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Short Communication

# *Toxoplasma gondii* diagnosis in ovine aborted fetuses and stillborns in the State of Pernambuco, Brazil

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ABSTRACT

The aim of this research was to study the contribution of *Toxoplasma gondii* to reproductive failure using nested PCR and histopathological examination of fetuses, stillborns and placentas. We examined 245 organs of fetuses and 28 placentas from 35 abortions and stillborns from naturally occurring miscarriages in sheep in the State of Pernambuco, Brazil. At necropsy, fragments of brain, cerebellum, medulla, lung, heart, spleen, liver and placenta were taken for nested PCR and histopathological tests. Pathological examination revealed macroscopic lesions, suggesting *T. gondii* infection in 5/35 (14.3%) of the placentas. The histopathological examination revealed no lesions characteristic of toxoplasmosis in the organs investigated. In the five placentas, lesions consistent with toxoplasmosis were observed as an inflammatory non-suppurative infiltrate, along with multiple necrosis and mineralization. Nested PCR showed three aborted fetuses and two stillborns (14.3%) to test positive for *T. gondii*, with DNA amplification in all organs and the placenta, especially the heart and the placenta, which are the tissues of choice. This study substantiates the theory that *T. gondii* is involved in miscarriages and stillbirths and in the placentas of naturally infected sheep in Brazil. Such findings have not previously been described in the national literature.



## 1. Introduction

*Toxoplama gondii* has been described as one of the most significant causes of reproductive disorders in flocks of sheep around the world (Dubey, 1986). Miscarriages are the main kind of reproductive failure, generating considerable economic losses (Silva and Silva, 1988; Buxton et al., 2007). Laboratory diagnosis of the infection is of fundamental importance because reproductive failure can result from a variety of other infectious diseases (Vidotto, 1992; Amato Neto et al., 1995).

In pregnant sheep, during acute infection, the placenta is invaded by tachyzoites, in the free form and inside trophoblasts, resulting in necrosis and mineralization of the placenta. Transplacental infection of the fetus may occur and miscarriage, with or without invasion of the fetus, may result (Jones et al., 2000). In females that have been pregnant for up to 90 days, infection accounts for the occurrence of embryonic death, miscarriage, stillbirth and neonatal mortality (Dubey and Towle, 1986; Barberan and Marco, 1997).

The diagnosis of congenital toxoplasmosis can be performed by identifying the agent using histological slides

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and the polymerase chain reaction (PCR) with aborted fetuses and placentas (Pereira-Bueno et al., 2004).

The aim of this research was to study the contribution of *T. gondii* to reproductive failure using nested PCR and histopathological examination of fetuses, stillborns and placentas from naturally occurring miscarriages in sheep in the State of Pernambuco, Brazil.

## 2. Methods

All experiments met or exceeded the standards set by the International Guiding Principles for Biomedical Research Involving Animals and all protocols were approved by the Federal Rural University of Pernambuco's Ethical Committee (CEUA-UFRPE, protocol # 021-2009).

### 2.1. Samples

Two hundred and forty-five organs and 28 placentas from 35 fetuses and stillborns from sheep raised in farms in the State of Pernambuco, Brazil, were obtained from naturally occurring miscarriages which were brought under refrigeration to the Federal Rural University of Pernambuco's Infectious Diseases Laboratory.

### 2.2. Anatomopathological examination

Pathological examination of the fetuses and the collection of samples were carried out according to the procedures outlined by Pérez et al. (2003). Fragments of brain, cerebellum, medulla, lung, heart, spleen, liver and placenta were collected for the nested PCR and histopathological examinations.

The histological techniques used were those described by Prophet et al. (1992). Histological findings were classified as absent, unrelated lesions, consistent with or peculiar to toxoplasmosis.

