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Isolation and genotyping of *Toxoplasma gondii* in sheep meat from Northern Parana state, Brazil

Isolamento e genotipagem de Toxoplasma gondii em carne ovina no norte do estado do Paraná, Brasil

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ABSTRACT

Introduction: Toxoplasmosis is caused by *Toxoplasma gondii*, an intracellular protozoan that can infect humans and all warm-blooded animals. **Objective:** The present study aimed to isolate and genetically characterize *T. gondii* in sheep meat. **Methods:** The study was conducted in a sheep slaughterhouse, and samples of masseter muscles, tongue, heart, diaphragm, and blood were collected from 50 sheep. The sheep sera were subjected to an indirect immunofluorescence assay (IFA) to detect anti-Toxoplasma gondii IgG antibodies. The cutoff point used was 1:64. Tissue samples from seropositive animals were subjected to mouse bioassay. Inoculating mice determined the virulence of isolated strains. Genotyping was performed using multilocus PCR-RFLP with 11 genetic markers. Kaplan-Meier was used to build a survival curve. **Results:** IFAT showed 12% (6/50) of seropositivity, and the antibody titers were 64 (4/50, 8%) and 256 (2/ 50, 4%). The isolates were named TgShBrPr01 and TgShBrPr02. Genetic characterization of the isolate TgShBrPr01 showed the genotype ToxoDB #183. The isolated TgShBrPr02 did not amplify all markers; however, the available typing data indicate a new genotype. The survival curve of TgShBrPr01 showed a high virulence strain and killed all the Swiss Webster mice infected with 10^2 and 10^3 *T. gondii* tachyzoites. **Conclusion:** This study isolated two new atypical (nonclonal) *T. gondii* strains from sheep, underscoring the potential risk of this transmission source to humans, especially when meat is consumed raw or undercooked.

Descriptors: Toxoplasmosis; Apicomplexa; Bioassays.

RESUMO

Introdução: A toxoplasmose é ocasionada pelo *Toxoplasma gondii*, um protozoário intracelular que pode infectar humanos e todos os animais de sangue quente. **Objetivo:** O presente estudo teve como objetivo isolar e caracterizar geneticamente o *T. gondii* em carne ovina. **Metódos:** A pesquisa foi conduzida em um frigorífico de ovinos e amostras de masseteres, língua, coração, diafragma e sangue de 50 ovinos foram obtidas. Os soros de ovinos foram submetidos a reação de imunofluorescência indireta (RIFI) para detecção de anticorpos IgG anti-T. *gondii*. O ponto de corte utilizados foi de 1:64 . Amostras de tecido de animais soropositivos foram submetidas a bioensaio em camundongos suíços. A genotipagem foi realizada por meio do PCR-RFLP multilocus com 11 marcadores genéticos e Kaplan-Meier para construir uma curva de sobrevivência. **Resultados:** A RIFI apresentou 12% (6/50) de soropositividade, e os títulos de anticorpos foram 64 (4/50, 8%) e 256 (2/50, 4%). Foram obtidos dois isolados denominados TgShBrPr01 e TgShBrPr02. A caracterização genética do isolado TgShBrPr01 mostrou o genótipo ToxoDB #183. O TgShBrPr02 isolado não amplificou todos os marcadores; no entanto, os dados de genotipagem disponíveis indicam um novo genótipo. A curva de sobrevivência do TgShBrPr01 apresentou uma cepa de alta virulência com letalidade dos camundongos suíços infectatos com 10^2 e 10^3 taquizoítos de *T. gondii*. **Conclusão:** Este estudo isolou cepas atípicas de ovelhas, o que destaca o potencial desta fonte de transmissão para humanos, seja a carne consumida crua ou mal cozida.

Descritores: Toxoplasmose; Apicomplexa; Bioensaio.

INTRODUCTION

Toxoplasmosis is caused by *Toxoplasma gondii,* an intracellular protozoan belonging to the phylum Apicomplexa[1]. This parasite can infect humans and all warm-blooded animals[1-3]. Humans may become infected by ingesting food and water contaminated with sporulated oocysts and eating tissue cysts from undercooking meat[3-4]. Additionally, sheep meat is considered one of the most common transmission sources for human beings[5].

The frequency of *T. gondii* is high in livestock[6], and toxoplasmosis causes about 20% of foodborne diseases in Europe[7] and has been ranked among the top three foodborne infections in the United States[8].

The high frequency of toxoplasmosis in sheep could be related to the lower resistance of this species to *T. gondii* and the sheep farming conditions, which may expose these animals to a high probability of ingesting sporulated oocysts[6]. Moreover, it has been recognized as a cause of abortions and stillbirths in sheep worldwide[9]. Accordingly, this study aimed to isolate and genetically characterize *T. gondii* from sheep meat.

