DOI: 10.1515/ap-2017-0054

Acta Parasitologica, 2017, 62(2), 466–470; ISSN 1230-2821

DE GRUYTER

Toxoplasma gondii in backyard pigs: seroepidemiology and mouse bioassay

Erika Fernanda Torres Samico-Fernandes*, Marcela Fernanda Torres Samico-Fernandes, Pedro Paulo Feitosa de Albuquerque, Jonatas Campos de Almeida, André de Souza Santos, André da Rocha Mota, Orestes Luiz de Souza Neto and Rinaldo Aparecido Mota

Rua Dom Manoel de Medeiros s/n. Dois Irmãos, Recife – PE. Brazil. CEP: 52171-900. Laboratório de Doenças Infectocontagiosas, Departamento de Medicina Veterinária/Universidade Federal Rural de Pernambuco, Brazil

Abstract

The aim of this study was to evaluate the occurrence of *Toxoplasma gondii* in backyard pigs destined for human consumption in Pernambuco state, Brazil. Blood and tissue samples (liver, heart, brain, lung and diaphragm) were collected from 224 pigs from legal slaughterhouses and tested for *T. gondii* infection. Antibodies to *T. gondii* were found in the sera of 37.9% (85/224) by using the immunofluorescence antibody test (cut-off – 1:64). Tissue samples from seropositive pigs were bioassayed in mice. Tissue samples from seropositive pigs and from mice of the bioassay were submitted to histopathology, immunohistochemistry, polymerase chain reaction (PCR) and sequencing; 14.1% of pig tissue samples and 27.7% of bioassayed mouse samples were positive for *T. gondii* DNA, but all pig and mouse tissues were negative in histopathology analysis and immunochemistry. By using a risk assessment questionnaire, there was significant difference ($p<0.001$) in seroprevalence of 21.2% (reproducer) and 3.1% (finishing pig). These data serve as indicative of the sanitary conditions and risk of *T. gondii* infection for backyard pigs. Preventive measures must be implemented by health services to avoid toxoplasmosis human cases due to ingestion of pig meat.

Keywords

Backyard pigs, direct diagnosis, serology, sequencing, *Toxoplasma gondii*

Introduction

Toxoplasma gondii is an obligate intracellular protozoan and is considered one of the most common parasitic diseases worldwide (Dubey 2010). Domestic and wild felids have an important role in the epidemiology of toxoplasmosis as definitive hosts, shedding in your feces millions of oocysts. Human being, domestic animals, livestock and wild animals serve as intermediate hosts, and the infection occurs by ingestion of food or water with oocysts, and raw or undercooked meat that contain cysts (Dubey 2010). Postnatal infection in pigs may occur by contact with oocysts present in contaminated food and water (Kiljestra *et al.* 2008), and there are cases of transplacental transmission (Dubey 2009). *T. gondii* serosurveys in pigs has been reported in several regions of Brazil and prevalence may range from 1.32% to 90.4% (Dubey 2009, 2010). Pigs raised on no-technified system may ingest viscera of slaughtered animals, birds and rodents, enhancing the risk infection (Dubey 2009). *T. gondii* tissue cysts may be found in liver, heart, brain, tongue, diaphragm, and skeletal muscle (Swierzy *et al.* 2014).

There are different routes of *T. gondii* transmission to human populations, depending of factors such as cultural habits, religion and environmental conditions (Demar *et al.* 2007). The ingestion of raw or undercooked meat from pork containing tissue cysts has been highlighted as the major main sources of human infection by this protozoan, and may represent 50% or more of toxoplasmosis human cases (Dubey 2010, Torgerson *et al.* 2014). Thus, pigs are considered a significant source of *T. gondii* infection to humans in several countries (Dubey 2010) and thereby have an important role in the maintenance of *T. gondii* chain infection by their products and sub-products (Bezerra *et al.* 2012, Oliveira *et al.* 2014).

