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Toxoplasma gondii infection in dairy ewes: Vertical transmission and influence on milk production and reproductive performance

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ABSTRACT

The present study aimed to evaluate the transmission of toxoplasmosis (vertical and venereal) and its influence on milk production and reproductive problems of Lacaune sheep seropositives for *Toxoplasma gondii*. Males and females were serologically selected using indirect immunofluorescence method in three steps of the study. Step 1: In order to evaluate the influence of toxoplasmosis on milk production, the volume of milk produced by 40 sheep (22 seronegatives and 18 seropositives for *T. gondii*) was weekly measured throughout the lactation period. There were no significant differences between these two groups; in other words, toxoplasmosis did not affect milk production. Step 2: In order to assess *T. gondii* venereal transmission, five samples of semen from seropositive rams (n = 5) were tested by endpoint and real time PCR with two days of interval; however, these semen samples were PCR negatives for *T. gondii*. Step 3: To evaluate reproductive problems, 12 seropositive animals out of a flock of 68 pregnant ewes showed signs of reproductive problems, such as abortion or fetal resorption. *T. gondii* transplacental transmission was evaluated on blood drawn from newborn lambs (n = 41), and their respective seropositive mothers (n = 30) after single, double or triple births. Serological tests showed that 65.8% of the lambs had antibodies against this protozoan, indicating a high transmission from ewe to fetus during pregnancy. Therefore, it is concluded that toxoplasmosis in sheep may impair reproduction with a high percentage of vertical transmission.

1. Introduction

Toxoplasmosis is a parasitic disease of humans, wild, and domestic animals caused by *Toxoplasma gondii*, a cosmopolitan protozoan of the Sarcocystidae family with animals of the Felidae family as definitive hosts [1]. Toxoplasmosis is usually subclinical [2], but the most common signs in symptomatic patients are hyperthermia, dyspnea, and neurological problems. The infection can trigger severe diseases, particularly when transmitted congenitally between humans or immunocompromised individuals, causing losses on production and reproduction performance [3]. *T. gondii* is described as a major causative agent of abortion and in this case, the placenta is invaded by tachyzoites, resulting in mineralization and necrosis [4]; thus, the interference on fetal development may result in abortion, mummified fetus, or debilitated and weak newborns [5–10].

The prevalence of toxoplasmosis varies from region to region, suffering interference of climate and geographical factors, as well as human and animal habits [5]. Its transmission to humans and animals mainly occurs through the consumption of tissue cysts from undercooked meat, oocysts intake from pastures and food, which are all influenced by poor hygiene [11,12]. Some authors estimate that one third of the human population has antibodies against *T. gondii* [13]. In sheep, researchers have highlighted that the main form of transmission is by ingestion of oocysts [14]. Lopes [2]

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isolated *T. gondii* from testes, epididymis, seminal vesicles and prostate of sheep experimentally infected by oocysts and tachyzoites using bioassays and PCR. In another study, Lopes et al. [15] have reported similar results as Teale et al. [16], where *T. gondii* was isolated from seminal fluid of rams experimentally inoculated, suggesting *T. gondii* venereal transmission. Therefore, this study aimed to evaluate the influence of toxoplasmosis on milk production, reproductive problems, and forms of *T. gondii* transmission between seropositives dairy ewes.

2. Materials and methods

2.1. Animals

This study was conducted in a rural property located in the Western region of Santa Catarina State, Southern Brazil. Males and females were tested on three different steps to evaluate the influence of toxoplasmosis on milk production (Step 1), possible venereal transmission (Step 2), as well as transplacental transmission and occurrence of reproductive problems (Step 3). All animals of each step were confined in collective pens (24 m^2 each) with wood shaving floors and fed with silage, concentrate (50:50), and water *ad libitum*.

T. gondii-seropositive Lacaune sheep were selected by an indirect immunofluorescence assay (IFA) searching for immunoglobulin G (IgG) anti-*T. gondii* in serum samples with microscope slides containing tachyzoites of *T. gondii* RH strain. Sera samples were previously diluted in PBS (pH 7.2) up to 1:64 (standard titration in the IFA technique for *T. gondii*), and incubated for 30 min at 37 °C in a humidified chamber. A secondary antibody (anti-sheep-IgG conjugated to fluorescein; Sigma-Aldrich®) was added and incubated for 30 min at 37 °C in a humidified chamber. Sera samples from positive and negative sheep were used as controls. Samples IFA positives at 1:64 or higher were considered positives and subjected to titration (1:128; 1:256; 1:512; and 1:1024), according to the literature [17]. Reactions with peripheral or diffuse fluorescence of tachyzoites were considered positives, as well as fluorescence with apical or polar reactions were considered negatives [17].