METHODS

Local of study and sampling:

The current study was approved by the Animal Ethics Committee of Universidade Estadual de Londrina (CEUA / UEL) permit No 206. The survey was conducted at a sheep slaughterhouse in northern Parana state, Brazil (23 ° 18'36 "S, 51 ° 09'46" W). Samples of the masseters, tongue, heart, diaphragm, and blood from 50 sheep were obtained. The tissue samples were collected and kept in plastic bags properly labeled, and the blood samples were collected in tubes without EDTA. Afterward, the samples were stored in a cooler box and sent to the laboratory for processing. The serum samples were obtained through centrifugation at 3,200 rpm for five minutes and stored at −20 °C.

Serological test:

The presence of IgG antibodies against *T. gondii* was performed by indirect immunofluorescence assay (IFA) using anti-Sheep IgG–FITC antibody (Sigma- Aldrich, St. Louis, Missouri, USA)[10]. Tachyzoites of the RH strain were used to perform tests. Serum samples from previously known positive and negative animals were included as controls. A cut-off titer of ≥64 was used.

Mouse bioassay:

Tissue samples from seropositive animals were subjected to mouse bioassay[11]. Briefly, 50 g of tissues (10 g each of the heart, diaphragm, liver, tongue, and masseter) were collected. Each sample was homogenized in a blender for 30 seconds in 250 mL of saline solution (0.14 M NaCl). After homogenization, 250 mL of pepsin solution was added and incubated at 37 °C for one hour. The homogenate was filtered through two layers of gauze and centrifuged at 2000 x g for 10 min. The supernatant was discarded, and the sediment was resuspended in 20 mL PBS (pH 7.2); 15 mL of 1.2% sodium bicarbonate (pH 8.3) was added to the solution, and the solution was centrifuged at 2000 x g for 10 min. The supernatant was discarded, and the sediment was resuspended in 5 mL of antibiotic saline solution (1,000 U penicillin and 100 µL of streptomycin/mL of saline solution) and inoculated into the pool of tissue subcutaneously into three Swiss Webster mice (1 mL/mouse). Swiss Webster mice were observed daily, and those who developed any symptoms (bristly hair, tearing, weight loss, diarrhea, and abdominal distension) were euthanized for collection of lung, liver, and peritoneal fluid and tachyzoites. These materials were used for 18S rRNA PCR[12]. Mice that survived after six weeks post-inoculation (p.i.) were euthanized. IFAT was used as the serological test for anti-*T. gondii*, and brain and lung tissues were analyzed for the presence of cysts or tachyzoites, respectively. Mice were considered positive when titers were ≥ 16. A brain squash specimen was prepared between the slide and the coverslip for microscopic examination.

The virulence of isolated strains was determined by Swiss Webster mice inoculation. Live tachyzoites tat dilutions of 10^1, 10^2, and 10^3 were prepared and inoculated into three mice each for survival curve evaluation. Animals were observed three times per day for 30 days, and those who developed any symptoms were euthanized. Detection of *T. gondii* was made as described above.

Toxoplasma gondii genotyping:

Genotyping was performed using multilocus PCR-RFLP with 11 genetic markers (SAG1, 5'-3 ' SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, e Apico)[13]. DNA from strains GT1, PTG, CTG, TgCgCa1, MAS, TgCatBr5, TgCatBr64, and TgRsCr1 was used as positive controls. The target DNA sequences were first amplified by multiplex-PCR using external primers for all markers, followed by a nested PCR for individual markers. After this, the amplified fragments were digested with appropriate restriction endonucleases. All digested PCR products were analyzed by agarose gel electrophoresis, stained with Sybr Safe (Invitrogen®, USA), visualized under UV light, and photographed using a gel documentation system (Safe Imager, Invitrogen®, USA). The results were compared and classified according to genotypes deposited in ToxoDB (http://toxodb.org/toxo). The evolutionary history was inferred by a reticulated network using the neighbor-joining method by SplitsTree 4.0[14].

Statistical analysis:

Kaplan-Meier was used to build a survival curve in GraphPad Prism version 6.

RESULTS

IFAT showed 12% (6/50) of seropositivity, and the antibody titers were 64 (4/50, 8%), and 256 (2/ 50, 4%) (Table 1). Two positive samples of the tissue pool (33%, 2/6) had *T. gondii* in the mouse bioassay. One sample of tachyzoites was obtained; the other one was positive by mouse IFAT and tissue PCR-RFLP. The samples were from two sheep under six months of age and had titers of 256 (Table 1). The isolates were named TgShBrPr01 and TgShBrPr02. Genetic characterization of the isolate TgShBrPr01 showed the genotype ToxoDB #183. The isolated TgShBrPr02 did not amplify all markers; however, the available typing data indicated a new genotype (Table 2). The phylogenetic tree is shown in Figure 1.

The survival curve of TgShBrPr01 showed a high virulence strain and killed all the Swiss Webster mice infected with 10^2 and 10^3 *T. gondii* tachyzoites until 11 (10^2) and 12 (10^3) days post infection (Figure 2). The Swiss Webster mice showed lethargy, ascites and prominent piloerection.

