. gondii* from pigs and chickens from the state of Paraiba, Brazil. Blood samples from 483 chickens and 120 pigs were collected. In these blood samples, the Indirect Fluorescente Antibody Test (IFAT) was performed for anti-*T. gondii* antibodies. For positive animals it was accomplished the mouse bioassay for isolation of the parasite. In the isolated obtained, PRC-RFLP genotyping was performed using 12 markers: SAG1, SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L258, PK1, CS3 e Apico. In chickens, it was observed the prevalence of 31.5% (152/483) for *T. gondii* and regarding the 71 animals submitted to the bioassay, 33 (46.5%) were isolated. In pigs, it was verified 12.5% (15/120) of the positive animals in the IFAT and it was possible to isolate the parasite in 80% (12/15) of the bioassays performed. It was possible to reveal the complete genotypes in 30 of the 33 isolates of chickens submitted to genotyping. Nine genotypes were identified, from these; three were identified as novel in six isolates. In the pig's evaluation, 12 isolates from this research were used, and also 13 isolates from previous studies. It was obteined the complete genotype from 19 isolates that were arranged in 12 genotypes, three of them unpublished. In both genotypes of pigs and chickens, the genotype # 013 was the most frequent. It was not observed in any clonal type I, II and III. It was observed the genotypes BrIII, # 13, # 116 and three unpublished genotypes present in pigs and chickens. It can be concluded that the prevalence of *T. gondii* in chickens and pigs is high in the state of Paraiba. The genetic diversity of the parasite is also high and there are genotypes that often circulate among pigs and chickens in the state of Paraiba.

Keywords: Bioassay, Genetic markers, IFAT, PCR-RFLP.

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INTRODUÇÃO

A toxoplasmose é uma enfermidade de distribuição mundial, causada pelo protozoário *Toxoplasma gondii*, que acomete o homem, animais domésticos e selvagens (DUBEY; BEATTIE, 1988). Os felinos domésticos e silvestres são os hospedeiros definitivos, sendo o gato (*Felis catus domesticus*), o principal.

Os hospedeiros susceptíveis podem se infectar por *T. gondii* por meio das seguintes formas primárias: transmissão transplacentária, ingestão de tecidos de animais contendo cistos infectantes e ingestão de água e alimentos contaminados com fezes de gatos contendo oocistos esporulados (DUBEY; BEATTIE, 1988).

Em suínos, assim como em outras espécies, os principais problemas causados por *T. gondii* são de ordem reprodutiva, como abortamento, natimortalidade e mumificação fetal, em fêmeas que se infectam pela primeira vez durante a gestação (TSUTSUI et al., 2003). Portanto, o parasita pode ter um importante papel na suinocultura. Esta espécie animal é considerada, entre os animais de produção, a mais importante fonte infecção de Toxoplasmose para o ser humano (DUBEY, 2010; TENTER et al., 2000).

As galinhas são consideradas resistentes à toxoplasmose, nesta espécie a doença cursa predominantemente na forma subclínica. Entretanto, alguns relatos de toxoplasmose em galinhas são descritos na literatura e os principais sintomas observados são torcicolo, incapacidade de ficar em pé, decúbito lateral (Dubey et al. 2007a). As galinhas de criações domésticas são consideradas importantes na epidemiologia desta doença, pois são fontes de transmissão tanto para o homem quanto para o gato, favorecendo, através deste último, a disseminação da doença pela eliminação de oocistos no meio ambiente (DUBEY, 2010).

É importante o sorodiagnóstico entre animais de produção, por sinalizar a contaminação do espaço rural, uma vez que estes animais estão em contato direto com o meio ambiente por longos períodos (BONNA et al., 2006). Tendo em vista a importância dos suínos e galinhas na transmissão de *T. gondii*, vários estudos soroepidemiológicos já foram descritos em várias partes do mundo, inclusive no Brasil. Em suínos, Valença et al. (2011) observaram uma prevalência de 26,9% de soropositivos criados em granjas tecnificadas no estado de Alagoas. Já em Pernambuco, Fernandes et al. (2011) encontraram soropositividade em 9,78% dos suínos estudados,

enquanto que na Paraíba em um estudo de prevalência realizado em 2013, Feitosa et al. (2014) encontraram 19,5% de animais soropositivos.

Estudos de soroprevalência apontam as galinhas de criação doméstica com alto índice de positividade para *T. gondii*. Dubey et al. (2007b), realizaram um estudo em galinhas provenientes do Pará e Rio Grande do Sul e verificaram 46,4% de animais soropositivos, assim como Oliveira et al. (2008) verificaram 53,3% de animais sororreagentes em todos os estados do Nordeste, exceto na Paraíba, que não participou desse estudo.

Estudos demonstram ampla variabilidade genética dos isolados brasileiros quando comparados aos da Europa e dos Estados Unidos, onde são encontrados mais comumente os Tipos clonais clássicos I, II e III. No Brasil, a maioria das amostras encontradas são atípicas e algumas linhagens são consideradas clonais brasileiras e designadas BrI, BrII, BrIII e Br IV. Levando em consideração a taxa de mortalidade em camundongos infectados, estas linhagens foram classificadas como virulenta (BrI), não virulenta (BrIII) e virulenta intermediária (BrII e BrIV) (PENA et al., 2008).

Devido à importância da toxoplasmose como zoonose, é de suma importância a investigação de genótipos provenientes de infecções animais, com o objetivo de verificar a correlação entre a variante encontrada e suas propriedades biológicas, bem como rastrear epidemiologicamente o agente para identificação de fontes de infecção ou vias de transmissão (OWEN; TREE, 1999).

Sabendo da importância das galinhas na transmissão da toxoplasmose para os seres humanos e animais, e ainda da incipiência de pesquisas sobre esta doença no estado da Paraíba, este estudo foi realizado objetivando conhecer a situação soroepidemiológica das galinhas caipiras abatidas neste estado, e, por meio do isolamento do parasita, determinar o risco real do consumo e manipulação dessas carnes cruas ou mal passadas pelas populações humana e animal. Sequencialmente, o estudo molecular desses isolados juntamente com os isolados de suínos, acrescentará novas informações sobre a distribuição dos genótipos de *T. gondii* e sua diversidade no Brasil.

Esta Tese de Doutorado é composta por três capítulos constituídos por artigos científicos originais. O Capítulo I é composto por um artigo publicado na revista Parasitology Research - Qualis A2, e descreve pela primeira vez um estudo epidemiológico no estado da Paraíba acerca do *T. gondii* em galinhas, sendo realizados a sorologia e isolamento do parasita. O Capítulo II é composto por um artigo submetido à revista Parasitology Research – Qualis A2, e neste, foi realizada a caraterização

genética dos isolados de *T. gondii* obtidos de galinhas no estado da Paraíba. O Capítulo III é composto por um artigo submetido à Veterinary Parasitology – Qualis A2, e objetivou realizar a sorologia, isolamento e caraterização genética de *T. gondii* em suínos abatidos no estado da Paraíba.

REFERÊNCIAS

BONNA, I.C.F; FIGUEIREDO, F.B; COSTA, T.; VICENTE, R.T.; SANTIAGO, C.A.D.; NICOLAU, J.L.; NEVES, L.B.; MILLAR, P.R.; SOBREIRO, L.G.; AMENDOEIRA, M.R.R. Estudo Soroepidemiológico da infecção por *Toxoplasma gondii* em suínos e frangos para abate em região rural do Rio de Janeiro. **Revista Brasileira de Ciência Veterinária**, v. 13, p. 186-189, 2006.

DUBEY, J.P.; BEATTIE, C.P. **Toxoplasmosis of animals and man**. Boca Raton, Florida: CRC Press, 1988, p. 220.

DUBEY J.P.; WEBB, D.M.; SUNDAR, N.; VELMURUGAN, G.V.; BANDINI, L.A.; KWOK, O.C.H., SU, C. Endemic avian toxoplasmosis on a farm in Illinois: clinical disease, diagnosis, biologic and genetic characteristics of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*), and a goose (*Anser anser*). **Veterinary Parasitology**, 148, 207-212, 2007a.

DUBEY, J.P.; SUNDAR, N.; GENNARI, S.M.; MINERVINO, A.H.; FARIAS, N.A.; RUAS, J.L.; DOS SANTOS, T.R.; CAVALCANTE, G.T.; KWOK, O.C.; SU, C. Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from the northern Pará state and the southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations. **Veterinary Parasitology**, 143, 182–188, 2007b.

DUBEY, J.P. *Toxoplasma gondii* infections in chickens (*Gallus domesticus*): Prevalence, Clinical Disease, Diagnosis and Public Health Significance. **Zoonoses Public Health**, 57, 60-73, 2010.

FEITOSA, T.F.; VILELA, V.L.R.; MELO, L.R.B.; ALMEIDA NETO, J.L.; SOUTO, D.V.; MORAIS, D.F.; ATHAYDE, A.C.R.; AZEVEDO, S.S.; PENA, H.F.J. *Toxoplasma gondii* and *Neospora caninum* in slaughtered pigs from Northeast, Brazil. **Veterinary Parasitology**, 202, 305-309, 2014.

FERNANDES, M.F.T.S.; SIMÕES, S.; FARIA, E.B.; PINHEIRO JÚNIOR, J.W.; MOTA, R.A. Anticorpos igG anti-*Toxoplasma gondii* em suínos abatidos em matadouros da região metropolitana do Recife, Pernambuco, Brasil. **Arquivos do Instituto Biológico**, 78, 425-428, 2011.

OLIVEIRA, L.N.; COSTA JUNIOR, L.M.; DE MELO, C.; RAMOS SILVA, J.; BEVILAQUA, C.M.; AZEVEDO, S.; MURADIAN, V.; ARAUJO, D.; DUBEY, J.P.; GENNARI, S.M. *Toxoplasma gondii* isolates from free-range chickens from the northeast region of Brazil. **International Journal for Parasitology**, 95, 235-237, 2008.

OWEN, M.R.; TREE, A.J. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. **International Journal of Parasitolology**, 85, 382-384, 1999.

PENA, H.F.J., GENNARI, S.M., DUBEY, J.P., SU, C. Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil. **International Journal for Parasitology**, 38, 561–569, 2008.

TENTER, A.M; HECKEROTH, A.R.; WEISS, L.M. *Toxoplasma gondii:* from animals to humans. **International Journal for Parasitology**, 30(12-13), 1217-1258, 2000.

TSUTSUI V.S.; NAVARRO I.T; FREIRE R.L.; FREITAS J.C.; PRUDENCIO L.B; DELBEM A.C.B.; MARANA E.R.M. Soroepidemiologia e fatores associados à transmissão do *Toxoplasma gondii* em suínos do norte do Paraná, Brasil. **Archives of Veterinary Science**, 8, 27-34, 2003.

VALENCA, R.M.B.; MOTA, R.A.; MOTA, R.A.; ANDERLINI, G.A.; GUERRA, M.M.P. Prevalência e fatores de risco associados à infecção por *Toxoplasma gondii* em granjas suinícolas tecnificadas no Estado de Alagoas. **Pesquisa Veterinária Brasileira**, 31, 121-126, 2011.

CAPÍTULO I

Soroepidemiologia e isolamento de *Toxoplasma gondii* em galinhas caipiras no Semiárido da Paraíba, Brasil

Artigo Publicado na Revista Parasitology Research (Qualis – A2)

Primeiro estudo sobre soroepidemiologia e isolamento de *Toxoplasma gondii* em galinhas caipiras no semiárido da Paraíba, Brasil.

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Resumo

Este trabalho objetivou realizar a soroprevalência e o isolamento de *T. gondii* a partir de galinhas caipiras no estado da Paraíba, Nordeste brasileiro. Para isso, foram coletadas amostras sanguíneas de 483 galinhas provenientes de cinco munícipios do estado da Paraíba. Nestas amostras sanguíneas foi realizado o teste de Reação de Imunofluorescência Indireta para anticorpos anti-*T. gondii*. Os animais positivos para o parasita foram abatidos, o cérebro e o coração foram coletados para realizar o bioensaio em camundongos. Nas propriedades visitadas foi aplicado um questionário epidemiológico e utilizadas de regressão logística univariável e multivariada para avaliar os fatores de risco. Verificou-se uma prevalência de 31.5% (152/483) de galinhas soropositivas para *T. gondii* e 86.1% (56/65) de propriedades positivas. Dos 71 animais submetidos ao bioensaio em camundongos, foram obtidos 33 (46.5%) isolados de *T. gondii.* Observou-se que quanto maior os títulos de anticorpos dos animais, maiores as chances de isolamento do parasita. Muitos isolados foram virulentos para os camundongos; 21 dos 33 isolados foram letais para todos os animais inoculados. Os fatores de risco observados utilizando o modelo de regressão logística multivariada foram: tipo de criação extensiva (Odds ratio 5.41; p=0.027), semi-extensiva (Odds ratio

4.81; p= 0.043), propriedades localizadas em área urbana (Odds ratio 1.90; p= 0.002) e presença de gatos (Odds ratio 1.95; p=0.001) para infecção por *T. gondii* em galinhas caipiras. Os resultados indicam que a prevalência de *T. gondii* em galinhas é alta no estado da Paraíba, que muitos parasitas permaneciam viáveis nos animais estudados e que a presença do protozoário está diretamente relacionada com o manejo desses animais.

Palavras-chave: Bioensaio, Diagnóstico, Toxoplasmose, zoonose

Introdução

A toxoplasmose é uma antropozoonose causada pelo *Toxoplasma gondii,* parasita intracelular obrigatório de distribuição cosmopolita. Esse parasita pode afetar aves e mamíferos, inclusive o homem. A infeção ocorre após a ingestão de água ou alimentos contaminados com oocistos esporulados eliminados nas fezes de felinos, sendo o gato (*Felis catus domesticus*) o principal, ou através da ingestão de carne crua ou mal passada infectada com cistos desse protozoário (Dubey 2010a). Estudos apontam que a principal via de transmissão da toxoplasmose para humanos é pelo consumo de alimento contaminado, especialmente carne mal cozida contendo bradizoítos (Cook et al. 2000).

