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# *Genotyping of* Toxoplasma gondii *strains detected in pork sausage*

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ABSTRACT

*DNA was extracted from 70 pork sausage samples obtained from 55 commercial establishments in the city of Botucatu, Sao Paulo, Brazil. Nested-polymerase chain reaction with primers specific for* ***Toxoplasma gondii*** *SAG2 locus was used and detected the parasite in 19 (27.14%) sausage samples. Digestion of amplicons with restriction enzymes HhaI and Sau3AI showed that 14 (73.68%) samples contained parasites genotyped as Type I, and 5 (26.32%) as Type III.*

***Key words: Toxoplasma gondii****; SAG2 genotyping; food-borne pathogen; sausage; swine.*

## INTRODUCTION

Toxoplasmosis is a worldwide-distributed zoonosis caused by the protozoan *Toxoplasma gondii*. This parasite presents a highly clonal populational structure[1] made up of three lineages, Types I, II and III. Several reports genetically classified strains of *T. gondii* isolated from humans[2-5]. However, there are few reports on the genotyping of *T. gondii* obtained from domestic animals.

Different strains of the parasite may be better adapted to several intermediate hosts[6]. Epidemiological surveillance is important to analyze greater collection of *T. gondii* strains from multiple sources of infection, in order to evaluate possible associations between parasite Types and severity of the disease in humans and animals[7, 8]. *T. gondii* infection generally is not a problem for healthy individuals, but congenitally-infected people, immunosupressed and AIDS patients may develop important lesions[9].

Meat-producing animals can be infected with *T. gondii*[10], but pork was considered as the most important source of *T. gondii* for humans in some countries[11]. In Brazil, as in other countries, technical improvement in swine meat production led to a reduction of *T. gondii* prevalence in young pigs[12]. However, it has been pointed out that older animals show higher prevalence of the parasite [13], and these animals are frequently used for the production of sausages, salami and cured

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meats, with potential risk of human infection after the intake of these foodstuffs[9].

The objective of the present trial was to evaluate the genotypes of T. gondii strains in fresh pork sausage samples obtained in Botucatu, Sao Paulo, Brazil.

## MATERIALS AND METHODS

**Fresh pork sausage samples:** Seventy fresh pork sausage samples (weighting at least 50 g each) were collected from 55 commercial establishments in the city of Botucatu-SP. Samples were identified and transported under refrigeration to the NUPEZO (*Núcleo de Pesquisas em Zoonoses* / Center of Research in Zoonoses) laboratory, where they were analyzed. Previous trial[14] showed that none of these parasites were isolated in mice; probably, salt content of these sausages kill *T. gondii* present in the samples, making it impossible to isolate the parasite and conduct pathogenicity studies.

**DNA extraction from sausage samples:** In order to concentrate de parasite in each sample, sausages (20 g each) were cut in to small pieces, ground in a blender with 100 ml of normal saline solution (0.85% NaCl). Tissue homogenate was mixed with 100 ml of acidic pepsin and incubated for 1 h at 37ºC, centrifuged and neutralized[15]. The pellet was resuspended to a final volume of 5 ml with TNE buffer (200 mM NaCl, 20 mM Tris, 50 mM EDTA), and DNA was extracted by digestion with proteinase K and SDS, followed by purification with phenol-chloroform and precipitation with ethanol[16]. Briefly, 250 ml samples of digested sausages were homogenized using 250 ml extraction buffer (TNE buffer plus proteinase K 1 mg/ml and 2% SDS) and incubated at 56ºC for 1h. Buffered phenol (500 ml) was added and the samples were centrifuged at 13,000 g for 3 min. Resulting aqueous layer was transferred to another microtube, added to phenol: chloroform:isoamyl alcohol, homogenized, and centrifuged at 13,000 g for other 3 min. Resulting aqueous layer was transferred to another microtube, mixed with 36 ml of 2 M sodium acetate and 472 ml of cold ethanol, and stored at -20ºC for 16-24 h. Samples were then centrifuged at 13,000 g for 10 min, added of 470 ml of cold ethanol, and centrifuged again at 13,000 g for 10 min. DNA samples were resuspended in 50 ml of ultra-pure water, incubated at 56ºC for 30 min, and stored at -20ºC until PCR was to be performed.