2.1.1. Step 1: Influence on milk production

Forty adult Lacaune females in the beginning of the lactation period with similar body weight (56.2 ± 3.9 kg), age (24.2 ± 1.1 months), and number of offsprings were divided into two groups: seropositives (n = 18) and seronegatives (n = 22) for *T. gondii*. Blood samples for serological tests were collected 7 days post-partum. The animals were confined into four collective pens (n = 10), and received the same diet twice a day (silage and concentrate at proportion of 50:50). The food provision was proportionally calculated based on their body weight, therefore isoenergetic and isonitrogenous diets were prepared.

Automated milking was performed twice a day (05:00 a.m. and 05:00 p.m.). The volume of milk produced was individually weighed once a week, up to seven weeks using a specific equipment (True-test®, Auckland, New Zealand). In the subsequent weeks, milk samples were collected until the end of lactation, totaling 126 days of lactation). Milk composition (fat, protein, and lactose) was determined using an infrared analyzer (Bentley 2000®) on days 7, 21, 35, 56, 84, and 126 of the experiment.

2.1.2. Step 2: Presence of T. gondii in semen

In order to find *T. gondii* positive rams, eleven breeding males underwent serological testing by IFA. Five of them were seropositives with titers ranging from 1:128 to 1:2048. Semen collection was carried out using an artificial vagina and a collection dummy. Five semen samples were collected from each male within two days of interval. The ejaculate was stored in tubes and frozen at −20 °C.

Semen samples collected from *T. gondii* seropositives and seronegatives animals were tested for the presence of *T. gondii* DNA through endpoint PCR, qPCR, and nested PCR. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA), according to manufacturer's recommendations. The extracted products were evaluated for concentration and purity using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). DNA templates (50 ng) from each sample with absorbance ratio > 1.7 (ranging between 260/280 nm) was used. The primers used for endpoint PCR, qPCR and nested PCR (external and internal primers) were designed using the Primer Express software (Applied Biosystems, USA), and synthesized by IDT (Brazil). For nested-PCR the internal primers used were: forward 5′-TTCATTGATGCTGTCGATCGA-3′ and reverse 5′-ACAAACGCCCGAGACAAAAC-3′ to amplify a fragment of 100 base pairs; and the external primers were forward 5′-GAGCAGTATGATTGGGTGTCATG-3′ and reverse 5′-TGCTTAAGTTCAGCGGGTAACC-3′ to amplify a fragment of 320 base pairs.

2.1.2.1. PCR. DNA (50 ng) extracted from each semen sample was used as template for PCR analysis. In addition, *T. gondii* extracted from tissue cysts of VEG strain (kept in mice) was used as positive control, and distilled water as negative control. PCR mix was composed of: 2 μl of DNA, specific buffer including magnesium chloride, DNTP, 200 nM of each primer (primer int), Platinum Taq DNA polymerase, and distilled water up to 25 μl of total volume. The reaction conditions were: denaturation at 95 °C for 60s, followed by 35 cycles of 95 °C for 30s, 60 °C for 60s and 72 °C for 2min. All the reactions were performed in a thermal cycler PTC-100TM (MJ Research Inc, USA). The PCR products were analyzed on agarose gel (1%).

2.1.2.2. qPCR. A pair of primers was used (internal primers) for the amplification of a specific *T. gondii* DNA fragment (100 pb). PCR mix consisted of 2 μl of DNA, 200 nM of each primer, fluorophore, and other components of SYBR® Green PCR Master Mix (Applied Biosystems, USA). The reaction conditions consisted of 40 cycles at 95 °C for 30s, 60 °C for 60s, and 72 °C for 2min in Step One Plus Real-Time PCR System (Applied Biosystems, USA). The results were evaluated for the presence/absence of amplified specific DNA fragment according to the melting curve of each sample.

2.1.2.3. Nested-PCR. Two pairs of primers were used: an external primer for the amplification of a specific *T. gondii* DNA fragment (320 pb), considered the external nested-PCR fragment; as well as an internal primer for the amplification of a specific internal fragment (100 pb), containing the fragment amplified by the external primer. Thus, it was possible to assure high sensitivity and specificity in the molecular diagnosis regarding the presence/absence of the target DNA. PCR mix was composed of 2 μl of DNA template (initial concentration of 50ng/stage 1of nested-PCR) or the amplified product from stage 1 (as DNA template of stage 2 of nested-PCR), 200 nM of each primer, 2,5U of platinum TaqDNA polymerase (Invitrogen, Brazil), buffer, and distilled water up to 25 μl. The conditions for both reactions were: 40 cycles at 95 °C for 30s, 60 °C for 60s and 72 °C for 2min in a Step One Plus Real-Time PCR System (Applied Biosystems, USA). The amplified products of the second stage from nested-PCR were assessed by electrophoresis on agarose gel (1%).

2.1.3. Step 3: Occurrence of reproduction problems and transplacental transmission

Reproductive problems (abortion or fetal resorption) were evaluated in a flock of 68 pregnant ewes, considered positives (with