A toxoplasmose pode causar inúmeros prejuízos à saúde humana e animal. Em seres humanos, destaca-se o risco da doença em gestantes, pois a infecção por este parasita pode causar vários transtornos à mãe e ao feto, podendo ocasionar aborto espontâneo, coriorretinite, calcificações cerebrais, hidrocefalia e outras séries de apresentações clínicas (Remington et al. 2001). Pode-se ressaltar também a importância desse protozoário em pacientes com o vírus da AIDS, devido à possibilidade de causar encefalite toxoplásmica nestes indivíduos (Rey 2008). No Brasil, este protozoário está amplamente difundido e o país apresenta um dos mais altos índices de soroprevalência em humanos do mundo (Gilbert et al. 2008; Dubey 2010a).

A toxoplasmose em galinhas cursa predominantemente na forma subclínica, apresentando pouca importância clínica para essa espécie. Entretanto, Dubey et al. (2007a) relataram um surto de toxoplasmose clínica em galinhas de postura e gansos numa fazenda de Illinois, EUA. Os sinais clínicos relatados foram de alterações neurológicas manifestando-se por torcicolo, incapacidade de se manter em estação e

decúbito lateral. As galinhas de criações domésticas são consideradas importantes na epidemiologia desta doença, pois são fontes de transmissão tanto para o homem quanto para o gato, favorecendo, através deste último, a disseminação da doença pela eliminação de oocistos no meio ambiente (Dubey 2010b). Além disso, a presença de *T. gondii* em galinhas caipiras é um dos melhores indicadores de contaminação ambiental com oocistos do parasita, porque esses animais tem o hábito de ciscar e se alimentar do solo, sendo considerados ótimos animais sentinelas (Dubey et al. 2006; Dubey 2010b).

Estudos de soroprevalência apontam as galinhas de criação doméstica com alto índice de positividade para *T. gondii*. Dubey et al. (2007b) realizaram um estudo em galinhas provenientes do Pará e Rio Grande do Sul e verificaram 46,4% de animais soropositivos. Oliveira et al. (2008) verificaram 53,3% de animais sororeagentes em todos os estados do Nordeste, exceto a Paraíba, que não participou desse estudo. Alguns trabalhos relatam alto índice de isolamento de *T. gondii* dos tecidos de galinhas caipiras. Dubey et al. (2006) conseguiram isolar este protozoário de 72,7% de animais soropositivos em um estudo conduzido no estado do Amazonas e Beltrame et al. (2012) também obtiveram um índice de isolamento alto, de 75%. Isso demonstra que os animais tinham cistos viáveis capazes de infectar humanos, assim como animais.

Sabendo da importância das galinhas na transmissão da toxoplasmose para os seres humanos e animais, e ainda da incipiência de pesquisas sobre esta doença no estado da Paraíba, este trabalho teve como objetivo estudar a situação soroepidemiológica de galinhas caipiras neste Estado e realizar o isolamento do parasita para determinar o risco real do consumo dessas carnes cruas ou mal passadas pela população humana e animal.

2. Material e Métodos

Caracterização da Área

O estado da Paraíba está localizado no Nordeste brasileiro, apresenta temperaturas elevadas o ano inteiro, variando entre 20 e 28 ºC, com pequenas diferenças regionais. O Estado apresenta variações climáticas que variam de úmido, na região próxima ao litoral, ao semiárido, clima predominate no interior, com precipitações anuais entre 350 e 700 mm^3 (Araújo 2011).

Galinhas

Foram utilizadas 483 galinhas provenientes de cinco munícipios do estado da Paraíba, Patos, Monteiro, Olho D'água, Esperança e Malta

Durante as visitas, amostras de sangue foram coletadas através da veia braquial e os animais devidamente identificados com números correspondentes nos tubos de coleta. As amostras sanguíneas foram armazenadas em isopor com gelo e encaminhados ao Laboratório de Doenças Parasitárias dos Animais Domésticos (LDPAD) da Universidade Federal de Campina Grande (UFCG), Patos-PB, para realização do exame sorológico de *T. gondii*. Após os resultados desse exame, a equipe retornava às propriedades para retirar os números dos animais soronegativos e recolher os animais soropositivos para posterior abate. Apenas as aves que os proprietários concordaram com o abate foram recolhidas (71/152). Após o abate, o coração e cérebro dos animais foram coletados e acondicionados individualmente em sacos plásticos identificados, armazenados em isopor com gelo e encaminhados ao LDPAD para realização do bioensaio.

Exame Sorológico e Isolamento de *T. gondii*

Os soros das galinhas foram examinados para pesquisa de anticorpos anti-*T. gondii* através da RIFI (Camargo 1974), usando amostra RH de taquizoítas de *T. gondii* fixados em lâmina. O ponto de corte utilizado foi 1:16 (Casartelli-Alves et al. 2014).

Os tecidos (cérebro e coração) dos animais soropositivos foram cortados em pequenos fragmentos, sendo removidos a gordura e o tecido conectivo e utilizados para bioensaio em camundongo segundo protocolo de Dubey (1998). Para cada animal positivo, foram utilizados em média três camundongos albinos Swiss com dois meses de idade, alojados na mesma caixa, provenientes do Biotério do Laboratório de Tecnologia Farmacêutica, Universidade Federal da Paraíba.

Os animais que vieram a óbito foram examinados para pesquisa de *T. gondii* nos tecidos, como descrito previamente por Dubey e Beattie (1988). Os camundongos que sobreviveram até seis semanas pós-inoculação foram examinados sorologicamente para pesquisa de anticorpos anti-*T. gondii,* através da RIFI com ponto de corte 1:16. Então, os soropositivos permaneciam no experimento até dois meses pós-inoculação (PI), quando eram eutanasiados e examinados para pesquisa de *T. gondii*; Já os soronegativos eram eutanasiados após o resultado da sorologia e submetidos ao mesmo exame. Os

camundongos foram considerados positivos quando cistos eram observados em seus tecidos (Dubey et al. 2002).

Questionário epidemiológico

Os proprietários foram entrevistados individualmente e questionados sobre as condições das propriedades, tipo de criação, tipo de alimentação, ambiente onde o animal vive e presença de gatos.

Análise estatística

Para a análise de fatores de risco associados com a frequência de soropositividade e isolamento foram utilizados os dados coletados nos questionários epidemiológicos. A análise de fatores de risco foi conduzida em duas etapas: análise univariável e análise multivariável. Na análise univariável, cada variável independente foi cruzada com a variável dependente (soropositividade e isolamento), e aquelas que apresentaram valor de $p \leq 0,20$ pelo teste de qui-quadrado (Zar 1999) foram selecionadas para a análise multivariável, utilizando-se regressão logística múltipla (Hosmer & Lemeshow 2000). O nível de significância adotado na análise múltipla foi de 5%. Todas as análises foram realizadas com o programa SPSS 20.0 *for Windows*.

O Comitê de Ética e Pesquisa da UFCG autorizou todos os procedimentos realizados neste trabalho, sob protocolo 14/2013.

3. Resultados

Neste estudo, 31.5% (152/483) das galinhas examinadas foram soropositivas para *T. gondii* através da RIFI. Das 65 propriedades visitadas, 56 (86.1%) apresentaram pelo menos um animal soropositivo. Observou-se frequências de 25 a 36.8% de animais soropositivos para *T. gondii* por município (Tabela 1). Não houve diferença estatística em relação à prevalência de animais soropositivos entre os cinco municípios estudados ($p= 0.284$).

Os títulos de anticorpos anti *T. gondii* variaram de 1:16 a 1:4092, sendo mais frequente o 1:64 (44/152). Dos animais positivos, 71 foram submetidos ao bioensaio para isolamento em camundongos, obtendo-se 33 isolados. Observou-se que a porcentagem de isolamento aumentava à medida que os títulos de anticorpos eram

maiores, chegando a 100% de isolamento nos animais que tinham títulos superiores a 2048 (Tabela 2).

Tabela 1. Prevalência de anticorpos anti-*T. gondii* através da RIFI em galinhas caipiras do estado Paraíba, Brasil.

Municípios	Propriedades		Galinhas	
	Examinados	Positivos(%)	Examinados	Positivos(%)
Esperança	10	9 (90)	97	26 (26.8)
Malta	14	13 (92.8)	95	35 (36.8)
Monteiro	15	12 (80)	98	35 (36.7)
Olho D'água	13	11 (84.6)	96	24 (25)
Patos	13	11 (84.6)	97	32 (33)
Total	65	56 (86.1)	483	152 (31.5)

Tabela 2. Frequência do isolamento de *T. gondii* de galinhas caipiras através do bioensaio em camundongos. no estado da Paraíba, Brasil

Título de anticorpos	No. de galinhas positivas	No. de galinhas		
		Bioensaios	Isolados de *T. gondii*	%
16	19	7	2	28.6
32	24	6	2	33.3
64	44	20	8	40
128	26	16	6	37.5
256	17	12	6	50
512	10	6	4	66.6
1024	7	3	2	66.6
2048	4	2	2	100
4096	1	1	1	100
Total	152	71	33	46.8

Dos 33 isolados, 27 (81.8¨%) foram letais para pelo menos um dos camundongos inoculados, estes vieram a óbito entre os dias 16 e 45 p.i. por toxoplasmose aguda, confirmada através da presença do parasita nos tecidos dos animais. Vinte e um isolados (63.6%) foram letais para todos os animais inoculados e em seis isolados (18.2%) todos os camundongos resistiram até os 42 dias p. i. quando então foi realizada a sorologia e os positivos foram eutanasiados no dia 60 p. i., com detecção do parasita em seus tecidos.

Os resultados da análise univariável dos fatores de risco para *T. gondii* estão apresentados na Tabela 3. Não foram observadas associações significativas entre as variáveis sexo (p=0.701) e tipo de alimentação (p=0.055) com a infecção por *T. gondii.* Em relação ao ambiente onde os animais viviam, todas as propriedades os animais eram criados soltos ou em piquetes de terra.

As variáveis tipo de criação extensiva (Odds ratio 5.41; p=0.027), semi-extensiva (Odds ratio 4.81; p= 0.043), propriedades localizadas em área urbana (Odds ratio 1.90; p= 0.002) e presença de gatos (Odds ratio 1.95; p=0.001) foram consideradas fatores de risco para infecção por *T. gondii* de acordo com a regressão logística multivariável (Tabela 4).

Tabela 3. Análise univariável para os fatores de risco associados com a soropositividade para *Toxoplasma gondii* em galinhas no Estado da Paraíba, Brasil.

Variável/categoria	N° total de galinhas	N° de galinhas soropositivos RIFI (%)	*P*
Sexo			
Macho	61	21 (34.4)	
Fêmea	422	131 (31)	0,701
Tipo de alimentação			
Ração	23	2 (8.7)	
Restos de comida	120	39 (32.5)	
Ração + Restos de comida	340	111(32.6)	0,55
Tipo de criação			
Intensiva	23	2 (8.7)	
Semi-intensiva	114	35 (30.7)	
Extensiva	346	115 (33.2)	0,48
Localização da propriedade			
Rural	302	76 (25.2)	
Urbana	181	76 (42)	0,002
Presença de gatos			
Não	225	56 (24.9)	
Sim	258	96 (37.2)	0,005

Tabela 4. Fatores de risco associados com a soropositividade para *Toxoplasma gondii* em galinhas do Estado da Paraíba, Brasil, determinados por regressão logística múltipla.

Variável	Odds ratio (IC 95%)	P
Criação Extensiva	5.41 (1.21-24.08)	0,027
Criação Semi-extensiva	4.81 (1.05-22.04)	0,043
Localização urbana	1.90 (1.27-2.85)	0,002
Presença de gatos	1.95 (1.30-2.92)	0,001

4. Discussão

A soroprevalência encontrada neste estudo (31.5%) foi considerada alta, assemelhando-se a observada por Beltrame et al. (2012), que encontraram prevalência de 38.8% (198/510) no estado do Espírito Santo e Tilahu et al. (2013) de 38.4% (48/125) em Adis Abeba (Ethiopia), ambos utilizando o MAT como teste de diagnóstico. Porém, outros pesquisadores encontraram índices de soroprevalência ainda mais elevados, 80% na ilha de Fernando de Noronha, Pernambuco (Costa et al. 2012) utilizando o MAT e 74.4% (102/137) em galinhas de quintal no Rio Grande do Sul utilizando a RIFI (Camillo et al. 2015). Estes trabalhos que apresentaram altos índices de animais soropositivos não realizaram cálculo amostral e utilizaram um pequeno número de animais, podendo ter influenciado na alta soropositividade. Além disso, Qin et al. (2015) relata que as diferenças nas soroprevalências entre as regiões dependem de vários fatores, incluindo diferenças de temperatura, precipitação pluviométrica e sensibilidade da técnica utilizada.

Observou-se que, quanto maior o título de anticorpos que o animal apresentava, maior o percentual de isolamento de *T. gondii*, corroborando com Holsback et al. (2012) que verificaram em seu estudo com galinhas de criação doméstica que as aves com altos títulos de anticorpos devem ser priorizadas, pois as chances de isolamento do parasita são maiores. Em outras espécies, como ovelhas, caprinos e gatos também já foram observadas essa associação (Gebremedhin et al. 2014; Pena et al. 2006).

