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Nussenblatt, Robert; Khan, Assis; Sibley, L. David; Belfort, Rubens
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High prevalence of unusual genotypes of *Toxoplasma gondii* infection in pork meat samples from Erechim, Southern Brazil

RUBENS BELFORT-NETO¹, VERONIQUE NUSSENBLATT², LUIZ RIZZO³,
CRISTINA MUCCIOLI¹, CLAUDIO SILVEIRA⁴, ROBERT NUSSENBLATT⁵, ASSIS KHAN⁶,
L. DAVID SIBLEY⁶ and RUBENS BELFORT-Jr.¹

¹Departamento de Oftalmologia, Universidade Federal de São Paulo, Rua Botucatu, 820
04023-062 São Paulo, SP, Brasil

²University of Maryland, School of Medicine, 655 West Baltimore Street, Baltimore Maryland, USA, 21201-1559

³Departamento de Imunologia, ICB, Universidade de São Paulo, Av. Professor Lineu Prestes, 1730
05508-900 São Paulo, SP, Brasil

⁴Clínica Silveira, Av. Sete de Setembro, 1510, 99700-000 Erechim, RS, Brasil

⁵National Eye Institute – NIH Bldg 10 Room 10N 202 – 10 center drive NSC 1858, Bethesda, Maryland, USA, 20892-1858

⁶Washington University School of Medicine, Mc.Donnell Pediatric Research Bld., Department of Molecular Biology
Box 8230, 660 South Euclid Ave, St. Louis, MO, USA, 63110-1093

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ABSTRACT

Toxoplasmosis is the most common cause of infectious uveitis in Brazil, with a higher frequency in the South of the country. We have collected samples from porcine tongue and diaphragm obtained in both large and small abattoirs and used molecular biological technique to determine the prevalence of infection and RFLP analysis to type the parasites. Seventeen out of 50 (34%) samples from the diaphragm and 33 out of 50 (66%) samples from the tongue demonstrated a positive PCR reaction for *T. gondii* and restriction analysis of four of the positive samples revealed that all had a type I genotype at SAG2. However, when other unlinked loci were analyzed, these strains had a type III genotype at markers BTUB, SAG3, and GRA6. One of the strains (8T) had a type II allele at SAG3, indicating it has a combination of alleles normally seen in the clonal lineages. Our sampling indicates a high prevalence of infection and suggests that unusual genotypes of *T. gondii* are found in Brazil even among domesticated pigs.

Key words: ocular toxoplasmosis, *Toxoplasma gondii*, genotypes.

INTRODUCTION

Toxoplasmosis is one of the most common causes of infectious uveitis – inflammation of the inner part of the eye – in humans and studies have shown a high frequency of toxoplasmic ocular lesions in the city of Erechim, Southern Brazil (Glasner et al. 1992, Silveira et al. 2001). The eye can be affected in both congenital and acquired infections (Holland 1996, Nussenblatt and Belfort 1994) and in this region of Brazil, the in-

fection is usually transmitted by eating uncooked meat containing toxoplasma organisms. Pork is considered one of the most important sources of infection and it has been shown that porcine diaphragm and tongue frequently contain parasite cysts (Dubey et al. 1986).

Recently developed special techniques in molecular biology have allowed for the identification of *T. gondii* DNA in different organs in humans and in various animals thought to harbor the parasite. Restriction fragment length polymorphism (RFLP) analysis of the organism has indicated that three major subgroups are predominant in North America and Europe, and it has been shown

*Member Academia Brasileira de Ciências
Correspondence to: Rubens Belfort Jr.
E-mail: epm@belfort.med.br

that human infection is mainly caused by types I and II (Ajzenberg et al. 2004, Howe and Sibley 1995). Our goal was to determine the prevalence of *T. gondii* contamination in commercially available pork meat obtained from Erechim, RS, Brazil and the genotype of those parasites.

MATERIALS AND METHODS

Porcine tongue and diaphragm obtained in both large and small abattoirs in the Erechim area were studied.

The samples were kept in a saline solution and were later ground and frozen on site. Extraction of DNA was performed afterwards in São Paulo. Samples were prepared separately, using disposable material, with time intervals and cleaning after each sample to avoid cross-contamination. PCR was performed using two specific primers for *T. gondii* –

CGCTGCAGGGAGGAAGACGAAAGTTGAG
and

AGCGCTGCAGACACAGTGCATCTGGATT
– respectively from the 5' and 3' ends of a 533 bp fragment from the *T. gondii* genome. After optimizing the PCR reaction using tissue culture parasites, the PCR reaction using these samples was performed in a 50 ml reaction mixture containing 0.5 mM of each primer, 100 mM dNTP (Pharmacia Biotech), 60 mM Tris + HCl (pH 9.0), 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.5 U Taq PLATINUM (Applied Biosystems). Amplification was performed using a PerkinElmer/Applied Biosystems 9600 thermo cycler by 10 min incubation at 94°C, followed by 38 cycles of 1.5 min at 94°C, 1 min at 56°C, 1 min at 72°C and a final 10 min incubation at 72°C. The PCR products were analyzed using Southern blot methodology.

Six samples (four positive for *T. gondii* and two negative) were sent to Washington University for genotype analysis. DNAzol (Molecular Research Center, Inc.) treated tissue samples were extracted with equal volume of chloroform. Polyacryl carrier (Molecular Research Center, Inc.) was added (5 µL) to the aqueous phase and DNA was precipitated by addition of equal volume of ethanol and centrifugation at 5,000xg for 10 min. DNAs were subjected to nested PCR analysis for the unlinked markers SAG2, BTUB, SAG3, and GRA6 (Khan et al. 2005). Following amplification, samples were digested with appropriate restriction enzymes,

resolved on agarose gel and alleles scored by comparison to the type strains RH (type I), ME49 (type II) and CTG (type III).