**SAG2 typing of sausage samples:** DNA samples were submitted to nested PCRs for the 3' and 5'ends of SAG2 gene[4], followed by digestion with restriction enzymes Sau3AI e Hha1, respectively. Initially PCR and nested PCR were performed in a thermal cycler (Biorad) in 50 ml samples placed in microtubes containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 1.25 mM dNTPs, 1.5 units of Taq-polymerase, 10 ml of extracted DNA (PCR) or 1 ml of amplicom (nested PCR), water to 50 ml, and 10 pM of the primers SAG2.F4 and SAG2.R4 (PCR for the 3' end), SAG2.F and SAG2.R2 (nested PCR for the 3'end), SAG2.F3 and SAG2.R3 (PCR for the 5'end), and SAG2.F2 and SAG2.R (nested PCR for the 5'end). Two rounds of 35 amplification cycles were performed for 30 s at 94ºC, 60 s at 63ºC (3'end) or 65ºC (5'end), and 60 s at 72ºC. Seven ml of each amplicom was digested with 1 ml of restriction enzymes Sau3AI e Hha1, for nested PCR to 3' and 5' ends, respectively, for 2 hours at 37ºC. Fragments were detected in 1.5% agarose gel electrophoresis stained with ethidium bromide. RH, ME49, and M7741 strains of *T. gondii* were used as controls for Types I, II and III, respectively. Water negative control templates were added to each batch of PCR and nested PCR assays. The risk of contamination was minimized by the use of different locations in each phase of molecular analysis, and by the use of small amounts of reagents and disposable labware.

## RESULTS AND DISCUSSION

*T. gondii* DNA was found in 19 (27.14%) of 70 sausage samples examined. Nested-PCR for 3' and 5' ends of SAG2 gene demonstrated that after digestion of the amplicons with enzymes Sau3AI and HhaI, 14 (73.68%) and 5 (26.32%) samples presented Type I and Type III parasites, respectively.

Only few other studies reported genotypes of *T. gondii* from pig or pig products. In one of them, in 43 strains isolated from adult sows from Iowa, USA, was found 36 Type II strains and 7 Type III strains[7], and in other one it was found in 25 isolates from market age pigs, that 20 were type III and five were type II [17]. Other authors, found 20 (34.48%) samples positive

for *T. gondii* DNA among 58 meat products containing pork meat in the United Kingdom, with 17 (85.00%) SAG2 Type I samples and 3 (15.00%) mixed SAG2 Type I + II samples [9], probably due the mixing of meat from different animals in the same product.

These results demonstrated the difference on the genetic distribution of *T. gondii* in different geographic regions. In the present trial, no Type II strain was found, and Type I was the most prevalent one. Dubey et al[18] found only Type I and Type III *T. gondii* in brain and heart samples of poultry from Brazil, and three of these samples were from Botucatu, the same site of our study. In other two studies with samples from Brazil, it was found only Type I and Type III strains in poultry, and in one sample, mixed Type I+III infection was observed, confirming the differences on genetic distribution of the parasite in North America, the United Kingdom and Brazil[19,20]. Of 37 isolates of viable *T. gondii* found in Paraná, Brazil, 22 were type III and 15 were type I[21].

Another interesting finding was that some Type III isolates from poultry in Brazil are virulent to mice in primary isolation[19], a result that is in disagreement with other trials, where SAG2 Type III *T. gondii* was found to be non-virulent to mice[22]. In our trial with strains from pork sausage, it was not possible isolate the parasite in order to conduct virulence studies[14]. However, in the study of *T. gondii* strains isolated from dogs in the same city[23], five Type III strains were found, and all of them killed all four mice inoculated, in a period ranging from 5 to 20 days. Further studies should be conducted in order to evaluate the extension of genetic differences between samples classified as Type III in the United States and in Brazil, as well as a possible relationship between their clinical behavior.

In spite of the decrease in the prevalence of the infection in pigs due to large scale technification in swine production, many regions in Brazil still have small, familiar breeding units, where sanitary conditions and direct contact with other animals ands men enable the maintenance of *T.gondii*. Thus, typing and virulence studies using *T. gondii* samples may contribute for the production of a clearer picture in relation to the distribution of different parasite clones between human and animal populations[8]. The present study describes the first genotyping of *T. gondii* DNA detected in pig samples in Brazil.

RESUMEN

Se extrajo el ADN de muestras de chorizo de cerdo obtenidas de 55 establecimientos comerciales en la ciudad de Botucatu, Sao Paulo, Brasil. Se utilizó la reacción de polimerasa en cadena (PCR) en un ensayo con oligonucleotideos específicos para el locus SAG2 de *Toxoplasma gondii*, en el cual se detectó el parásito en 19 (27,14%) de las muestras de chorizo. La digestión de los amplificados con las enzimas de restricción HhaI y Sau3AI demostró que 14 muestras (73,68%) tenían cepas de *T. gondii* Tipo I y 5 (26,32%) del Tipo III.

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