Although *T. gondii* is widely prevalent in pigs throughout Brazil (Dubey *et al.* 2012), data on occurrence of *T. gondii* in backyard pig populations in northeastern Brazil are limited. The aim of the present study was to assess the occurrence of

*Corresponding author: erikasamico@hotmail.com

T. gondii in pigs destined for human consumption in Pernambuco state, northeastern Brazil.

Materials and Methods

Samples

The present study was developed according to Brazilian laws and was approved by the Animal Use Ethics Committee (protocol number 015533/2012-71).

In this study were used 224 pigs of several ages (from six months to two years) and different surrounding towns, coming from legal slaughterhouses in Pernambuco state (Table 1). Blood samples were obtained during the bleeding of pigs, and in slaughter line were collected the tissue samples: liver, heart, brain, lung and diaphragm. Tissue samples were stored in Ziploc® bags and kept at +4º C until the analysis.

Serological test and mouse bioassay

Immunofluorescence Antibody Test (IFAT) was performed to investigate antibodies (IgG) against *T. gondii* (Camargo 1974). Ten microliters of diluted serum were placed in wells on *T. gondii* slides contained RH tachyzoites as antigen. The samples were placed over the antigen in the slides and incubated at 37º C for 30 minutes. The slides were washed, and incubated with anti-pig IgG serum conjugated with fluorescein isothiocyanate (Sigma Chemical, USA) containing 0.001% of Evan's Blue (Sigma Chemical, USA). In all slides, were included positive and negative controls. Titers ≥64 were considered positive, and positive serum was retested using twofold serial dilutions.

Tissue samples from seropositive pigs were submitted to peptic digestion as described by Dubey (1988). Briefly: 50 g of liver, heart, brain, lung and diaphragm were cutted with a scalp, following by addition of 250 mL of saline solution (NaCl) and 250 mL of acidic solution of pepsin (pH = 1.1–1.2). The material was incubated at 37°C for one hour. The homogenate was filtered through two layers of gauze and centrifuged at 1200xg for 10 min. The supernatant was draw off and the sediment was resuspended with a solution of bicarbonate at 1.2% (pH = 8.3) and centrifuged at 1200 × g for 10 min. The supernatant was draw off again and the sediment was resuspended in 3 ml of an antibiotic saline solution (1,000 U of penicillin and 100 µL of streptomycin/ml of saline solution). This product was inoculated subcutaneously in three Swiss Webster mice (1 ml per mouse). The mice were observed daily for six weeks and then euthanized to collect blood for IFAT, and lung and brain for imprint and molecular diagnosis.

DNA extraction, Polymerase Chain Reaction (PCR) and sequencing

DNA was extracted from tissue samples (liver, heart, brain, lung and diaphragm) of seropositive pigs by IFAT, as well as from tissue of mice submitted to bioassay. DNA extraction was performed using the commercial kit "Qiagen DNA Easy Blood and Tissues Kit" (Qiagen®), according to the manufacturer's instructions. The DNA obtained was analyzed and quantified in agarose gel (0.8%) with a 1Kb molecular weight marker, stained with blue green, visualized in ultraviolet light and photodocumented to confirm quality.

The amplification reactions were performed in a final volume of 12.5µL containing: 2.5µL of genomic DNA; 0.5µM of each primer; 2.75µL of Milli-Q ultrapure water and 6.25µL of

MasterMix (mixture for PCR – Qiagen), following the manufacturer's instructions. The thermal profile of reaction stages was performed using a MJ-96G thermocycler (Biocycle Co. Ltd, China) (Homan *et al.* 2000). The primer pairs used were TOX-4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX-5 (CGCTGCAGACACAGTGCATCTGGATT) to amplify a region with 529 base pairs (bp). The amplified products were detected by electrophoresis in agarose gel (2%), stained with blue green, visualized under ultraviolet light and photodocumented. Positive control of the reaction was a suspension of RH strain tachyzoites (10^4 tachyzoites/mL), and ultrapure water was used as negative control. PCR-positive samples were purified using the QIAquick® commercial gel extraction kit and were submitted to sequencing reactions. Samples were sequenced using the Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) in 96-well plates, in accordance with the manufacturer's instructions. DNA templates were sequenced by means of the fluorescent dideoxy terminator method in an ABI PRISM 3100 genetic analyzer for automatic sequencing (Applied Biosystems, USA). The Jalview software (available at: http://www.jalview.org/) was used to perform the identity analysis. The sequences were analyzed and edited using the BioEdit software. The GenBank sequences were aligned with the sample sequences using the Mega 5.05 software and the Clustal cutoff value of 50%.