O percentual de bioensaios positivos para *T. gondii* foi 46.5% (33/71), semelhante ao relatado por Holsback et al. (2012), que obtiveram um índice de 40.7% (11/27) de isolamento utilizando o cérebro e coração de galinhas soropositivas provenientes do Mato Grosso do Sul. Porém, outros pesquisadores observaram índices superiores, como Dubey et al. (2007a) que obtiveram isolamento de 100% num surto de Toxoplasmose em Illinois, EUA e Beltrame et al. (2012) que observaram índice de 78,1% de isolamento no Espírito Santo, Brasil. Essa diferença pode ser explicada pelos tecidos utilizados para isolamento, pois estes últimos autores utilizaram para o bioensaio os músculos da coxa além do coração e cérebro das galinhas soropositivas. Apesar do coração ser o órgão mais parasitado pelo protozoário em galinhas, Dubey et al. (2007a) fala da importância de se utilizar macerados de diferentes órgãos, isolados ou misturados, para aumentar a sensibilidade do isolamento, destacando-se os músculos da coxa que são fortemente parasitados pelo protozoário.

A virulência de uma cepa de *T. gondii* é definida com base na mortalidade dos camundongos inoculados e o tempo entre a inoculação e a morte desses animais, e esta depende de vários fatores como a via de inoculação, quantidade de parasita no inócuo e o tipo de animal utilizado como modelo experimental. Neste estudo, a maioria dos isolados foram considerados virulentos, pois observou-se que 63.6% (21/33) foram letais para todos os camundongos inoculados. No Brasil, a virulência dos isolados costuma ser alta, diferente do que é observado em outros países, que apresentam cepas avirulentas do parasita. Beltrame et al. (2012) observaram que 44 dos 48 isolados obtidos de galinhas de criação livre foram letais para todos os camundongos inoculados; Vitaliano et al. (2014) obtiveram 15 isolados de animais selvagens provenientes de vários estados brasileiros e todos os camundongos inoculados morreram de toxoplasmose aguda; Já Gebremedhin et al. (2014) realizaram estudo com galinhas caipiras na Etiópia e obtiveram 29 isolados, todos considerados avirulentos.

Não foi observada associação entre a soropositividade para *T. gondii* em machos e fêmeas, corroborando com outros estudos realizados em caprinos, ovinos, búfalos, suínos e animais selvagens (Ragozo et al. 2009; Correia et al. 2015; Brasil et al. 2015; Feitosa et al. 2013 e Pimentel et al. 2009).

Apesar de não se verificar diferença estatística em relação ao tipo de alimentação das galinhas, a quantidade de animais positivos que comiam apenas ração foi baixa (8.7%) em comparação aos que comiam restos de comida (32.5%), isso porque nas criações domésticas, alvos do estudo, os proprietários tinham o costume de oferecer aos animais restos de frutas, carnes e vísceras cruas que não são utilizadas para consumo humano. Assim, as galinhas ficam expostas a oocistos e cistos teciduais que podem estar presentes nestes restos de comida. Essa prática de fornecer refugo da alimentação humana às galinhas, reforça o papel desses animais como sentinelas, não apenas da contaminação ambiental, mas também da exposição dos seres humanos ao *T. gondii*.

A criação intensiva mostrou-se a mais segura para evitar a contaminação das galinhas por *T. gondii*, quando comparadas a criação extensiva e semi-extensiva. Na criação extensiva, os animais estão sujeitos a contato com fezes de felídeos, solo e água contaminada, passando mais tempo no mesmo ambiente, aumentando as chances de se infectar pelo parasita. Além disso, nenhuma das propriedades visitadas possuía saneamento básico, nem coleta de lixo regular. Outros estudos também observaram que o número de animais positivos nas criações intensivas são menores que as extensivas, Xu et al. (2012) encontraram uma prevalência de 5,6% e 18,8% em galinhas de sistema

intensivo e extensivo, respectivamente, utilizado o MAT. Millar et al. 2012 realizaram uma pesquisa no estado do Rio de Janeiro e observaram que 14.8% das galinhas poedeiras criadas intensivamente apresentavam anticorpos anti-*T. gondii*, enquanto que as criadas extensivamente apresentavam 51.4% de animais reagentes.

A presença de gatos é considerada importantíssima na infecção por *T. gondii* em galinhas de criação extensiva. Essa correlação também foi relatada por outros autores como Bonna et al. (2006) em criações de suínos e frangos no Rio de Janeiro, Brasil e por Millar et al. (2012) em galinhas poedeiras criadas em sistema semiextensivo.

Foi observado que as galinhas de propriedades localizadas em áreas urbanas são mais susceptíveis a infecção por *T. gondii* do que os animais que vivem em propriedades rurais, corroborando com Silva et al. (2003), que também verificaram menor porcentagem de galinhas positivas para *T. gondii* em áreas rurais no Sul do Brasil. Pode-se atribuir esse resultado a maior densidade populacional dos gatos na cidade, com isso maior concentração de fezes de gatos infectados contendo oocistos no solo, aumentando a probabilidade de infecção nas galinhas. Weigel et al. (1995) afirmou que a quantidade de felinos nas propriedades é mais importante do que a presença desses, uma vez que quanto maior esse número, maior será a eliminação de oocistos de *T. gondii* nas fezes.

Conclusão

A alta prevalência de anticorpos anti-*T. gondii* indica que há grande contaminação ambiental com oocistos do protozoário. Além disso, a carne das galinhas caipiras é importante para a epidemiologia da toxoplasmose na região estudada, podendo ser fonte de infecção para os seres humanos e animais. A infecção por *T. gondii* está diretamente relacionada com a forma de criação dos animais, com isso, a forma de controle dessa doença depende do conhecimento dos proprietários sobre suas formas de transmissão.

Referências

Araújo SMS (2011) A Região Semiárida do Nordeste do Brasil: Questões ambientais e possibilidades de uso sustentável dos recursos. Rios Eletrônica – Revista Científica da FASETE 5:89-98

Beltrame MAV, Pena HFJ, Ton NC, Lino AJB, Gennari SM, Dubey JP, Pereira FEL (2012) Seroprevalence and isolation of *Toxoplasma gondii* from free-range chickens from Espírito Santo state, southeastern Brazil. Vet Parasitol 188:225-230

Bonna ICF, Figueiredo FB, Costa T, Vicente RT, Santiago CAD, Nicolau JL, Neves LB, Millar PR, Sobreiro LG, Amendoeira MRR (2006) Estudo Soroepidemiológico da infecção por *Toxoplasma gondii* em suínos e frangos para abate em região rural do Rio de Janeiro. Rev Bras Ci Vet 13:186-189

Brasil AWL, Parentoni RN, Feitosa TF, Bezerra CS, Vilela VLR, Pena HFJ, Azevedo, SS (2015) Risk factors for *Toxoplasma gondii* and *Neospora caninum* seropositivity in buffaloes in Paraiba State, Brazil. R Bras Parasitol Vet 24:459-463

Camargo ME (1974) Introdução às técnicas de imunofluorescência. R Bras Patol Clín 10:143-171

Camillo G, Cadore GC, Ferreira MST, Maciel JF, Pivoto F, Braunig P, Sangioni LA, Vogel FSF (2015) *Toxoplasma gondii* and *Neospora Caninum* Antibodies in Backyard Chickens in Rio Grande do Sul, Brazil. Braz J Poult Sci 17:263-265

Casartelli-Alves L, Boechat VC, Macedo-Couto R, Ferreira LC, Nicolau JL, Neves LB, Millar PR, Vicente RT, Oliveira RVC, Muniz AG, Bonna ICF, Amendoeira MRR, Silva RC, Langoni H, Schubach TMP, Menezes RC (2014) Sensitivity and specificity of serological tests, histopathology and immunohistochemistry for detection of *Toxoplasma gondii* infection in domestic chickens. Vet Parasitol 204:346-351

Cook AJ, Gilbert RE, Buffolano W, Zufferey J, Petersen E, Jenum PA, Foulon W, Semprini AE, Dunn DT (2000) Sources of *Toxoplasma* infection in pregnant woman: European multicentre case–control study. Br Med J 321:142-147

Correia ELB, Feitosa TF, Santos FA, Azevedo SS, Pena HFJ, Gennari SM, Mota RA, Alves CJ (2015) Prevalence and risk factors for *Toxoplasma gondii* in sheep in the State of Paraíba, Northeastern Brazil. Rev Bras Parasitol Vet 24:383-386

Costa DGC, Marvulo MFV, Silva JSA, Santana SC, Magalhães FJR, Lima Filho CDF, Ribeiro VO, Alves LC, Mota RA, Dubey JP, Silva JCR (2012) Seroprevalence of *Toxoplasma gondii* in Domestic and Wild Animals From the Fernando de Noronha, Brazil. J Parasitol 98:679-680

Dubey JP, Beattie CP (1988) Toxoplasmosis of animals and man. Boca Raton, Florida

Dubey JP (1998) Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. Vet Parasitol 74:75–77

Dubey JP, Graham DH, Blackston CR, Lehmann T, Gennari SM, Ragozo AMA, Nishi SM, Shen SK, Kwok OCH, Hill DE, Thulliez P (2002) Biological and genetic characterization of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from São Paulo, Brazil: Unexpected findings. Int J Parasitol 32:99–105

Dubey JP, Gennari SM, Labruna MB, Camargo LM, Vianna MC, Marcet PL, Lehmann T (2006) Characterization of *Toxoplasma gondii* isolates in free-range chickens from Amazon, Brazil. J Parasitol 92:36-40

Dubey JP, Webb DM, Sundar N, Velmurugan GV, Bandini LA, Kwok OCH, Su C (2007a) Endemic avian toxoplasmosis on a farm in Illinois: clinical disease, diagnosis, biologic and genetic characteristics of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*), and a goose (*Anser anser*). Vet Parasitol 148:207-212

Dubey JP, Sundar N, Gennari SM, Minervino AH, Farias NA, Ruas JL, Dos Santos TR, Cavalcante GT, Kwok OC, Su C (2007b) Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from the northern Pará state and the southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations. Vet Parasitol 143:182–188

Dubey JP (2010a) Toxoplasmosis of Animals and Humans. Boca Raton, Florida

Dubey JP (2010b) *Toxoplasma gondii* infections in chickens (*Gallus domesticus*): Prevalence, Clinical Disease, Diagnosis and Public Health Significance. Zoonoses Public Health 57: 60-73

Feitosa TF, Vilela VLR, Bezerra LRB, Almeida Neto JL, Oliveira DVS, Morais DF, Athayde ACR, Azevedo SS, Pena HFJ (2014) *Toxoplasma gondii* and *Neospora caninum* in slaughtered pigs from Northeast, Brazil. Vet Parasitol 202:305-309

Gilbert RE, Freeman K, Lago EG, Bahia-Oliveira LM, Tan HK, Wallon M, Buffolano W, Stanford MR, Petersen E (2008) Ocular sequelae of congenital toxoplasmosis in Brazil compared with Europe. PLoS Negl Trop Dis 2:277

Gebremedhin E Z, Tesfamaryam G, Duguma R, Tilahun G, Di Marco V, Vitale M (2014) Majority of *T. gondii* seropositive chickens (*Gallus domesticus*) in Central Ethiopia carries the infective parasite. Acta Veterinaria Scand 24: 56:60

Holsback L, Pena HFJ, Ragozo AMA, Lopes EG, Gennari S M, Soares RM (2012) Serologic and molecular diagnostic and bioassay in mice for detection of *Toxoplasma gondii* in free ranges chickens from Pantanal of Mato Grosso do Sul. Pesq Vet Bras 32:721-726

Hosmer DW, Lemeshow S (2000) Applied Logistic Regression, seconded. John Wiley & Sons, New York

Millar PR, Alves FMX, Teixeira VQ, Vicente RT, Menezes EM, Sobreiro LG, Pereira VLA, Amendoeira MRR (2012) Occurrence of infection with *Toxoplasma gondii* and

factors associated with trans-mission in broiler chickens and laying hens in different raisingsystems. Pesq Vet Bras 32: 231–236.

Oliveira LN, Costa Junior LM, Melo C, Silva JR, Bevilaqua CM, Azevedo SS, Muradian V, Araujo D, Dubey JP, Gennari SM (2008) *Toxoplasma gondii* isolates from free-range chickens from the northeast region of Brazil, Int J Parasitol 95:235-237

Pena HFJ, Soares R, Amaku RM, Dubey JP, Gennari S (2006) *Toxoplasma gondii* infection in cats from São Paulo state, Brazil: Seroprevalence, oocyst shedding, isolation in mice, and biologic and molecular characterization. Res Vet Sci 81:58-67

Pimentel JS, Dubey JP, Gennari MS, Marvulo MFV, Vasconcellos AS, Morais ZM, Silva JCR, Evêncio Neto J (2009) Inquérito sorologico para toxoplasmose e leptospirose em mamiferos selvagens neotropicais do zoológico de Aracaju, Sergipe. Pesq Vet Bras 29:1009-1014

Qin SY, Zhou DH, Cong W, Zhang XX, Lou ZL, Yin MY, Tan QD, Zhu XQ (2015) Seroprevalence, risk factors and genetic characterization of *Toxoplasma gondii* in free-range white yaks (*Bos grunniens*) in China. Vet parasitol 211:300-302.

Ragozo AMA, Yai LEO, Oliveira LN, Dias RA, Gonçalves HC, Azevedo SS, Dubey JP, Gennari SM (2009) Isolation of *Toxoplasma gondii* from goats from Brazil. J Parasitol 95:323-326

Remington JS, Macleod RP, Thulliez, Desmonts G (2001) Toxoplamosis. In: Remington JS, Klein JO (ed) Infectious Diseases Of The Fetus And Newborn Infant. W. B. Saunders Company, Philadelphia, pp. 205-2346.