RESULTS

Seventeen out of 50 (34%) samples from the diaphragm and 33 out of 50 (66%) samples from the tongue demonstrated a positive PCR reaction for *T. gondii*, while 28% had positive PCR reactions in both the tongue and diaphragm (data not shown).

Restriction analysis and DNA sequencing were performed on six random samples. Re-amplification was negative on the two previously negative samples (negative controls). All four positive samples had a type I genotype at SAG2 (Table I). However, when other unlinked loci were analyzed, these strains had a type III genotype at markers BTUB, SAG3, and GRA6 (Table I). One of the strains (8T) had a type II allele at SAG3, indicating that it has a mixed profile (Table I). Although a high incidence of PCR positive samples were found, we were unable to infect interferon-gamma deficient mice with either a macerated suspension, the meat or with serum from the animals (data not shown). This suggests that the parasite load in these samples is small.

DISCUSSION

These results suggest that a significant proportion of meats commercially available in Erechim, Southern Brazil, are contaminated with *T. gondii*. This poses a real public health risk to those who live in that environment. Recently a study in the UK (Aspinall et al. 2002) assessed the prevalence of *T. gondii* by polymerase chain reaction and parasite typing by genotype in commercial meat products and demonstrated that 27 of the 71 samples harbored parasites. Restriction analysis and DNA sequencing showed that 21 of the contaminated meats contained parasites genotyped as type I at the SAG2 locus, whilst six of the samples contained parasites of both types I and II (Aspinall et al. 2002). A recent study conducted in São Paulo and Erechim has shown that in both sites only type I was identified (Vallochi et al. 2005).

SAG2 has previously been shown to be highly sensitive means of genotyping strains: based on a combination of polymorphism in the 5' and 3' ends of the gene, it is possible to classify strains of the three con-

TABLE I
Genotypes of *T. gondii* samples detected in porcine samples from Erichim, Brazil.

Strain or sample	5'-SAG2 ^a	3'-SAG2 ^b	BTUB		SAG3	GRA6	Overall genotype
	<i>Sau</i> 3AI	<i>Hha</i> I	<i>Taq</i> I	<i>Bsi</i> EI	<i>Nci</i> I	<i>Mse</i> I	
RH	1 ^c	1	1	1	1	1	I
ME49	1	2	2	2	2	2	II
CTG	2	1	1	2	3	3	III
6T	1	1	1	2	3	3	I/III
7T	1	1	1	2	3	3	I/III
8T	1	1	1	2	3	2	I/II/III
9T	1	1	1	2	3	3	I/III

^a only detects a unique allele in type III.

^b only detects a unique allele in type II.

^c Alleles as defined in Khan et al. 2005.

ventional clonal lineages into one of the three genotypes (Howe et al. 1997). However, as shown in the present study, typing at SAG2 alone does not detect strains with recombinant genotypes. In the present case, all of the strains would have been misidentified as type I, when in fact they contain alleles of different genotypes at different genetic loci. This pattern of apparently recombinant genotypes is highly unusual as has not been seen frequently in isolates studied previously. Typically, 95% of strains isolated in North America and Europe fall into one of three highly clonal genotypes (Howe and Sibley 1995). However, it has recently been proposed that a greater degree of genetic diversity is found among strains in some geographic areas and possibly among non-domesticated animals (Ajzenberg et al. 2004). Previous studies of strains isolated in Brazil has shown that they contain unusual, recombinant genotypes when analyzed using markers based on polymorphisms identified in the North American and European clonal lineages (Ferreira et al. 2006, Fux et al. 2000). Our data indicate that unusual genotypes may be found in Brazil even among domesticated pigs. Consequently, the unusual clinical presentation of ocular toxoplasmosis in Brazil may be related to the presence of these unusual genotypes. Another recent published paper studied retinal scars from human eyes after enucleation and showed that parasites containing the type I genotype were found (Vallochi et al. 2005). This study used SAG2 region, amplified by polymerase chain reaction and analyzed by restriction fragment length polymorphism, so they could be analyzing

recombinant strains and further testing will be necessary to establish this.

The cause for such a high incidence and severity of toxoplasmic infection in the south of Brazil is still uncertain. One possibility is that it is due infection by different strains of *T. gondii* than in other parts of the world; another possibility is that a high contamination of food (mainly pork) can lead to chronic re-infection. Our results have supported such theories since there has been 66% of contamination on the pork samples.

RESUMO

Toxoplasmose é a causa mais comum de uveíte infecciosa no Brasil, com maior frequência no sul do país. Coletamos amostras de diafragma e língua de porcos em pequenos e grandes abatedouros e utilizamos biologia molecular para determinar a taxa de infecção e "DNA genotyping" para tipar os parasitas. Dezesete das 50 amostras de diafragma (34%) e 33 das 50 amostras de língua (66%) foram positivas na reação de PCR para *T. gondii*. A análise restritiva e o sequenciamento do DNA em quatro amostras revelaram que todas apresentam genótipo tipo I no SAG2. No entanto, quando outros loci não ligados foram analisados, estas mesmas amostras se mostraram como tipo III nos marcadores BTUB, SAG3 e GRA6. Uma das amostras (8T) mostrava-se como tipo II no SAG3, indicando um perfil misto. Estas amostras demonstraram não só uma alta taxa de infecção, mas também genótipos incomuns que não foram observados com frequência em estudos prévios. Nosso trabalho sugere que genótipos incomuns de *T. gondii* podem ser encontrados no Brasil, até mesmo em porcos domesticados.

Palavras-chave: toxoplasmose ocular, *Toxoplasma gondii*, genótipos.

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