Histopathology and immunohistochemistry (IHC)

Tissue fragments from pigs and mice from bioassay were fixed in buffered formalin (10%) and processed using the usual methods for histological analysis (Luna 1968). Briefly: after fixation, the fragments were dehydrated in alcohol with increasing concentrations (from 70% to 100%), followed by diaphonization in xylene, a paraffin bath at 60ºC and finally, inclusion in paraffin. Next, sections of 5µm thickness were cut, deparaffinized, rehydrated and stained using hematoxylin-eosin (HE). The entire slide was analyzed for presence of tissue cysts or histopathological lesions (Olympus BX40, objective 40x).

The immunohistochemistry technique was performed (Galiza *et al.* 2014) with some modifications: we used the labeled streptavidin biotin (LSAB) alkaline phosphatase technique (Dako Corporation, USA). The histological sections with 5 µm fixed on silanized slides were dewaxed and hydrated. Inactivation of endogenous peroxidase was performed using 3% hydro-

gen peroxide solution in distilled water at room temperature for 30 minutes. Washes were performed in PBS (0.01 M; pH 7.4). For antigen retrieval, the sections were subjected to heating in a water bath with citrate buffer (pH 6.0; 10 mM) and were then placed in a microwave oven for 20 minutes at high power and washed twice in PBS afterwards. Non-specific binding was blocked by means of incubation with 5% skim milk in PBS for 30 minutes at room temperature. After the slides had been washed with distilled water, they were immersed in PBS and then incubated with primary *T. gondii* antibody (goat polyclonal antiserum #210–70, VMRD, USA) diluted 1:200 in PBS (pH 7.4), in a moist chamber at 4 ºC for overnight. After this period, the slides were washed in PBS and the commercial LSAB alkaline phosphatase (Dako Corporation, USA) kit was used. Subsequently, the sections were washed in PBS and the Permanent Red (Dako Corporation, USA) (chromogen) was used in accordance with the manufacturer's recommendations. Finally, the samples were counterstained with Harris hematoxylin (Sigma Chemical, USA), dehydrated, cleared in xylene (Vetec®, Brasil) and mounted using balsam (Labsynth, Brasil), under a cover slip.

Statistical analysis

To identify risk factor was used an epidemiological questionnaire concerning to: age and gender of animals, type of feeding, breeding system used and presence of cats on farms. Data were analyzed statistically based on the relative and absolute frequency distribution. In addition, univariate analysis was performed using the Chi-squared test. The results obtained by PCR and serology were the dependent variable. The level of significance adopted was 5% (Thrusfield 2004). Statistical calculations were done using the Statistical Package for the Social Sciences (SPSS) version 19.0. The Cohen´s Kappa Coefficient (kappa) was used to assess the agreement between the techniques. Kappa values were interpreted as described by Landis and Koch (1977): 0.00 – 0.20 = weak agreement; 0.21 – 0.40 = regular agreement; 0.41 – 0.60 = moderate agreement; 0.61 – 0.80 = good agreement; 0.81–1.00 = very good agreement. Negative values were interpreted as equal to 0.00.