Rey L (2008) Parasitologia. Guanabara Koogan, Rio de Janeiro:

Silva D S, Bahia-Oliveira LMG, Shen SK, Kwok OCH, Lehmann T, Dubey JP (2003) Prevalence of *Toxoplasma gondii* in chickens from an area in Southern Brazil highly endemic to humans. J Parasitol 89:394–396

Tilahun G, Tiao N, Ferreira LR, Choudhary S, Oliveira S, Verma SK, Kwok OCH, Molla B, Saville WJA, Medhin G, Kassa T, Aleme H, Gebreyes WA, Su C, Dubey JP (2013) Prevalence of *Toxoplasma gondii* from Free-Range Chickens (*Gallus domesticus*) from Addis Ababa, Ethiopia. J Parasitol 99:740-741

Vitaliano SN, Soares HS, Minervino AHH, Santos ALQ, Werther K, Marvulo MFV, Siqueira DB, Pena HFJ, Soares RM, Su C, Gennari, SM (2014) Genetic characterization of *Toxoplasma gondii* from Brazilian wildlife revealed abundant new genotypes. Int J Parasitol: Parasites and Wildlife 3:276-283

Zar JH (1999) Biostatistical Analysis, fourth ed. Prentice-Hall, Upper Sad-dle River.

Weigel RM, Dubey JP, Siegel AM (1995) Risk factors for transmission of *Toxoplasma gondii* on swine farms in Illinois. J. Parasitol 81:736-741

Xu P, Song X, Wang W, Wang F, Cao L, Liu Q (2012) Seroprevalence of *Toxoplasma gondii* Infection in Chickens in Jinzhou, Northeastern China. J Parasitol 98:1300-1301

CAPÍTULO II

First report of typical Brazilian *Toxoplasma gondii* genotypes from isolates of freerange chickens (*Gallus gallus domesticus*) circulating in the state of Paraíba, northeast Brazil

Artigo submetido à Publicação na Revista Parasitology Research (Qualis – A2)

First report of typical Brazilian *Toxoplasma gondii* genotypes from isolates of freerange chickens (*Gallus gallus domesticus*) circulating in the state of Paraíba, northeast Brazil

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Abstract

This study evaluated, for the first time, the genetic diversity of *Toxoplasma gondii* isolates from free-range chickens from the state of Paraíba, northeast Brazil. Tissue samples from thirty-three chickens from properties in five municipalities of Paraíba (Esperança, Olho d'Água, Malta, Monteiro, and Patos) were bioassayed in mice. The brains of mice infected with *T. gondii* cysts were used for DNA extraction and genotyping. Genotyping was performed using 11 PCR-RFLP markers and 15 microsatellite (MS) markers. Complete genotyping results were obtained for 29 isolates, with nine genotypes detected by RFLP and 15 genotypes identified by MS. Three genotypes (#273, #274, and #277) have only been recently identified from pigs in the region. Brazilian clonal types BrII and BrIII were identified from one isolate each. Clonal types I, II, and III were not detected by RFLP. Genotype #13 (Caribbean 1), detected in 48.3% (14/29) of isolates from four of the five municipalities investigated, was the most prevalent genotype in the state of Paraíba. However, the MS analysis showed that of these 14 isolates, only four were unique genotypes, and considering the distance between the municipalities from where they were collected, it is possible that

only seven are independent isolates while the others are clones. The other genotypes were restricted to different microregions. The results indicate that the Caribbean 1 lineage of *T. gondii* is circulating widely in northeast Brazil. The genotypic diversity of *T. gondii* in the state of Paraíba is high and microsatellite analysis revealed this diversity with higher resolution than PCR-RFLP.

Keywords: toxoplasmosis, diversity, genotyping, PCR-RFLP, microsatellites.

Introduction

Toxoplasma gondii is a protozoan in the phylum Apicomplexa that is capable of infecting all species of birds and mammals (Tenter and Johnson 1997; Elmore et al. 2010). Felids are the definitive hosts where the sexual reproduction of the parasite occurs and are responsible for the production and excretion of oocysts in the feces. Accidental ingestion of oocysts from the environment is one of the most common forms of parasite transmission. Other routes of transmission include the ingestion of undercooked meat containing viable cysts and vertical transmission through transplacental passage of tachyzoites to the fetus. The different routes of transmission and the possibility of *T. gondii* permanently infecting its hosts make this parasite one of the most prevalent and widespread worldwide (Dubey et al. 2012; Sullivan and Jeffers 2012).

Most humans infected with *T. gondii* are asymptomatic. The prevalence seems to depend on the contamination of vegetables and infection of animals that live on the ground and are used for human food (Dubey et al. 2012; Gangneux and Dardé 2012). Free-range chickens are the best indicator for soil contamination with *T. gondii* oocysts because they feed from the ground and in doing so become infected (Furuta et al. 2007; Dubey 2009). In addition, the isolation and genotyping of *T. gondii* from chickens can provide information on the strains that are circulating in a region.

The early studies of *T. gondii* using the SAG2 gene showed a limited genetic diversity and only three clonal lineages: I, II, and III (Howe and Sibley 1995). However, later studies using a larger number of molecular markers revealed the existence of many recombinant or atypical lineages, especially in South America (Ajzenberg et al. 2002; Dubey et al. 2007a; Ferreira et al. 2008). These markers generate valuable informationabout the diversity of the parasite, are simple, and have a high resolution in the identification of *T. gondii* isolates (Su et al. 2010). PCR-RFLP (polymerase chain

reaction–restriction fragment length polymorphism) is the most used genotyping method, having contributed to the genotypic characterization of *T. gondii* isolates from animals and humans worldwide (Howe and Sibley 1995; Su et al. 2010), even though microsatellite (MS) analysis has a higher resolving power with two levels of discrimination, the first one identifying the lineages and the second one distinguishing closely related samples belonging to the same lineage (Ajzenberg et al. 2010).

There are few data available regarding *T. gondii* genotypes found in northeast Brazil, especially in the state of Paraíba. A high prevalence of *T. gondii* antibodies was found in a previous study with free-range chickens, and many parasites remained viable in the tissues of the animals (Feitosa et al. 2016). Thus, this study evaluated, for the first time, the genetic diversity of *T. gondii* isolates from free-range chickens raised in the state of Paraíba, northeast Brazil.

Materials and Methods

Samples

Thirty-three *T. gondii* isolates from free-range chickens raised in five municipalities in the state of Paraíba, northeast Brazil, and described by Feitosa et al. (2016) were used in the study. The brains of mice positive on microscopy for *T. gondii* were used for genotyping. The samples were stored at −70 °C until DNA extraction.

DNA extraction

After thawing, a 300-µl aliquot of brain homogenate from each sample was separated, washed three times with TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA), and centrifuged at 12,000 g for 5 min. DNA extraction was performed using WIZARD® Genomic Purification Kit (cat. A 1125; Promega, Madison, WI, USA)

Isolate genotyping

Genotyping of isolates was performed using 11 PCR-RFLP genetic markers: SAG1, SAG2 (5´3´SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Su et al. 2010) and marker CS3 (Pena et al. 2008). Clonal type I (RH), type II (PTG), and type III (CTG) samples and reference samples Cougar, MAS, and TgCatBr5 were used as positive controls. First, the target DNA sequences were amplified by multiplex PCR using the outer primers for all markers followed by nested

PCR separately for each marker. The PCR products were diluted 1:1 in ultrapure water before being amplified. All protocols have been described elsewhere (Su et al. 2010; Pena et al. 2008).

To determine the RFLP pattern of each sample, 3 μL of nested PCR products were mixed with 17 μL of digestion reaction containing buffer and one unit of a given restriction enzyme. The samples were incubated at the proper temperature for each restriction enzyme according to the manufacturer's recommendations. The restriction enzymes used are described in Su et al. (2006), except for CS3, which is described in Pena et al. (2008). The digested PCR products were resolved by electrophoresis in a 2.0–3.0% agarose gel containing 10 μL of SYBR® Safe for each 100 mL of solution with a molecular weight marker with multiple 100-base pair (bp) fragments. The bands were visualized under UV light on an image analyzer (Alpha Innotech Corporation, San Leandro, CA, USA).

The isolates were compared and classified according to the genotypes available in the ToxoDB database (http://toxodb.org/toxo/) and based on recent studies. The phylogenetic relationships of *T. gondii* isolates genotyped by PCR-RFLP were examined using SplitsTree4 software (Huson 1998; Huson and Bryant 2006).

The isolates were also genotyped using 15 microsatellite markers: TUB2, W35, TgMA, B18, B17, M33, IV.1 and X1.1, N60, N82, AA, N61, N83, M48, and M102 (Ajzenberg et al. 2010). The data were analyzed using GeneMapper® 4.1 software (Applied Biosystems, Foster City, CA, USA).

Results

Of the 33 isolates analyzed by PCR-RFLP, complete genotyping results were obtained for 29 samples with RFLP and MS markers (Supplementary material). Nine different RFLP genotypes and 15 MS genotypes were detected (Table 1). Two isolates from the state capital (Patos), TgCkBrPB9 and TgCkBrPB30, belonged to Brazilian clonal lineages BrII (#11) and BrIII (#08), respectively. Clonal archetypal lineages I, II, and III were not detected by RFLP.

Genotypes #48 (TgCkBrPB4,5,6) and #88 (TgCkBrPB7,8) had been previously identified in chickens, but from different Northeast states and as single isolates. Genotype #116 (TgCkBrPB1,2) had been previously detected in chickens and pigs in North and Northeast Brazil, respectively. Genotypes #273 (TgCkBrPB11,12,14), #274 (TgCkBrPB26), and #277 (TgCkBrPB7,8) have only recently been reported in Brazil in pig isolates from the state of Paraíba (Feitosa et al., unpublished data).

Genotype #13, detected in 48.3% (14/29) of isolates, was the most prevalent and well-distributed genotype in the state of Paraíba. These isolates came from four municipalities located 70–262 km apart, whereas the other genotypes were restricted to different microregions. Nevertheless, the MS analysis indicated the possibility of a high level of circulation of genotype #13 (Caribbean 1) clones due to the occurrence of identical genotypes in isolates from the same municipality and from nearby properties, including isolates TgCkBrPB15,16 (Esperança), TgCkBrPB10,13 (Monteiro), TgCkBrPB20,25 (Malta), and TgCkBrPB22,23,24,27 (Malta) (Suppl. material). Similarly, isolates TgCkBrPB1,2 (genotype #116) and TgCkBrPB7,8 (genotype #277) also appear to be clones from the same sample.

The genetic diversity of the 29 *T. gondii* isolates from chickens is summarized in Figure 1. The analysis showed a great divergence of these isolates from type II strains and a clear clustering with type III and type BrIII strains.

Table 1 PCR-RFLP genotyping of *Toxoplasma gondii* isolates from free-range chickens from Paraíba state, northeastern Brazil, and lethality in mice.

		PCR-RFLP Genótipos												ToxoDB RFLP-genótipos
Isolado ID[#]	Cidade		5´3´											
		SAG1	SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	CS3	
TgCkBrPB9	Patos	I	I	II	III	III	III	I	III	I	II	I	I	#011 Type BrII
TgCkBrPB30	Patos	I	III	III	III	III	III	II	III	III	III	III	III	#008 Type BrIII
TgCkBrPB1, 2	Olho d'água	I	III	III	III	I	III	II	III	III	III	III	III	#116
TgCkBrPB3, 10, 13, 15,16, 17, 18, 19, 20 22, 23, 24, 25 27	Esperança e Malta Patos, Olhod'água,	I	I	I	I	I	III	II	III	III	I	III	III	#013
TgCkBrPB4, 5, 6,21	Patos	I	III	III	III	III	III	III	III	III	III	III	I	#048
TgCkBrPB28	Patos	I	I	I	III	III	III	II	I	III	I	III	I	#088
TgCkBrP7,8	Patos	I	III	III	I	III	III	II	III	III	I	III	III	Novo

TgCkBrPB11 12,14	Monteiro	I	I	I	III	I	II	u-1	III	III	I	III	III	Novo
TgCkBrPB26	Malta	I	III	III	III	I	III	II	I	III	III	III	II	Novo

1

2

3

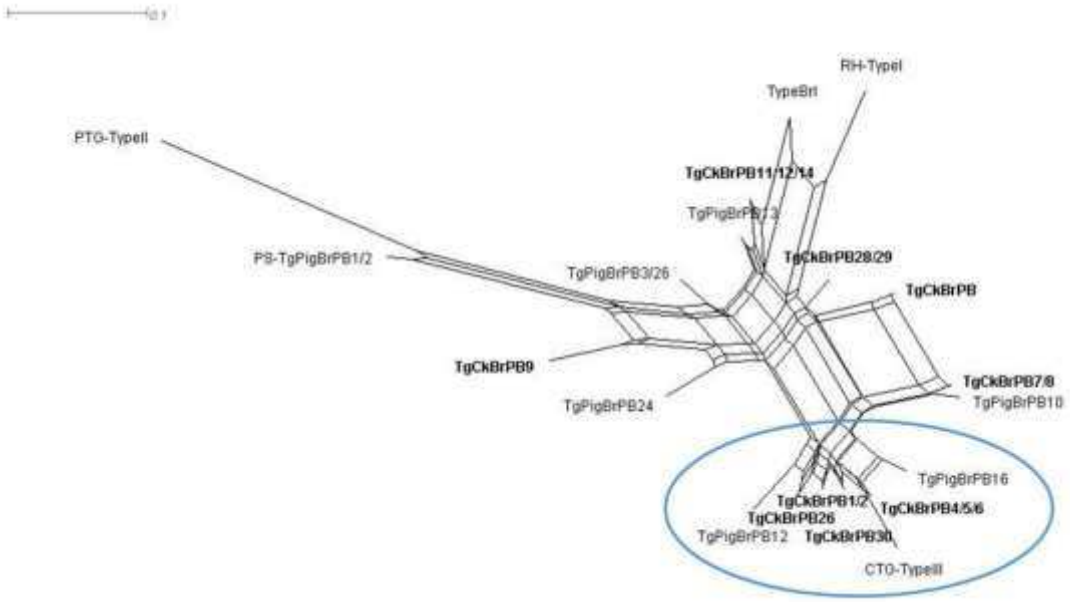

Fig 1. Phylogenetic network of the *T. gondii* isolates from chickens from Paraíba state, Northeastern Brazil, using PCR-RFLP data. The samples in bold were obtained in the present study. The following were included: archetypal reference genotypes (GTI = type I; PTG = type II; and CTG = type III); typical Brazilian genotypes (type BrI, type BrII and type BrIII) and isolates from pigs that had previously been described in the same region.