### 2.3. Molecular diagnosis

All samples of placental and fetal tissue were submitted to DNA extraction using Qiagen DNA Easy Blood and Tissues Kit (Qiagen®, Hilden – Germany), in accordance with the manufacturer's instructions. After DNA extraction, amplification reactions were performed at a final volume of 12.5 (L containing: 2.5 μL f genomic DNA, 0.5 μL of each primer at 10 μM, 2.5 μL of Mili-Q ultrapure water and 6.25 μL of MasterMix (mixture for PCR – Promega), according to the supplier's recommendations. The thermal profile of the reaction stages was drawn up using a thermocycler MJ-96G (Biocycle Co. Ltd., Hangzhou – China) according to the protocol described by Spalding et al. (2006).

All negative and control samples were submitted to nested PCR, using 1 μL of the simple PCR product and added to the reaction mixture to provide a final volume of 12.5 (L containing 10 μM of each primer, 4.75 μL of Mili-Q ultrapure water and 6.25 μL of MasterMix, according to the supplier's recommendations. The reaction cycles consisted of an initial DNA denaturation at 95 °C (4 min), followed by 35 cycles at 95 °C for 1 min of denaturation, 62 °C for 30 s of annealing, 72 °C for 1 min of extension and a final extension period of 10 min, at 72 °C.

The primer pairs used are fragments of the B1 gene. For the first amplification, TOXO-C1/TOXO-N1 was used, amplified to 197 bp. For the second amplification, TOXO-C2/TOXO-N2 was used, amplified to 97 bp (Burg et al., 1989; Spalding et al., 2006).

Amplified products were detected by electrophoresis in 2% agarose gel stained with ethidium bromide, viewed under ultraviolet light and photo-documented. DNA sequencing was used to confirm the identity of the amplified fragments. The DNA fragments analyzed showed values similar or identical to those of the sequences already in the GenBank, which ranged from 93 to 99%, with $E = 1e - 100$.

## 3. Results

Nested PCR confirmed three miscarriages and two stillborns 5/35 (14.3%) to test positive for *T. gondii*. The parasite was detected in all fetal and placental organs of these five animals, with percentages ranging from 100% in the heart and placenta, 80% in spleen, brain, liver and lung, and 60% in cerebellum and medulla, making a total of 32/40 (80%) tissue samples testing positive. The 30/35 (85.7%) fetuses and stillborns remaining tested negative according to both techniques (Table 1).

Macroscopic examination allowed the fetuses and stillborns to be classified according to their state of conservation, 10/35 (28.6%) being considered fresh and 25/35 (71.4%) autolyzed. Examination of the five fetuses testing positive according to nested PCR revealed 3/5 (60%) to be fresh and 2/5 (40%) autolyzed. No macroscopic findings peculiar to toxoplasmosis were observed in the organs, 42.3% of which were considered non-specific for autolysis. There were pulmonary edemas in 10% and hemorrhagic areas in the heart and brain of 6.7%. The five placentas from the fetuses testing positive according to the molecular examination exhibited lesions peculiar to toxoplasmosis, such as infiltrated mononuclear cells, multiple necrosis and areas of mineralization.

## 4. Discussion

Reproductive disorders, especially miscarriages and stillbirths are the most common problems described as being associated with congenital toxoplasmosis in sheep around the world (Weissmann, 2003). In Brazil, serological studies of the frequency of anti-*T. gondii* antibodies have shown the extent to which toxoplasmosis is present in

**Table 1**
Comparative results of histopathological technique and nested PCR in 40 samples (35 fetal and 5 placental) of the 5 confirmed cases for *T. gondii*.

| | | Nested PCR | | |
|---|---|---|---|---|
| | | Pos. | Neg. | Total |
| Histopathological | Pos. | 5 (12.5%) | 0 (0%) | 5 (12.5%) |
| | Neg. | 27 (67.5%) | 8 (20%) | 35 (87.5%) |
| | Total | 32 (80%) | 8 (20%) | 40 (100%) |

Pos., number of positive samples; Neg., number of negative samples.