Results

Antibodies against *T. gondii* were found in 37.9% (85/224) with higher frequency pigs aged over one year (39.4% (54/137) than, and younger animals (≤ 6 months, 35.6% (31/87). Relative to gender: 36.5% (34/93) of positive males, and 38.9% (51/131) of positive females. Of the samples positive by IFAT, 37.6% (32/85) exhibited titers of 64, 28.2% (24/85) exhibited titers of 128; 21.1% (18/85) had titers of

Table I. Analysis of risk factors associated with *Toxoplasma gondii* infection in Backyard pigs from Pernambuco state, northeastern Brasil, considering the Immunofluorescence Antibody Test (IFAT) and the Polymerase Chain Reaction (PCR)

VARIABLE	IFAT	Univariate Analysis	P	PCR	Univariate Analysis	P
	Positive	OR (CI 95%)	value	Positive	OR (CI 95%)	value
Age						
Termination	05/158 (3.1%)		<0.001*	05/55 (9.09%)		
Reproduction	14/66 (21.2%)	8.2 (2.8 – 23.9)		12/30 (40.0%)	1.7 (0.9 – 3.0)	0.072
Gender						
Female	09/131 (6.9%)	–	0.305	08/50 (16.0%)	1.0 (0.6 – 1.7)	0.935
Male	10/93 (10.7%)	1.6 (0.6 – 4.2)		09/35 (25.7%)	–	
Type of feeding						
Leftovers	05/87 (5.7%)	–		03/33 (9.09%)	–	
Ration	04/37 (10.8%)	1.9 (0.5 – 7.8)	0.498	04/12 (33.3%)	10/40 (25.0%)	0.720
Mixed	10/100 (10.0%)	1.8 (0.6 – 5.5)				
Breeding System						
Intensive	04/46 (8.7%)	4.1 (0.3 – 4.0)		04/14 (28.5%)	–	
Extensive	09/125 (7.2%)	–	0.664	07/51 (13.7%)	1.5 (0.7 – 3.2)	0.464
Mixed	06/53 (11.3%)	1.6 (0.5 – 4.8)		06/20 (30.0%)	1.4 (0.6 – 3.2)	
Presence of cats						
Yes	09/87 (10.3%)	3.7 (0.4 – 30.3)		08/32 (25.0%)	–	
No	09/104 (8.6%)	3.0 (0.3 – 24.8)	0.436	08/41 (19.5%)	1.1 (0.5 – 2.5)	0.913
Unknown	01/33 (3.1%)	–		01/12 (8.3%)	–	

OR – Odds Ratio; CI – confidence interval; *significant association (p<0.05)

256; 11.7% (10/85) showed titers of 512 and 1.17% (1/85) exhibited titers of 1024.

By PCR , *T. gondii* DNA was found in 14.1% (12/85) of pig tissue samples in fragments of heart (4/12) and brain (8/12) of pig and (5/18—hearts of 2 and brains of 3) of mouse tissue from bioassay. In sequencing analysis on direct double strain, it was confirmed the molecular identity of the samples, exhibiting similarity of 100% for *T. gondii*, in relation to the sequence DQ779192.1 stored at GenBank. There were no positive samples in histopathology analysis or immunochemistry. Table I display the results of the statistical analysis.

No significant differences were found for gender, although the frequency of positive females was higher than positive males. There was significant difference ($p < 0.001$) when analyzed the reproductive age of pigs: seroprevalence of 21.2% and 3.1% were found for reproducer and finishing pig, respectively (Table I).

The kappa values obtained were: 0.218 to PCR versus IFAT; 0.285 to PCR versus bioassay and 0.394 to IFAT versus bioassay. All kappa values were classified as regular agreement.

Discussion

The prevalence of seropositive pigs in the present study (38.0%) was similar to those reported by previous studies carried out in Brazil (Cademartori *et al.* 2014). Backyard pigs are subjected to greater risks of infection and are more exposed to infectious forms of the parasite in soil, water and food, due to failure in sanitary management. Seroprevalence studies in intensive pigs production systems report a lower prevalence of antibodies anti-*T.gondii* (12.5 – 13.4%) explained by effective sanitary management on these properties, resulting in a safer production (Piassa *et al.* 2010, Fernandes *et al.* 2012).

In this study an association between age and seropositivity to *T.gondii* antibodies ($p < 0.05$), was similar to others studies. *In natura* meat comes from younger animals, while meat from discarded old pigs is used in the manufacture of sausage and this group was exposed to *T.gondii* for a longer period (Silva *et al.* 2010), especially in a management system with inadequate sanitary controls. Therefore, the breeding system (intensive versus 10 extensive) and the degree of technological advancement have been considered as risk factors for *T.gondii* infection for pigs (Feitosa *et al.* 2014).