Discussion

Genotyping results revealed a high genetic diversity of *T. gondii* in chickens from the state of Paraíba, northeast Brazil, supporting previous studies that reported similar findings. This diversity is best represented by the MS analysis, which due to its higher resolving power identified 15 genotypes in the 29 isolates analyzed against the nine RFLP genotypes. Pena et al. (2013) identified 11 genotypes in 44 isolates from free-range chickens from the state of Espírito Santo and Sousa et al. (2016) found four genotypes in five isolates from the state of Maranhão. In Brazil, these findings are common, unlike other countries where the genetic diversity of *T. gondii* is low. For instance, Dubey and Su (2009) identified only 18 genotypes of 253 isolates in the USA, whereas genotyping of 149 isolates identified 58 genotypes in Brazil (Pena et al. 2008).

Despite the great genetic diversity of *T. gondii* previously identified in Brazil, Pena et al. (2008) suggested that there are four clonal types circulating among different hosts and regions in the country: BrI, II, III, and IV – the latter of which has only been found in southern Brazil. In the current study, only two isolates were types BrII and BrIII. These Brazilian clonal types have been identified in cats, chickens, dogs, sheep, and newborn humans in South and Southeast Brazil (Dubey et al. 2008; Silva et al. 2011; Dubey et al. 2007b; Carneiro et al. 2013), but this is the first time that clonal type BrII has been identified in the northeast and clonal type BrIII has only been recently identified from pigs in the region (Feitosa et al., unpublished data), highlighting its high level of circulation in Brazil.

The clonal spread of *T. gondii* can be explained by its modes of transmission, which include transmission by meat of intermediate hosts without the parasite passing through the definitive host and undergoing meiosis and genetic recombination. This recombination can occur in the intestine of the definitive host (cat) if it gets infected with different strains of the parasite simultaneously or in a very short time, enabling the effective crossing of gametes and the production of progenies that are genetically different from the original parental strains (Sibley and Ajioka 2008; Boothroyd and Grigg 2002). According to Feitosa et al. (2014), most domestic cats in the study region have free access to the outdoors and engage in hunting activity, providing the perfect environment for the occurrence of recombinant and novel genotypes in the study region.

Samples with the same genotype are not necessarily epidemiologically related and may comprise independent samples. The MS analysis can more accurately identify this relationship, because PCR-RFLP detects less variation in each locus when

compared to microsatellite genotyping. Ajzenberg et al. (2002) found 3–16 alleles per locus in a population of 83 *T. gondii* isolates and only 2–4 alleles for each PCR-RFLP marker, suggesting that the microsatellite technique has a superior discriminatory power than PCR-RFLP.

The genotype with the highest frequency was #13 (Caribbean 1), which accounted for approximately half of the isolates, indicating that it is circulating freely among chickens in Paraíba. Nevertheless, the occurrence of a large number of possible clones as indicated by the MS analysis and the origin of isolates underscores the role of chickens as indicators for environmental contamination with oocysts and the widespread distribution of oocysts in the soil after excretion by cats. In other Northeast states, including Ceará, Rio Grande do Norte, Pernambuco, Alagoas, Sergipe, and Bahia, this genotype has been identified in chickens (Dubey et al. 2008). Genotype #13 has also been identified in other animals, including monkeys and pigs, also in northeast Brazil (Pena et al. 2011; Clementino-Andrade et al. 2013). The only state in the northeast region where genotyping of *T. gondii* isolates has been conducted without any records of this genotype is Maranhão (Dubey et al. 2008; Sousa et al. 2016). Even though Maranhão is a northeast state, its climate characteristics differ from those of the other states, because unlike the others that have a predominantly semi-arid climate it is in a transition zone between the semi-arid and humid equatorial climate of the Amazon forest, which could result in different *T. gondii* epidemiology, routes of spread, and prevalent genotypes in the state.

Conclusion

Toxoplasma gondii has high genotypic diversity in the state of Paraíba, Brazil; genotype #13 can be considered a frequent clonal type in northeast Brazil; and due to the epidemiological features of transmission, the chances are great of finding novel nonarchetypal genotypes in the study region.

Conflict of Interest

The authors declare that they have no conflict of interest

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References

Ajzenberg D, Bañuls AL, Tibayrenc M, Dardé ML (2002) Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. Int J Parasitol 32:27–38. doi: 10.1016/S0020-7519(01)00301-0

Ajzenberg D, Collinet F, Mercier A, Vignoles P, Dardé ML (2010) Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. J Clin Microbiol 48:4641-4645. doi: 10.1128/JCM.01152-10

Boothroyd JC, Grigg ME (2002) Population biology of *Toxoplasma gondii* and its relevance to human infection: do different strains cause different disease? Curr Opin Microbiol 5(4):438-42. doi: 10.1016/S1369-5274(02)00349-1

Carneiro AC, Andrade GM, Costa JG, Pinheiro BV, Vasconcelos-Santos DV, Ferreira AM, Su C, Januário JN, Vitor RW (2013) Genetic characterization of *Toxoplasma gondii* revealed highly diverse genotypes for isolates from newborns with congenital toxoplasmosis in southeastern Brazil. J Clin Microbiol 51:901-907. doi: 10.1128

Clementino Andrade MM, Pinheiro BV, Cunha MM, Carneiro ACAV, Andrade Neto VF, Vitor RWA (2013) New genotypes of *Toxoplasma gondii* obtained from farm animals in Northeast Brazil. Res Vet Sci 94:587-589. doi: 10.1016/j.rvsc.2013.01.006

Dubey JP, Sundar N, Gennari SM, Minervino AH, Farias NA, Ruas JL, Dos Santos TR, Cavalcante GT, Kwok OC, Su C (2007a) Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from thenorthern Para state and the Southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations. Vet Parasitol 143:182-188. doi: 10.1016/j.vetpar.2006.08.024

Dubey JP, Gennari SM, Sundar N, Vianna MC, Bandini LM, Yai LE, Kwok CH, Su C (2007b). Diverse and atypical genotypes identified in *Toxoplasma gondii* from dogs in São Paulo, Brazil. J Parasitol 93:60-64. doi: 10.1645/GE-972R.1

Dubey JP, Velmurugan GV, Chockalingan A, Pena HFJ, Leifer CA, Gennari SM, Bahia-Oliveira LMG, Su C (2008) Genetic diversity of *Toxoplasma gondii* isolates from chickens from Brazil. Vet Parasitol 157:299-305. doi: 10.1016/j.vetpar.2008.07.036

Dubey, PB (2009) Toxoplasmosis in sheep – The last 20 years. Vet Parasitol 163:1-14. doi: 10.1016/j.vetpar.2009.02.026

Dubey JP, Su C (2009) Population biology of *Toxoplasma gondii*: what's out and where did they come from. Mem Inst Oswaldo Cruz 104:190-195. doi: 10.1590/S0074-02762009000200011/JCM.02502-12

Dubey JP, Lago EG, Gennari SM, Su C, Jones JL (2012) Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. Parasitol 139:1375-1424. doi: 10.1017/S0031182012000765

Elmore SA, Jones JL, Conrad PA, Patton S, Lindsay DS, Dubey J P (2010) *Toxoplasma gondii*: epidemiology, feline clinical aspects, and prevention. Trends Parasitol 26(4):190-196. doi: 10.1016/j.pt.2010.01.009

Feitosa TF, Vilela VLR, Dantas ES, Souto DVO, Pena HFJ, Athayde ACR, Azevedo SS (2014) *Toxoplasma gondii* and Neospora caninum in domestic cats from the Brazilian semi-arid: seroprevalence and risk factors. Arq Bras Med Vet Zootec 66:1060-1066. doi: 10.1590/1678-6696

Feitosa TF, Vilela VLR, Almeida-Neto JL, Santos A, De Morais DF, Athayde ACR, Azevedo SS, Pena HFJ (2016) First study on seroepidemiology and isolation of *Toxoplasma gondii* in free-range chickens in the semi-arid region of Paraíba state, Brazil. Parasitol Res 8:1-8. doi: 10.1007/s00436-016-5164-5

Ferreira IM, Vidal JE, Costa-Silva TA, Meira CS, Hiramoto RM, Penalva de Oliveira AC, Pereira-Chioccola VL (2008) *Toxoplasma gondii*: genotyping of strains from Brazilian AIDS patients with cerebral toxoplasmosis by multilocus PCR-RFLP markers. Exp Parasitol 118:221-7. doi: 10.1016/j.exppara.2007.08.006

Furuta PI, Mineo TW, Carrasco AO, Godoy GS, Pinto AA, Machado RZ (2007) Neospora caninum infection in birds: experimental infections in chicken and embryonated eggs. Parasitology 134:1931-1939. doi: 10.1017/S0031182007003344

Gangneux FR, Dardé ML (2012) Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev 25:264-296. doi: 10.1128/CMR.05013-11

Howe DK, Sibley LD (1995) *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis 172:1561-6

Huson DH (1998) Splits tree: a program for analyzing and visualizing evolutionary data. Bioinformatics 14:68-73

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254-267. doi: 10.1093/molbev/msj030

Pena HFJ, Gennari SM, Dubey JP, Su C (2008) Population structure andmousevirulence of *Toxoplasma gondii* in Brazil. Int J Parasitol 38:561-569. doi: 10.1016/j.ijpara.2007.09.004

Pena HF, Marvulo MF, Horta MC, Silva MA, Silva JC, Siqueira DB, Lima PA, Vitaliano SN, Gennari SM (2011) Isolation and genetic characterisation of *Toxoplasma gondii* from a red-handed howler monkey (Alouatta belzebul), a jaguarundi (Puma yagouaroundi), and a black-eared opossum (Didelphis aurita) from Brazil. Vet Parasitol 175(3-4):377-81. doi: 10.1016/j.vetpar.2010.10.015

Pena HFJ, Vitaliano SN, Beltrame MA, Pereira FE, Gennari SM, Soares RM (2013) PCR-RFLP genotyping of *Toxoplasma gondii* from chickens from Espirito Santo state, Southeast region, Brazil: new genotypes and a new SAG3 markerallele. Vet Parasitol 192:111-117. doi: 10.1016/j.vetpar.2012.10.004

Sibley LD, Ajioka JW (2008) Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. Annu Rev Microbiol 62:329-51. doi: 10.1146/annurev.micro.62.081307.162925

Silva RC, Langoni H, Su C, Da Silva AV (2011) Genotypic characterization of *Toxoplasma gondii* in sheep from Brazilian slaughterhouses: new atypical genotypes and the clonal type II strain identified. Vet Parasitol 175(1-2):173-177. doi: 10.1016/j.vetpar.2010.09.021

Sousa IC, Pena HF, Santos LS, Gennari SM, Costa FN (2016) First isolation and genotyping of *Toxoplasma gondii* from free-range chickens on São Luis island, Maranhão state, Brazil, with a new genotype described. Vet Parasitol 223:159-64. doi: 10.1016/j.vetpar.2016.04.041

Su C, Zhang X, Dubey JP (2006) Genotyping of *Toxoplasma gondii* by multilocusPCRRFLP markers: a high resolution and simple method for identification of parasites. Int J Parasitol 36:841-848. doi: 10.1016/j.ijpara.2006.03.003

Su C, Shwab EK, Zhou P, Zhu XQ, Dubey JP (2010) Moving towards anintegrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitol 137:1-11. doi: 10.1017/S0031182009991065

Sullivan WJJr, Jeffers V (2012) Mechanisms of *Toxoplasma gondii* persistence and latency. FEMS Microbiol Rev 36(3):717-33. doi: 10.1111/j.1574-6976.2011.00305.x

Tenter, A. M.; Johnson, A.M. Advances in parasitology, Elsevier, 1997

CAPÍTULO III

High genetic diversity in *Toxoplasma gondii* 1 isolates from pigs at slaughterhouses in Paraíba state, northeastern Brazil: circulation of new non-archetypal genotypes and Brazilian clonal lineages

Artigo submetido à Publicação na Revista Veterinary Parasitology (Qualis – A2)

High genetic diversity in *Toxoplasma gondii* isolates from pigs at slaughterhouses in Paraíba state, northeastern Brazil: circulation of new non-archetypal genotypes and Brazilian clonal lineages

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Abstract

Consumption of raw or undercooked pig meat containing *Toxoplasma gondii* cysts is considered to be an important means of transmission of this protozoon toanimals and humans. This study had the aims of serologically diagnosing, isolating and genotyping *T. gondii* from pigs that were slaughtered in the state of Paraíba, northeastern Brazil, for human consumption. Blood and tissue samples (heart, tongue and brain) were collected from 120 pigs at slaughterhouses in the state of Paraíba. Serological examinations were performed by means of the indirect immunofluorescent antibody test (IFAT), with a cutoff point of 1:64. The tissues from positive animals were subjected to bioassays in mice to isolate the parasite. Twelve isolates obtained in 60 this study and another 13 isolates that had previously been described were genotyped by means of the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), using 11 genetic markers (SAG1, 5´3´SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L258, PK1, CS3 and Apico). In addition, microsatellite analysis using 15 markers was performed. It was observed that 12.5% (15/120) of the animals were

positive in the IFAT test, with titers ranging from 64 to 2048. It was possible to isolate viable parasites in 80% (12/15) of the bioassays performed. Among the 25 isolates subjected to PCR-RFLP, complete genotypes were obtained from 19. Among these, there were 12 different genotypes, of which six were new. One isolate presented mixed infection. The type BrIII clonal lineage was found in one isolate, while genotype #013 (Caribbean 1), which is commonly encountered in northeastern Brazil, and it is probably a clonal lineage circulating in this region, was the most frequent genotype. These results demonstrate that *T. gondii* is widespread among pigs slaughtered in the state of Paraíba. They confirm that this parasite has high genetic diversity in this region and that non-archetypal genotypes commonly 74 circulate between different hosts and different regions in Brazil.