sheep, with 7–55% testing positive for the disease (Dubey, 2009). There is no data on *T. gondii*'s contribution to miscarriage and stillbirth in sheep and this is as yet the only study that has used a large number of tissue samples from naturally occurring miscarriages. In this study, a frequency of 14.3% of miscarriages associated with *T. gondii* infection was observed. This figure lies within the variation limits for frequency observed in other countries. The smallest percentage of miscarriages found was 10.6%, reported by Steuber et al. (1995), in Germany. Masala et al. (2003, 2007) found 11.1% and 18.1% respectively, in Italy. In the USA, a figure of 17.5% has been reported (Dubey and Kirkbride, 1990). In Spain, a frequency of 16.9% has been registered (Hurtado et al., 2001) with 23.2% miscarriages (Pereira-Bueno et al., 2004). This variation in the frequencies observed in different countries may be due to the use of different diagnosis techniques. Similar to the results obtained by Pereira-Bueno et al. (2004), miscarriages were usually found to occur in the middle or towards the end of gestation.

The use of techniques that detect or isolate the parasite in fetal or placental tissue confirms that miscarriage was caused by *T. gondii* (Owen et al., 1998a; Hurtado et al., 2001; Masala et al., 2003). In this study, the number of samples testing positive using PCR was higher than that for the histopathological examination. These results differ from those previously described by Pereira-Bueno et al. (2004), who studied tissues from sheep miscarriages in Spain and found a good correlation between the results of the nested PCR and histopathological examinations. On the other hand, Hurtado et al. (2001) demonstrated that nested PCR may be more sensitive and specific for toxoplasmosis diagnosis in sheep, which is in accordance with the results of this study. However, according to Pereira-Bueno et al. (2004), there are still few studies using PCR for toxoplasmosis diagnosis. Hence, further investigation is necessary to heighten the possibility of arriving at a correct diagnosis of the etiology of miscarriages in sheep.

In this study, nested PCR was able to detect parasitic DNA in 80% of the fetal and placental tissue from the five cases of confirmed miscarriages. These figures are higher than those found by Hurtado et al. (2001) and Pereira-Bueno et al. (2004), who reported negative findings using PCR and a histopathological examination, owing to the poor distribution of the parasite in fetal tissue, especially when brain tissue is used for the diagnosis. In this study, all the organs examined presented a percentage of 60–100%, the figures for the heart and placenta being especially high. It is usual for muscle (Masala et al., 2003), brain and lung (Hurtado et al., 2001) tissue to be recommended for diagnosis, although a number of studies have demonstrated the potential of placental tissue (Owen et al., 1998a,b; Hurtado et al., 2001; Masala et al., 2003; Pereira-Bueno et al., 2004). In this study, 100% of the placentas from the 5/35 animals testing positive using nested PCR also tested positive when the histopathological examination was used, owing to the presence of cysts.

Various studies agree that fetal and placental tissues are the best to use for the PCR technique. Spalding et al. (2002) tested the PCR technique on samples of human blood and placenta and Masala et al. (2003) on sheep fetuses and placentas, in order to diagnose congenital toxoplasmosis. These authors reported that the placental tissue is an excellent material for congenital toxoplasmosis diagnosis, in contrast to fetal serology, which may detect maternal antibodies arising from the intake of colostrum, resulting in false positives. In another study, Owen et al. (1998b) also confirmed that a larger number of parasites were found in the placentas.

Macroscopic examination enabled the state of conservation of the fetus to be classified and 71.45% of them were judged to be autolyzed. This figure is close to that reported by Engeland et al. (1998), who examined miscarriages in goats in Norway and reported that 65% of them were autolyzed. However, the autolysis found in most of the animals examined for this study may be due to the delay in collecting the fetuses and dispatching them to the laboratory, as well as poor conservation. This is one limitation of using a histopathological examination for diagnosis and suggests that this technique should be used as a complementary method for diagnosis of congenital toxoplasmosis.

Vertical transmission of *T. gondii* in sheep is still not fully understood and the PCR technique is highly useful for studies of this nature (Williams et al., 2005; Hide et al., 2009). The present study has provided evidence of the involvement of *T. gondii* in the aborted fetuses and placentas of naturally infected sheep in Brazil. No similar data have been described previously in the national literature.

## Conflict of interest statement

None.

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