The amplification of *T. gondii* DNA was observed in several tissue samples from seropositive pigs screened by IFAT. Of these animals, 14.1% (12/85) were positive by PCR. The presence of the parasite DNA in these tissues is a significant finding. Several studies have assessed the presence of *T. gondii* in pigs, and described a significant frequency of tissue cysts in commercial cuts and sausages from animals naturally or experimentally infected (Berger-Schoch *et al.* 2010, Fernandes *et al.* 2012). The result of the PCR does not mean that the parasite was viable in the sample. Although the bioassay in mouse performed in this study suggested the viability of the parasite, representing a probable risk to public health, especially if this meat is consumed undercooked (Oliveira *et al.* 2014).

There was no positive result in tissue samples analyzed by histopathology and immunohistochemistry. It is a difficult task to find cysts or tachyzoites using histopathology or IHC, because not always the tissue fragment chosen to be analyzed has parasite forms (Dubey *et al.* 2005). It is estimated that the number of *T.gondii* cysts per gram of tissue from food animal such as pigs, may be lower than one tissue cyst per 50 g (Dubey 1988).

Conclusion

The results obtained in this study provide an indication of the sanitary conditions and risk of *Toxoplasma gondii* infection for backyard pigs in the region analyzed. Preventive measures must be implemented by health authorities, to minimize negative effects of infection by this protozoan on backyard pigs, and to avoid toxoplasmosis human cases due to ingestion of pig meat.

Conflict of interest statement

The authors declare that no competing interests exist.

Acknowledgements. The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for their financial support for this project (APQ 1492-5.05/12).

References

Berger-Schoch A., Herrmann D., Schares G., Müller N., Bernet D., Doherr M., *et al.* 2010. Incidence and genotypes of *Toxoplasma gondii* in the muscle of sheep, cattle and pigs in Switzerland. *Veterinary Parasitology*, 1, 290–97. DOI: 10.1016/j.vetpar.2010.11.046

Bezerra R.A., Carvalho F.S., Guimarães L.A., Rocha D.S., Maciel B.M., Wenceslau A.A., *et al.* 2012. Genetic characterization of *Toxoplasma gondii* isolation from pigs intended for human consumption in Brazil. *Veterinary Parasitology*, 189, 153–61. DOI: org/10.1016/j.vetpar.2012.04.036

Cademartori B.G., Santos L.M.J.F., Oliveira A.F.C., Quevedo A.P., Oliveira A.P.A., Ramos T.S., *et al.* 2014. Isolation and pathogenicity of *Toxoplasma gondii* in naturally infected (rustic farm) pigs in southern Brazil. *Veterinary Parasitology*, 203, 207–21. DOI: 10.1016/j.vetpar.2014.02.009

Camargo M.E. 1974. Introduction to immunofluorescence technique. *Revista Brasileira de Patologia Clínica*, 10, 143–171(In Portuguese)

Demar M., Ajzenberg D., Maubon D., Djossou F., Panchoe D., Punwasi W., *et al.* 2007. Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical and parasitological aspects, *Clinical Infectious Diseases*, 45:88–95. DOI: 10.1086/521246. [PubMed] [Cross Ref]

Dubey J.P. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. 1988. *American Journal Veterinary Research*, 49, 910–3. PMID:3400928

Dubey J.P., Weigel R.M., Siegel A.M., Thulliezt P., Kitron U.D., Mitchell M.A., *et al.* 1995. Sources and reservoirs of *Toxoplasma gondii* infection on 47 swine farms in Illinois. *Journal Parasitology*, 81, 723–29. PubMed: 7472862

Dubey J.P. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues.1998. *Veterinary Parasitology*, 74, 75–77. DOI: 10.1016/S0304-4017(97)00135-0

Dubey J.P., Hill D.E., Jones J.L., Hightower A.W., Kirkland E., Roberts J.M., *et al.* 2005. Prevalence of viable *Toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. *Journal Parasitology*, 91, 1082–93. DOI: org/10.1645/GE-683.1