Keywords: Toxoplasmosis; swine; serology; isolation; PCR-RFLP; microsatellites; genotyping

Introduction

Toxoplasma gondii is an obligate intracellular protozoon that is very widespread around the world. Over the course of its evolution, it has developed a large variety of transmission routes. All homeothermic animals probably serve as intermediate hosts, while members of the family Felidae are the definitive hosts (Tenter et al., 2000 and Dubey 2010). Transmission can take place through ingestion of oocysts and tissue cysts that are present in water or food and also through tachyzoites transmitted transplacentally or through blood transfusion or organ transplantation (Hill and Dubey 2002).

In humans, infection by *T. gondii* is usually asymptomatic. However, in immunosuppressed individuals or in cases of congenital infection, the symptoms may be severe and may especially involve the nervous system (Dubey and Jones, 2008). In pigs, like in other animal species, the main problems caused by *T. gondii* are of reproductive nature, causing abortion, stillbirth and fetal mummification (Tsutsui et al., 2003). However, acute toxoplasmosis may also occur in newborn or newly weaned pigs (Thiptara et al., 2006 and Klein et al., 2010). Among production animals, pigs are considered to be the most important source of infection for humans (Dubey, 2009 and Tenter et al., 2000).

In South America, there is wide genetic variability among *T. gondii* isolates, compared with those in Europe and the United States, where the classical clonal types I, II and III are most commonly encountered. In Brazil, most of the samples encountered are non-archetypal and some lineages are considered to be Brazilian clonal types and have been designated BrI, BrII, BrIII and BrIV (Pena et al., 2008).

Serological diagnoses are important among 122 production animals. These indicate situations of contamination of rural spaces, given that these animals are in direct contact with the environment for long periods (Bonna et al., 2006). Isolation of this parasite through bioassays demonstrates the viability of cysts that are capable of infecting humans and other animals, and this is fundamental for diagnosing the zoonotic potential of *T. gondii* in production animals. Investigation of genotypes originating from *T. gondii* infection in animals has the aims of ascertaining the correlation between the variant encountered and the biological properties of this variant and epidemiologically tracking the agent in order to identify infection sources or transmission routes (Owen and Trees, 1999).

Therefore, this study had the aims of serologically diagnosing, isolating and genotyping *T. gondii* samples from natural infection in pigs that were slaughtered in the state of Paraíba, northeastern Brazil.

Materials and Methods

For the genotyping analysis, 25 samples of *T. gondii* that were isolated in mice and had originated from pigs slaughtered in the state of Paraíba, northeastern Brazil, were used. From these, 12 isolates were obtained in the present study, through analysis on samples from 120 pigs, using the indirect immunofluorescent antibody test (IFAT) (Camargo, 1974), with cutoff of 1:64, for investigating anti-*T. gondii* antibodies. The isolates were from brain, heart and tongue tissues of the seropositive animals and were obtained in accordance with protocols that had previously been described (Dubey, 2010). The only two municipalities in this region of Paraíba that had authorization for slaughtering these animals were Patos and Esperança, but the animals came from several neighboring municipalities (Figure 1). In 147 addition, 13 isolates from pigs (named in the present study as TgPigBrPB3-15) that were described by Feitosa et al. (2014) were incorporated into the present study for genotyping.

This experiment was conducted in accordance with the laws in force in Brazil and was approved by the ethics committee of the Federal University of Campina Grande (UFCG), under protocol 01-2012.

DNA extraction was performed on brain tissue from mice that was found to be positive through examination under a microscope. For this, the commercial kit Wizard® DNA Clean-Up System (Promega, Madison, WI, USA) was used.

For the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), 11 genetic markers were used: SAG1, SAG2 (5´3´ SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico (Su et al., 2010) and the marker CS3 (Pena et al. 2008). Samples of clonal types I (RH), II (PTG) and III (CTG) were included as positive controls; and Cougar, MAS and TgCatBr5 were included as references. Firstly, the DNA target sequences were amplified by means of multiplex PCR, using external primers for all markers, followed by nested PCR for all the markers separately. The PCR products were diluted 1:1 in ultrapure water before use in the nested PCR. All the protocols used had previously been described (Pena et al., 2008 and Su et al., 2010).

To investigate the RFLP pattern of each sample, 3 μL of the product from the nested PCR was mixed with 17 μL of digestion reaction and one unit of each restriction enzyme. The samples were incubated in accordance with the manufacturer's recommendations for what would be ideal for each enzyme. The digestion protocols described by Su et al. (2010) were used, except for the protocol for CS3, which was described by Pena et al. (2008). After digestion, the samples were subjected to horizontal electrophoresis on 2.0-3.0% agarose gel and the bands were viewed under ultraviolet light, using an image analyzer (Alpha Innotec Corp, San Leandro, CA, USA). The results thus obtained were compared and classified according to the genotypes present in the TOXODB database (http://toxodb.org/toxo/) and according to recent published papers. The phylogenetic relationships of the *T. gondii* isolates obtained were examined by means of SplitsTree4 (Huson, 1998, Huson and Bryant, 2006), using the PCR-RFLP results.

The isolates were also analyzed by means of 15 microsatellite markers: TUB2, W35, TgMA, B18, B17, M33, IV.1, X1.1, N60, N82, AA, N61, N83, M48 and M102, following protocols described by Ajzenberg et al. (2010). The analysis on the results was done using the Genemapper® 4.1 software (Applied Biosystems).

Results

Among the 120 pigs tested for *T. gondii*, 15 (12.5%) were seropositive, with titers ranging from 64 to 2048. The parasite was isolated in 80% of these seropositive pigs (12/15), with titers ≥ 64 in the IFAT, and these isolates were named TgPigBrPB16-27.

Eight of these isolates were considered to be lethal for at least one of the five mice that were infected with them. Only the isolate TgPigBrPB18 was capable of killing all of the mice thus infected. It was observed that with most of the isolates, at least one mouse resisted until the 42nd day post-inoculation (DPI), which was when serological tests were performed. The seropositive mice were sacrificed on the 60th DPI (Table 1). Details on the lethality of the isolates TgPigBrPB3-15 are described in summary form in Table 1 and can be seen in greater detail in Feitosa et al. (2014). Regarding the lethality of the 18 isolates with complete genotyping (excluding the isolate with mixed infection) in the present study, 40.3% of the mice (27/67) died between the 10th and 30th DPI with acute toxoplasmosis.

Complete genotypes were obtained from 19 of the 25 isolates, using 11 markers, by means of PCR-RFLP (Table 1). In these 19 isolates, 12 genotypes were revealed. These were distributed across nine municipalities in Paraíba, Brazil (Table 1). Six of them were new and the present study provided the first description in Brazilian studies, or in other studies, as far as we know. Genotype #203, from the isolate TgPigPB13, was described for the first time in Brazil, although it had been described previously in Gabon, Africa (Shwab et al., 2014). Genotypes #114 and #116 had only been described previously in the northern and northeastern regions of Brazil (supplementary material).

It was also highlighted that most of the genotypes are a combination of alleles I and III in the different markers and that allele II was only found in three markers (GRA-6, c22-8 and CS3). The isolate named TgPigBrPB22 presented mixed infection in seven *loci*, with a combination of two alleles in six markers (5'3' SAG, alt.SAG2, c22-8, c29-2, L358 and Apico).

Fifteen genotypes were obtained through the microsatellite analysis (supplementary material), which was expected, given that this technique has greater power of resolution.

Among the six new PCR-RFLP genotypes, four were represented in only one isolate. The isolates TgPigBrPB3 and TgPigBrPB26 which originated from the same municipality and were possibly clones, presented the same microsatellite profile (supplementary material).

No classical clonal lineage of types I, II or III was found through PCR-RFLP. The Brazilian clonal lineage type BrIII was detected in a single isolate (TgPigBrPB19), which corresponded to the classical type III, 222 according to microsatellite analysis (supplementary material). The genotype most frequently encountered was #013, which was identified in four isolates from different municipalities (TigPigBrPB17, 18, 20 and 27). Through microsatellite analysis, two genotypes (Caribbean 1) were identified and the isolates TgPigBrPB17, 20 and 27 presented the same profile; however, because of the distance between the three municipalities, it is possible that these were independente samples, which would thus demonstrate the high level of circulation of these genotypes in this region. These samples were compared by means of microsatellite analysis with another nine isolates of the genotype Caribbean 1, also from the northeastern region, and were considered to be unique genotypes (H.F.J. Pena, personal communication).

The phylogenetic network (Fig. 2) formed by these isolates, together with the Brazilian clonal types and other genotypes that have been described in isolates from pigs in the northeastern region of Brazil, showed a high degree of reticulation because of the high genetic diversity between these isolates. The greatest divergence observed was in relation to types II and BrII and the isolates studies presented greatest proximit in relation to types III and BrIII.

Table 1 PCR-RFLP genotyping of *Toxoplasma gondii* isolates from pigs from Paraíba state, northeastern Brazil, and lethality in mice.

				PCR-RFLP genótipo												ToxoDB RFLP-Genótipo
Isolado ID[#]	Cidade	Mortalidade [1]	Díada morte PI[2]	SAG1	5´3´ SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	CS3	
TgPigBrPB19	Patos	0/5	Nd	I	III	III	III	III	III	II	III	III	III	III	III	#008 **Tipo BrIII**
TgPigBrPB17	Malta	0/4	Nd	I	I	I	I	I	III	II	III	III	I	III	III	#13
TgPigBrPB18	Malta	2/2	25, 44													
TgPigBrPB20	Água Branca	2/5	10, 41													
TgPigBrPB27	São José do Bonfim	3/5	17, 22, 23													
TgPigBrPB11	Quixaba	5/5	23, 25, 28, 35, 53	I	III	III	III	I	III	II	III	III	III	III	III	#116
TgPigBrPB14	Areial	2/2	21, 23													
TgPigBrPB16	Esperança	1/1	31	I	III	III	I	III	III	III	I	III	I	I	III	#114
TgPigBrPB24	Patos	3/4	15, 16	I	I	II	III	III	III	III	I	III	III	III	II	#109
TgPigBrPB23	Patos	3/5	20, 21, 22	I	III	III	I	III	III	II	III	III	I	III	III	[3]TgCkBrPB7,8
TgPigBrPB9	Olho d'água	1/4	33	I	I	I	III	I	II	u-1	III	III	I	III	III	[3]TgCkBrPB11

TgPgBrPB21	Olho d'água	1/4	17													12,14
TgPigBrPB8	Quixaba	1/1	41	I	III	III	III	I	III	II	I	III	III	III	II	[3]TgCkBrPB26
TgPigBrPB3, 26	Água Branca	0/2	Nd	I	I	I	III	III	II	II	I	III	III	III	I	Novo
TgPigBrPB10	Olho d'água	4/5	27, 33, 35, 42	I	III	III	I	III	III	I	III	III	I	III	III	Novo
TgPigBrPB12	Esperança	5/5	18, 19,19, 20, 20	I	III	III	III	I	III	u-1	I	III	III	I	I	Novo
TgPigBrPB13	Campina Grande	2/4	21, 23	I	I	I	III	I	II	II	III	III	I	III	III	Gabon, Africa
TgPigBrPB22	Patos	3/4	15, 21, 21	I	I+III	I+III	I	III	III	II+III	III+I	III+I	I	III+I	III	Mista

1 Número de camundongos mortos/ total de camundongos infectados
2 Dias após a inoculação que cada camungondo morreu
3 Aguardando a numeração do ToxoDB

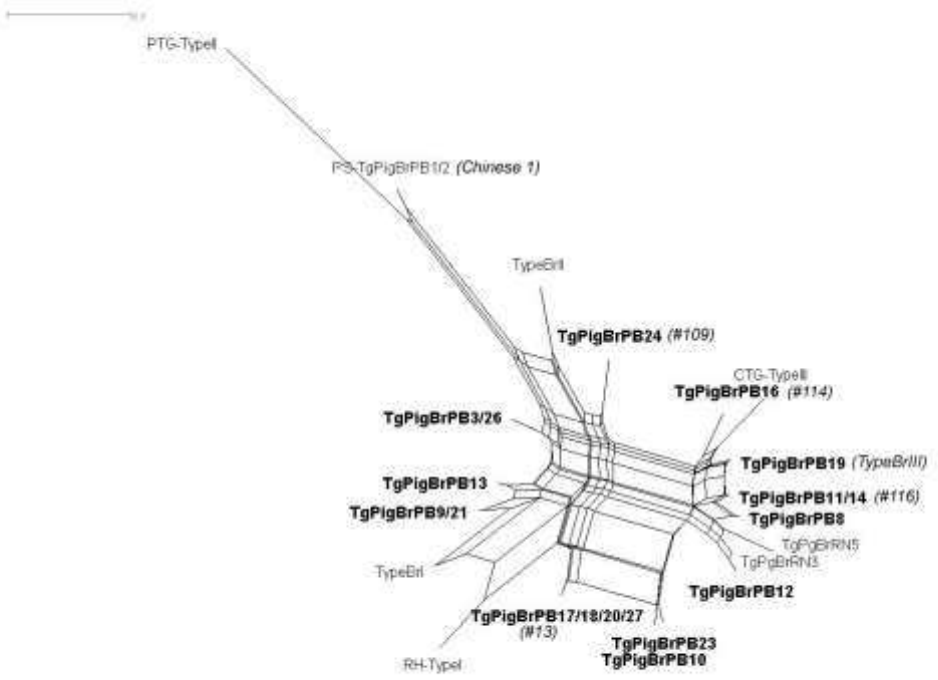

Fig.2. Phylogenetic network of the *T. gondii* isolates from pigs from northeastern Brazil, using PCR-RFLP data. The samples in bold were obtained in the present study. The following were included: archetypal reference genotypes (GTI = type I; PTG = type II; and CTG = type III); typical Brazilian genotypes (type BrI, type BrII and type BrIII) and other isolates from pigs that had previously been described. *Samples from this study with genotypes described for the first time.