Table 1. *Toxoplasma gondii* IFAT serology and mouse bioassay from sheep.

Animal	IFAT titers	Age (months)	Bioassay
01	256	<6	+
02	256	<6	+
03	64	<6	-
04	64	<6	-
05	64	>12	-
06	64	>12	-

Table 2. PCR-RFLP genotyping of *Toxoplasma gondii* from sheep meat.

Isolate ID	Genetic Markers											PCR-RFLP genotype
	SAG 1	5' + 3' SAG2	alt. SAG 2	SAG 3	BTUB	GRA 6	c22-8	c29-2	L 358	PK 1	Apico	
TgSh-BrPr01	I	III	III	III	III	III	III	I	I	I	III	#183
TgSh-BrPr02	II/III	I	II	II	II	NA	II	NA	II	II	II	NA

*NA - no amplification.

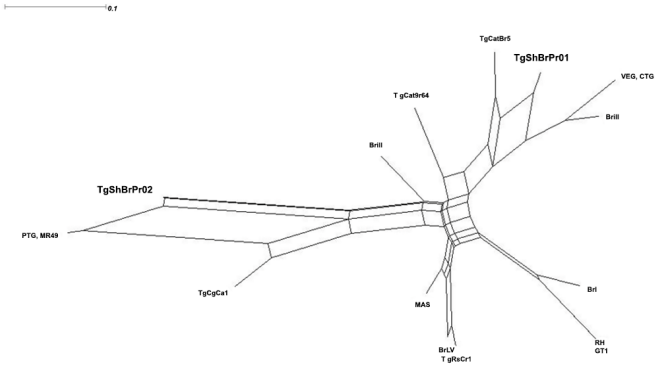

Figure 1. Phylogenetic network of Toxoplasma gondii isolates from sheep meat.

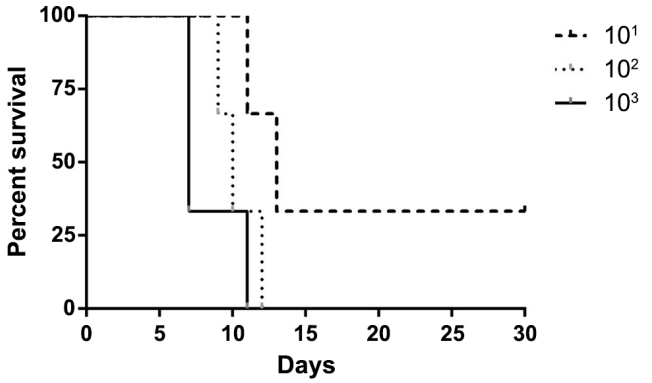

Figure 2. Survival curve of mice inoculated with tachyzoites of strain TgShBrPr01 of *Toxoplasma gondii.*

DISCUSSION

In this study, we isolated *T. gondii* from 33% of seropositive sheep, and the overall serum occurrence was 12%. In addition to the clinical and economic concerns derived from abortion outbreaks, the seroprevalence in sheep highlights the potential public health risk of the consumption of sheep meat containing tissue cysts[15-16]. The ingestion of sheep has been identified as a risk factor for seroprevalence among pregnant women[17], and it was also confirmed to be the cause of an outbreak of acute toxoplasmosis in Brazil[18]. There is a correlation between tissue cysts and anti-*T. gondii* antibodies in sheep[19]. The seroprevalence in sheep from Brazil ranged from 17.3 to 51.8% [20-25].

Genotypic characterization of the isolate obtained in the present study revealed the presence of non-clonal genotype #183. The phylogenetic analysis showed that the genotypes TgShBrPr01 and TgShBrPr02 had proximity to the reference strain Type III and II, respectively. Atypical genotypes are common in Brazil, where the genetic diversity of *T. gondii* is wide. The genetic structure of the *T. gondii* population has a large diversity of genotypes from animals, and the clonal expansion of atypical genotypes characterizes it[26]. New genotypes may be formed when a feline (definitive host) hunting superinfected intermediate hosts is infected simultaneously with different strains, or successively in a very short[27-28].

The lethality of the isolate TgShBrPr01 in our study was considered high and killed all mice inoculated with 10^2 and 10^3. Several factors interfere with the virulence of *T. gondii*, including infection route, parasite stage, infective dose, mouse lineage, inoculation mode, the host's immunity, and characteristics of the isolate[29-30]. In Brazil, other isolates were obtained from sheep. Some isolates were mouse-virulent[31-32]);

However, other studies found virulence isolates[23]. The virulence assays of *T. gondii* isolates worldwide demonstrated that the South American isolates were more virulent (lethal) to mice than North American, European, or Asian isolates[33].

CONCLUSION

In conclusion, this study isolated atypical strains from sheep, highlighting the potential of this transmission source for humans, whether meat is consumed raw or undercooked. Through genotypic characterization, two new atypical strains of T. gondii were discovered, one of which is phenotypically pathogenic to mice.

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