Dubey, JP. 2009. Toxoplasmosis in pigs – The last 20 years. *Veterinary Parasitology*, 164, 89–103. DOI: org/10.1016/j.vetpar.2009.05.018

Dubey JP. (Ed.) 2010. Toxoplasmosis of animals and humans. Second Edition. Florida, (CRC Press). pp.336

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

Feitosa T.F., Vilela V.L.R., Melo L.R.B., Neto J.L.A., Souto D.V.O., Morais D.F., *et al.* 2014. *Toxoplasma gondii* and *Neospora caninum* in slaughtered pigs from Northeast, Brazil. *Veterinary Parasitology*, 202:305– 09. DOI: 10.1016/j.vetpar.2014.03.015

Fernandes E.F.T.S., Fernandes M.F.T.S., Kim P.C.P., DE Albuquerque P.P.F., Neto O.L.S., Santos A.S., *et al.* 2012. Study of *Toxoplasma gondii* in slaughtered swine in the state of Pernambuco, Brazil. *Journal Parasitology*, 98:690–91. DOI: 10.1645/GE-3032.1

Galiza G.J.N., Tochetto C., Rosa F.B., Panziera W., Silva T.M., Caprioli R.A., *et al.* 2014. Usage of three immunohistochemical methods in the detection of aspergillosis and zygomycosis in animals. *Pesquisa Veterinária Brasileira*, 34, 637–42. DOI: org/10.1590/S0100-736X2014000700005

Homan W.L., Vercammen M., De Braekeleer J., Verschueren H. 2000. Identification of a 200 – to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. International *Journal Parasitology*, 30, 69–75. DOI: org/10.1016/S0020-7519(99)00170-8

Landis J.R., Koch G.G. 1977. The measurement of observer agreement for categorical data. *Biometrics*, 33, 159–74

Luna L.G. (Ed.). 1968. Manual of histologic staining methods of the armed forces institute of pathology. New York, (McGraw-Hill Book Company). (In Portuguese)

Oliveira P.A., Oliveira F.C., Faria L.M.J., Cademartori B.G., Pereira C.M., Coelho A.C.B., *et al.* 2014. Patogenicidade e virulência de *Toxoplasma gondii* isolado de suínos de criação artesanal no sul do Brasil. Pesquisa *Veterinária Brasileira*, 34, 1186–90. DOI: org/10.1590/S0100-736X2014001200007

Piassa F.R., Araújo J.B., Rosa R.C., Mattei R.J., Silva R.C., Langoni H., *et al.* 2010. Prevalence and risk factors for *Toxoplasma gondii* infection in certified and non-certified pig breeding farms in the Toledo microregion, PR, Brazil. *Revista Brasileira Parasitologia Veterinária*, 19, 152–56. DOI: org/10.1590/S1984-29612010000300005

Silva A.V., Da Silva R.C., Zamprogna T.O. 2010. *Toxoplasma gondii* in pigs with emphasis on the Brazilian contribution. *Scince Medical*, 20: 120–130. (In Portuguese)

Swierzy I.J., Muhammad M., Kroll J., Abelmann A., Tenter A.M., Luder C.G. 2014. *Toxoplasma gondii* within skeletal muscle cells: a critical interplay for food-borne parasite transmission. International *Journal Parasitology*, 44, 91–8. DOI: 10.1016/j.ijpara.2013.10.001. [PubMed] [Cross Ref]

Torgerson P.R., Silva N.R., Fevre E.M., Kasuga F., Rokni M.B., Zhou X.N., *et al.* 2014. The global burden of foodborne parasitic diseases: an update. *Trends Parasitology*, 30, 20–6. DOI: 10.1016/j.pt.2013.11.002.[PubMed] [Cross Ref]

Thrusfield M.V. (Ed.). 2004. Veterinary Epidemiology. Second Edition. São Paulo, (Roca), pp. 556. (In Portuguese)

Received: November 29**,** 2016
Revised: January 20, 2017
Accepted for publication: February 20, 2017