Discussion

The high frequency of seropositivity for *T. gondii* observed among the pigs in this study (12.5%) was similar to what had previously been found by Feitosa et al. (2014), who observed that 19.5% (37/190) of the pigs at slaughterhouses in the state of Paraíba were seropositive. Magalhães et al. (2016) found even higher seroprevalence(51.85%; 14/27) among pigs on the island of Fernando de Noronha, Pernambuco, Brazil. In these studies, the pigs were reared extensively and in an artisanal manner, without any concern for sanitary management. Cats 247 were frequently present, in direct contact with the animals. On the other hand, in studies conducted on technologically more advanced pig farms, the seroprevalence of *T. gondii* is much lower, as can be seen in the studies by Djokic et al. (2016) and Trevisani et al. (2013), who found prevalences of 6.9% and 6.5% in France and Brazil, respectively. This demonstrates that appropriate management for pigs is extremely important for diminishing the rate of infection of this host due to *T. gondii*.

As expected among the Brazilian isolates of *T. gondii* from different hosts, the lethality observed among mice was high (Dubey et al., 2012a, Beltrame et al., 2012 and Vitaliano et al., 2015). However, because the infecting dose could not be calculated at the time of the bioassay, it was not possible to determine the virulence of each sample. On the other hand, in the northern hemisphere, most studies have shown that the *T.gondii* strains isolated present low lethality in mice (Dubey et al., 2008a and Dubey et al., 2012b).

In the present study, clonal type BrIII was identified in one isolate. This Brazilian clonal lineage had previously been identified in several hosts, such as cats, sheep, humans and dogs, in different regions of Brazil (Pena et al. 2008, Ragozo et al., 2010, Carneiro et al., 2013 and Silva et al., 2014). Moreover, Carneiro et al. (2013) found that genotype in newborns with congenital *T. gondii* infection. However, this is the first record of identification of type BrIII in pigs, thus highlighting its widespread distribution among definitive and intermediate hosts in Brazil. Little is known about the circulation of *T. gondii* strains among humans, and the information that does exist originated only from the southeastern or southern regions of the country.

Genotype #013 had already been described in several *T. gondii* isolates in northeastern Brazil, both in domestic and wild animals (Dubey et al., 2008b, Ragozo et al., 2010, Pena et al., 2011 and Almeida et al., 2017). Clementino Andrade et al. (2013) also found that this genotype was present in an isolate from pig in Rio Grande do Norte, a

state that neighbors Paraíba, Brazil. It seems that #013 occurs homogenously among different animal species in the northeastern region of Brazil, thus suggesting that this is a clonal type that circulates in the northeastern region of Brazil.

Occurrence of several genotypes at the same locality increases the chances of *T. gondii* mixed infections which would explain the observation of a mixed genotype in the present study. This type of infection provides a situation favoring *T. gondii* gene recombination, since from the time when a felid becomes infected concomitantly with different samples of the parasite, crossing of gametes and production of new parasites that are genetically different from the infecting strains can occur, thus providing greater genetic diversity for the parasite (Dardé, 2008, Pena et al., 2008 and Sibley and Ajioka, 2008).

Genotyping studies on *T. gondii* isolates in Brazil began approximately one decade ago. More than 300 isolates and 100 genotypes have now been described in Brazil (Shwab et al., 2014), but it was not a surprise that six new genotypes were identified in isolates from pigs in the present study, given that most of the isolates studied so far have originated from the southern and southeastern regions of Brazil. Likewise, only recently, Olinda et al. (2016) described two cases of acute toxoplasmosis in pigs, also in Paraíba, in which the genotype Chinese 1 was reported in Brazil for the first time. Six out of the 12 genotypes identified in the present study can be considered to be unique to Brazil. The low technological level of pig breeding in the region studied, with the presence of cats and the habit of offering leftovers to the animals (Feitosa et al., 2014) provides an appropriate environment for infection with the different strains that are in circulation.

The results from the present study demonstrate the great genetic diversity of *T. gondii* in the state of Paraíba and corroborate what has been found in several genetic studies, which demonstrated its enormous diversity in different hosts and Brazilian states (Ragozo et al., 2010, Pena et al., 2013, Carneiro et al., 2013 and Silva et al.,2014). These results also indicate the possibility that further new genotypes might be identified in future studies.

Conclusion

It can be concluded that *T. gondii* is widely disseminated among pigs slaughtered for human consuming in the state of Paraíba and that its isolates present high lethality towards mice. Regarding genetic analysis, the genotypic diversity of the parasite in the

state of Paraíba is high and non-archetypal genotypes commonly circulate among different hosts in different regions of Brazil.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Almeida, J.C., Melo, R.P., De Morais, P.C., Da Silva, S.M., De Barros, L.D., Garcia, J.L., Porto, W.J., Mota, R.A., 2017. First isolation and RFLP genotyping of *Toxoplasma gondii* from crab-eating fox (*Cerdocyon thous*-Linnaeus, 1766). Acta Trop. 169, 26-29.

Ajzenberg, D., Collinet, F., Mercier, A., Vignoles, P., Dardé, M.L., 2010. Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. J. Clin. Microbiol. 48, 4641-4645.

Beltrame, M.A.V., Pena, H.F.J., Ton, N.C., Lino, A.J.B., Gennari, S.M., Dubey, J.P., Pereira, F.E.L., 2012. Seroprevalence and isolation of *Toxoplasma gondii* from free range chickens from Espírito Santo state, southeastern Brazil. Vet. Parasitol. 188, 225-230

Bonna, I.C.F, Figueiredo, F.B, Costa, T., Vicente, R.T., Santiago, C.A.D., Nicolau, J.L., Neves, L.B., Millar, P.R., Sobreiro, L.G., Amendoeira, M.R.R., 2006. Estudo soroepidemiológico da infecção por *Toxoplasma gondii* em suínos e frangos para abate em região rural do Rio de Janeiro. Rev. Bras. Ciênc. Vet. 13, 186-189.

Camargo, M.E., 1974. Introdução às técnicas de imunofluorescência. Rev. Bras. Patol. Clín. 10, 143-169.

Carneiro, A.C., Andrade, G.M., Costa, J.G., Pinheiro, B.V., Vasconcelos-Santos, D.V., Ferreira, A.M., Su, C., Januário, J.N., Vitor, R.W., 2013. Genetic characterization of *Toxoplasma gondii* revealed hi 347 ghly diverse genotypes for isolates from newborns with congenital toxoplasmosis in southeastern Brazil. J. Clin. Microbiol. 51, 901-907.

Clementino Andrade, M.M., Pinheiro, B.V., Cunha, M.M., Carneiro, A.C.A.V., Andrade Neto, V.F., Vitor, R.W.A., 2013. New genotypes of *Toxoplasma gondii* obtained from farm animals in Northeast Brazil. Res. Vet. Sci. 94, 587-589.

Dardé, M.L., 2008. *Toxoplasma gondii*, "new" genotypes and virulence. Parasite. 15, 366-371.

Djokic, V., Fablet C., Blaga R., Rose N., Perret C., Djurkovic-Djakovic O., Boireau P., Durand B., 2016. Factors associated with *Toxoplasma gondii* infection in confined farrow-to-finish pig herds in western France: an exploratory study in 60 herds. Parasit. Vectors. 24, 466-474.

Dubey, J.P., 2009. *Toxoplasma gondii* infections in chickens (*Gallus domesticus*): Prevalence, Clinical Disease, Diagnosis and Public Health Significance. Zoonoses Public Health. 57, 60-73.

Dubey, J.P. (2 Ed.), 2010. Toxoplasmosis of animals and humans. CRC Press, Maryland, 313pp.

Dubey, J.P., Jones, J.L., 2008. *Toxoplasma gondii* infection in humans and animals in the United States. Int. J. Parasitol. 38, 1257-1278.

Dubey, J.P., Huong, 372 L.T., Lawson, B.W., Subekti, D.T., Tassi, P., Cabaj, W., Sundar, N., Velmurugan, G.V., Kwok, O.C., Su, C., 2008a. Seroprevalence and Isolation of *Toxoplasma gondii* from Free-Range Chickens in Ghana, Indonesia, Italy, Poland, and Vietnam. J. Parasitol. 94, 68-71.

Dubey, J.P., Velmurugan, G.V., Chockalingan, A., Pena, H.F.J., Leifer, C.A., Gennari, S.M., Bahia-Oliveira, L.M.G., Su, C., 2008b. Genetic diversity of *Toxoplasma gondii* isolates from chickens from Brazil. Vet. Parasitol. 157, 299-305

Dubey, J.P., Lago, E.G., Gennari, S.M., Su, C., Jones, J.L., 2012a. Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. Parasitol. 139, 1375-1424.

Dubey, J.P., Hill, D.E., Rozeboom, D.W., Rajendran, C., Choudhary, S., Ferreira, L.R., Kwok, O.C., Su, C., 2012b. High prevalence and genotypes of *Toxoplasma gondii* isolated from organic pigs in northern USA. Vet. Parasitol. 188, 14-18.

Feitosa, T.F., Vilela, V.L.R., Melo, L.R., Almeida Neto, J.L., Souto, D.V., Morais, D.F., Athayde, A.C., Azevedo, S.S., Pena, H.F., 2014. *Toxoplasma gondii* and *Neospora caninum* in slaughtered pigs from Northeast, Brazil. Vet. Parasitol. 28, 305-309.

Hill, D., Dubey, J.P., 2002. *Toxoplasma gondii*: transmission, diagnosis and prevention. Clin. Microbiol. Infect. 8, 634-640.

Huson, D.H., 1998. Splits tree: a program for analyzing 397 and visualizing evolutionary data. Bioinformatics 14, 68-73.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254-267.

Klein, S., Wendt, S.M., Baumgärtner, W., Wohlsein, P., 2010. Systemic toxoplasmosis and concurrent porcine circovirus-2 infection in a pig. J. Comp. Pathol. 142, 228-234

Magalhães, F.J., Da Silva, J.G., Ribeiro-Andrade, M., Pinheiro, J.W. Júnior, Mota, A.R., 2016. High prevalence of toxoplasmosis in free-range chicken of the Fernando de Noronha Archipelago, Brazil. Acta Trop. 159, 58-61.

Olinda, R.G., Pena, H.F., Frade, M.T., Ferreira, J.S., Maia, L.Â., Gennari, S.M., Oliveira, S., Dantas, A.F., Riet-Correa F., 2016. Acute toxoplasmosis in pigs in Brazil caused by *Toxoplasma gondii* genotype Chinese 1. Parasitol. Res. 115, 2561-2566

Owen, M.R., Trees, A.J., 1999. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. Int. J. Parasitol. 85, 382-384.

Pena, H.F.J., Gennari, S.M., Dubey, J.P., Su, C., 2008. Population structure and mouse virulence of *Toxoplasma gondii* in Brazil. Int. J. Parasitol. 38, 561-569.

Pena, H.F., Marvulo, M.F., Horta, M.C., Silva, M.A., Silva, J.C., Siqueira, D.B., Lima, P.A., Vitaliano, S.N., Gennari, S.M., 2011. Isolation and genetic characterisation of *Toxoplasma gondii* from a red-handed howler monkey (*Alouatta belzebul*), a jaguarondi (*Puma yagouaroundi*), and a black-eared opossum (*Didelphis aurita*) from Brazil. Vet. Parasitol. 175, 377-81.

Pena, H.F.J., Vitaliano, S.N., Beltrame, M.A., Pereira, F.E., Gennari, S.M., Soares, R.M., 2013. PCR-RFLP genotyping of *Toxoplasma gondii* from chickens from Espírito Santo state, Southeast region, Brazil: new genotypes and a new SAG3 marker allele. Vet. Parasitol. 192,111-117.

Ragozo, A.M.A., Pena, H.F.J., Yai, L.E., Su, C., Gennari, S.M., 2010. Genetic diversity among *Toxoplasma gondii* isolates of small ruminants from Brazil: novel genotypes revealed. Vet. Parasitol. 170, 307-312.

Shwab, E.K., Zhu, X.Q., Majumdar, D., Pena, H.F.J., Gennari, S.M., Dubey, J.P., Su, C., 2014. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. Parasitology 141, 453-461.

Sibley, L.D., Ajioka, J.W., 2008. Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. Annu. Rev. Microbiol. 62, 329-351

Silva, L.A., Andrade, R.O., Carneiro, A.C., Vitor, R.W., 2014. Overlapping *Toxoplasma gondii* genotypes circulating in domestic animals and humans in Southeastern Brazil. PLoS One 27, 1-7.

Su, C., 447 Shwab, E.K., Zhou, P., Zhu, X.Q., Dubey, J.P., 2010. Moving towards anintegrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology 137, 1-11.

Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30, 1217-1258.

Thiptara, A., Kongkaew, W., Bhumibhamon, T., Bilmad, U., Anan, S., 2006. Toxoplasmosis in piglets in southern Thailand. Ann. N. Y. Acad. Sci. 1081, 336-338.

Trevisani, N., Vieira neto, A., Guths, M.F., Sartor, A.A., Souza, A.P., Moura, A.B., 2013. *Toxoplasma gondii*: sorologia e fatores de risco em suínos de granjas no Vale do Itajaí, Santa Catarina. Arch. Vet. Sci. 18, 14-19

Tsutsui V.S., Navarro I.T., Freire R.L., Freitas J.C., Prudencio L.B., Delbem A.C.B., Marana, E.R.M., 2003. Soroepidemiologia e fatores associados à transmissão do *Toxoplasma gondii* em suínos do norte do Paraná, Brasil. Arch. Vet. Sci. 8, 27-34.

Vitaliano, S.N., de Mendonça, G.M., de Sandres, F.A., Camargo, J. de S., de Tarso, P., Basano, S. de A., Silva, J.C., de Souza, V.K., Cartonilho, G., de Almeida, A.T., Gennari, S.M., Camargo, L.M., 2015. Epidemiological aspects of *Toxoplasma gondii* infection in riverside communities in the Southern Brazilian Amazon. Rev. Soc. Bras. Med. Trop. 48, 301-306.

CONCLUSÕES

Os resultados obtidos permitem concluir que:

- O *T. gondii* encontra-se amplamente distribuído em suínos e galinhas no estado da Paraíba, Brasil;
- Os isolados provenientes de suínos e galinhas do estado da Paraíba apresentam um perfil de grande mortalidade em camundongos;
- O genótipo #013 é mais frequente em suínos e galinhas do Estado da Paraíba, Brasil;
- A variabilidade genética deste parasito encontrada em suínos e galinhas é alta e
- Existem grandes chances de novos genótiposrecombinantes serem revelados na região estudada.

ANEXOS

ANEXO I – Comprovante de Publicação do Capítulo I

Parasitology Research
October 2016, Volume 115, Issue 10, pp 3983–3990

First study on seroepidemiology and isolation of *Toxoplasma gondii* in free-range chickens in the semi-arid region of Paraíba state, Brazil

Authors | Authors and affiliations

Thais Ferreira Feitosa, Vinicius Longo Ribeiro Vilela, João Leite de Almeida-Neto, Antonielson dos Santos, Dayana Firmino de Morais, Ana Célia Rodrigues Athayde, Sérgio Santos de Azevedo, Hilda Fátima de Jesus Pena

Original Paper

First Online: 09 June 2016

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Feitosa, T.F., Vilela, V.L.R., de Almeida-Neto, J.L. et al. Parasitol Res (2016) 115: 3983. doi:10.1007/s00436-016-5164-5

ANEXO II – Comprovante de Submissão do Capítulo II

Parasitology Research

New genotypes of Toxoplasma gondii from free range chickens (Gallus gallus domesticus) from Paraíba State, Brazil

--Manuscript Draft--

Manuscript Number:	
Full Title:	New genotypes of Toxoplasma gondii from free range chickens (Gallus gallus domesticus) from Paraíba State, Brazil
Article Type:	Short Communication
Funding Information:	Conselho Nacional de Desenvolvimento Científico e Tecnológico (no. 474737 / 2012-8 (H.F.J.P.).) — Dra Hilda Fátima de Jesus Pena
Abstract:	The objective of this study was to genetically characterise T. gondii isolates from free range chickens from State of Paraíba, Brazil. There were used 33 samples of chickens isolated from mice. These samples were obtained from properties located in five municipalities of Paraíba including Esperança, Mãe D'água, Malta, Monteiro and Patos. For DNA extraction and subsequent genotypic analysis, there were used brains of infected mice with T. gondii cysts. Strain typing was performed using 12 PCR-RFLP markers: SAG1, SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3. Nine genotypes were identified; three were identified as novel in six isolates. The Brazilian clonal types BrII and BrIII had one isolate each. Clonal types I, II or III were not observed. The most frequent genotype, found in 14 (46.7%) isolated distributed in four of the five municipalities studied was # 013. The other genotypes were restricted to different micro regions. It can be concluded that there is great genotypic diversity of T. gondii in the state of Paraíba and that the genotype # 013 is frequently found in the Brazilian northeast, which may be even considered a typical clonal type of this region.
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Corresponding Author's Secondary Institution:	
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	Hilda Fátima de Jesus Pena, Doctor
Order of Authors Secondary Information:	
Author Comments:	

ANEXO III – Comprovante de Submissão do Capítulo III

Elsevier Editorial System(tm) for Veterinary Parasitology

Manuscript Draft

Manuscript Number: No Vetpar-S-17-00166

Title: High genetic diversity showed for the first time in Toxoplasma gondii isolates from pigs from Paraiba state, Northeast Brazil, with new non-archetypal genotypes and clonal Brazilian lineages circulation

Article Type: Research paper

Keywords: Genotypic characterization; Diagnosis; PCR / RFLP; IFAT

Corresponding Author: Professor Thais Ferreira Feitosa, M.D.

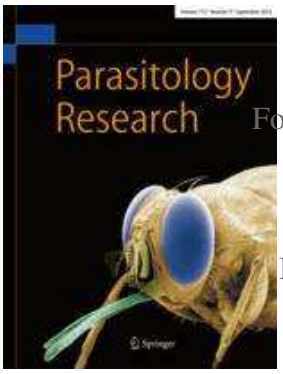
Corresponding Author's Institution: Universidade Federal de Campina Grande

First Author: Thais Ferreira Feitosa, M.D.

Order of Authors: Thais Ferreira Feitosa, M.D.; Vinicius Longo R Vilela, Doctor; João L Almeida-neto, Graduate; Lidio Ricardo B Melo, Magister; Dayana F de Morais, Magister; Ana Célia R Athayde, Doctor; Bruna F Alves, Magister; Hilda Fátima J Pena, Doctor; Fabiana Nakashima, Doctor

Abstract: The consumption of swine meat infected with Toxoplasma gondii cysts is considered an important route of transmission of this protozoan to humans and animals. Therefore, this study aimed to perform the serology, isolation and genotyping of slaughtered pigs in the state of Paraiba, Brazil. There were collected a total of 120 blood and tissue samples (heart, tongue and brain) from pigs from slaughterhouses in the state of Paraíba. The serological test was performed through Indirect Fluorescente Antibody Test (IFAT) with a cut-off point of 1:64. The tissues of the positive animals were submitted to the bioassay in mice for isolation of the parasite. The isolates obtained in this study and additionally 13 previously unreleased isolates from previous research were genotyped by PCR-restriction fragment-lengh polymorphism (PCR / RFLP) using 12 genetic markers (SAG1, SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, C29-2, L258, PK1, CS3 and Apico). It was observed a total of 12.5% (15/120) of positive animals in the IFAT with titers varying from 64 to 2048. It was possible to isolate the parasite in 80% (12/15) of the bioassays performed. From the 25 (12 of this research and 13 of previous studies) isolates submitted to PCR / RFLP, a complete genotype of 23 was obtained and 13 genotypes were revealed, four of them unpublished. One isolate presented mixed infection. The BrIII clonal lineage was found in one isolate while genotype # 013 (Caribbean I), commonly found in the Northeast region of Brazil, was the most frequent genotype. These results demonstrate that T. gondii is widely distributed among pigs slaughtered in the state of Paraíba, confirming the high genetic diversity of the parasite in the region studied and that non-archetypes genotypes commonly circulate among different hosts and regions of Brazil

ANEXO IV- NORMAS PARA A PUBLICAÇÃO NA PARASITOLOGY RESEARCH



Parasitology Research

Founded as Zeitschrift für Parasitenkunde
Editors: H. Mehlhorn; B. Chobotar
ISSN: 0932-0113 (print version)
ISSN: 1432-1955 (electronic version)
Journal no. 436

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Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

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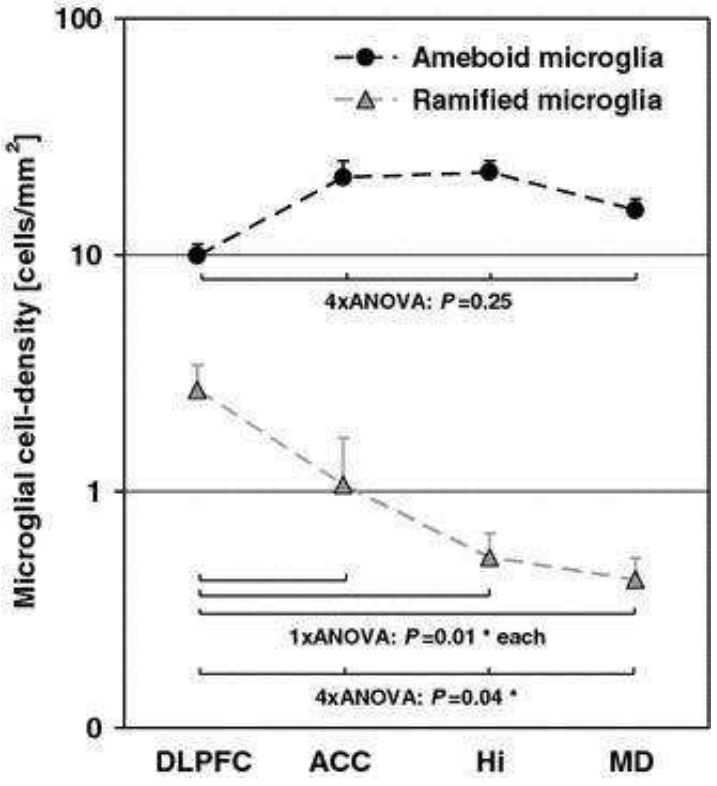

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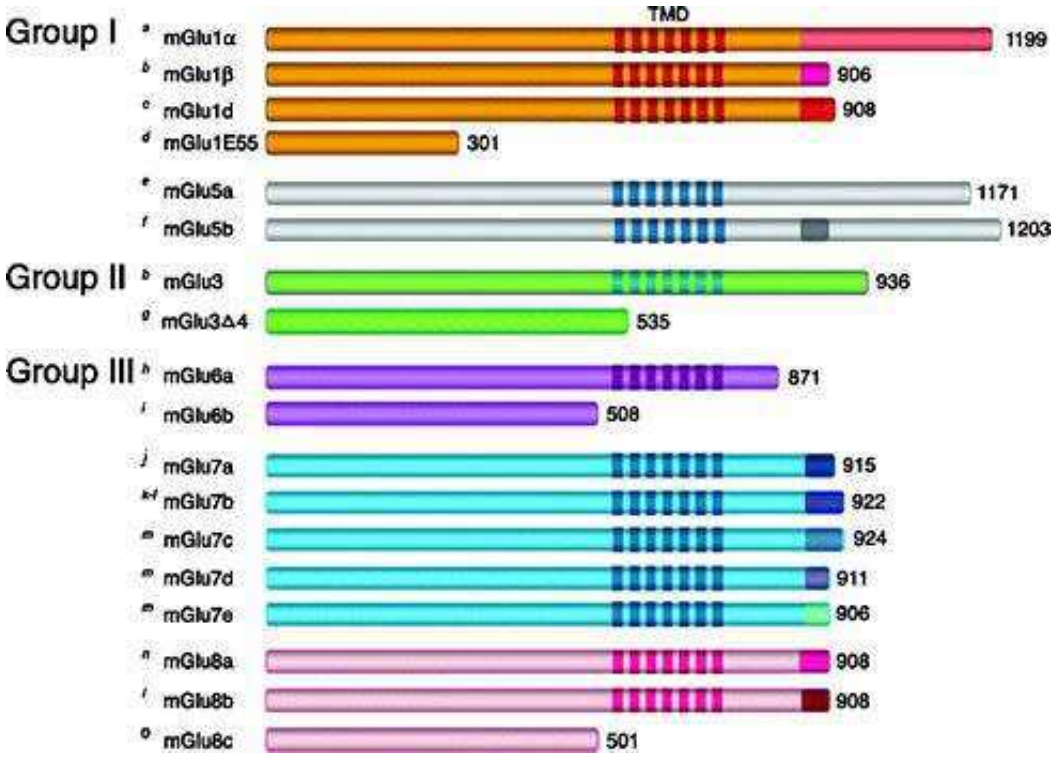

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ANEXO IV- NORMAS PARA A PUBLICAÇÃO NA VETERINARY PARASITOLOGY

VETERINARY PARASITOLOGY

An international scientific journal and the Official Organ of the American Association of Veterinary Parasitologists (AAVP)), the European Veterinary Parasitology College (EVPC) and the World Association for the Advancement of Veterinary Parasitology (WAAVP)

AUTHOR INFORMATION PACK

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ISSN: 0304-4017

DESCRIPTION

This journal is concerned with those aspects of helminthology, protozoology and entomology which are of interest to animal health investigators, veterinary practitioners and others with a special interest in parasitology. Papers of the highest quality dealing with all aspects of disease prevention, pathology, treatment, epidemiology, and control of parasites in all domesticated animals, fall within the scope of the journal. Papers of geographically limited (local) interest which are not of interest to an international audience will not be accepted. Authors who submit papers based on local data will need to indicate why their paper is relevant to a broader readership.

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AUDIENCE

Research Workers and Practitioners in veterinary medicine, Animal Health Investigators and others with a special interest in parasitology, veterinary pharmaceutical industry.

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Letters to the Editor
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Dr W. Pomroy

Institute of Veterinary, Animal and Biomedical Sciences Massey University

Private Bag 11 222 Palmerston North 4442 New Zealand w.pomroy@massey.ac.nz

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