Goats reinfected with *Toxoplasma gondii*: loss of viable prolificacy and gross revenue


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ABSTRACT

We determined the reproductive parameters and clinical disorders in pregnant goats infected and reinfected with *Toxoplasma gondii*, and posteriorly the loss of gross revenue due to congenital toxoplasmosis was estimated. Of the 25 non-pregnant females negative for *T. gondii*, 20 were orally inoculated (ME 49 strain) and of these, 15 pregnant females chronically infected were orally reinoculated (VEG strain) with *T. gondii* oocysts. Five groups were formed (n=5): GI, GII and GIII (reinoculations at 40, 80 and 120 days of gestation, respectively), GIV (inoculation) and GV (no inoculation). Clinical and serological exams were performed on days 0 (prior to inoculation), 3, 6, 9, 15 and 21 and every 7 days post-inoculation. Exams were also performed on day 3 and every 7 days post-reinoculation. Reproductive management was performed on all females and initiated when the females infected displayed IgG titers IFAT<1,024. From the average prolificacy indexes of each experimental group were estimated: total production of kilograms of live weight (total kg LW) of goats for slaughter, gross revenue and loss of gross revenue in U.S. dollars (US$), designed for a herd of 1,000 matrices. The unviable prolificacy indexes were 0.8 (GI), 1.2 (GII) and 0.2 (GIII). Clinical disorders affected 57.1% (GI), 75.0% (GII) and 16.7% (GIII) of the offspring of goats reinfected with *T. gondii*. Congenital toxoplasmosis in goats reinfected resulted in the loss of 26.5% of gross revenues, being GI (US$ 10,577.60 or 57.1%) and GII (US$ 12,693.12 or 60%) holders of the highest values and percentages of economic losses. It was found that congenital toxoplasmosis reinfection cause clinical disorders in goats chronically infected with *T. gondii* and their offspring with birth of unviable animals and loss of gross revenue, at different stages of pregnancy (40, 80 and 120 days of gestation), being in the initial and intermediate stages of pregnancy the largest estimates of these losses.

Keywords: caprine toxoplasmosis, clinical disorders, pregnant does

RESUMO

Nós determinamos os parâmetros reprodutivos e distúrbios clínicos em cabras gestantes infectadas e reinfectadas com Toxoplasma gondii, e posteriormente, foi estimada a perda de receita bruta debido à toxoplasmosose congênita. Das 25 fêmeas não prenhes negativas para T. gondii, 20 foram inoculadas oralmente (cepa ME 49) e, destas, 15 fêmeas gestantes infectadas cronicamente foram reinoculadas (cepa VEG), via oral, com oocistos de T. gondii. Cinco grupos foram formados (n = 5): GI, GII e GIII (reinoculações aos 40, 80 e 120 dias de gestação, respectivamente), GIV (inoculação) e GV (não inoculação). Exames clinicos e sorológicos foram realizados nos dias 0 (antes da inoculação), 3, 6, 9, 15 e 21 e a cada sete dias após a inoculação. Os exames também foram realizados nos dias 3 e a cada sete dias de pós-reinoculação. Manejo reprodutivo foi realizado em todas as fêmeas e iniciou-se quando as fêmeas infectadas exibiram títulos de anticorpos IgG<1.024. A partir dos índices médios de prolificidade...
INTRODUCTION

Goats are often infected by *Toxoplasma gondii*, an obligate intracellular protozoan that spreads throughout the body of the host, especially in the reproductive system, with abortion being one of the most prominent clinical signs of caprine toxoplasmosis (Dubey *et al*., 1980; Dubey, 1988; Abouzeid *et al*., 2010). This often results in loss of animals and in reproductive consequences that are difficult to estimate economically.

In Brazil, the high prevalence of antibodies against *T. gondii* observed in goat herds (Varaschin *et al*., 2011; Garcia *et al*., 2012), reflect the seriousness of toxoplasmosis in the field. In natural situations exact timing and duration of each phase of infection with *T. gondii* are difficult to determine, which does not rule out the possibility of toxoplasmic reinfection with or without clinical signs, especially when there is a risk of animals coming into contact with the different strains of existing *T. gondii*. This should be distinguished from reactivation of toxoplasmosis in animals.

Some aspects are relevant for understanding the toxoplasmic reinfection regarding the contact of the host with different genotypes of *T. gondii*: chronic infection does not prevent reinfection and acute disease with different strains (Araujo *et al*., 1997), immune protection conferred by the strain in infection can be violated by another strain in reinfection (Dao *et al*., 2001) and the response immune a strain of low virulence dont prevent a new response against another strain of greater virulence (Dzitko *et al*., 2006).

It is necessary to study the toxoplasmic reinfection in chronically infected goats and in reproductive age, including the economic impact on unviable animal production. In addition, the diagnosis of infection and reinfection with *T. gondii* requires caution, particularly in the identification of strains of this parasite, as there is a need for sophisticated laboratory techniques and the absence of the parasite does not mean a definitive result (Silva *et al*., 2014). Under natural conditions this becomes complicated by the diversity of strains of *T. gondii*. The aims of this study were to determine the reproductive parameters and clinical disorders in pregnant goats infected and reinfected with *T. gondii*, and posteriorly the loss of gross revenue due to congenital toxoplasmosis was estimated.

MATERIAL AND METHODS

The experiment was performed at the division of small ruminants, in the “Centro de Pesquisas em Sanidade Animal – CPPAR”, of the “Faculdade de Ciências Agrárias e Veterinárias – FCAV”, of the “Universidade Estadual Paulista "Julio de Mesquita Filho" – UNESP”, Jaboticabal Campus, São Paulo State, Brazil. The experimental period was from February 2010 to June 2011.

The study project was approved by the “Comissão de Ética no Uso de Animais – CEUA, FCAV / UNESP” (Protocol no. 010192), in June 2009.

Two Boer bucks (18 months and 4 years old) and 25 crossbred (Boer x Saanen) non-pregnant females of reproductive age (18 months to 3 years), seronegative for toxoplasmosis, neosporosis, brucelosis and leptospirosis were selected and remained in quarantine for 3 months. The goats were initially submitted to
serological exams: indirect immunofluorescence antibody test (IFAT) for the detection of antibodies against *T. gondii* (Camargo, 1964) and *Neospora caninum* (Conrad et al., 1993), acidified plate antigen test (Alton et al. 1988) and microscopy serum agglutination test (Cole et al., 1973) were performed for the diagnosis of *Brucella* and *Leptospira*, respectively. The females were kept in collective stalls, and the bucks were housed individually. Food and water were provided *ad libitum*, and the dry matter intake was of 5% of body weight, 70:30 forage (70% corn silage and Tifton hay 30%): concentrate (diet with 20% gross protein), mineral (Ca: P in the ratio 2:1) and 10% leftover.

We opted for a heterologous challenge to distinguish reacutization of toxoplasmosis, and strains ME49 - type II (avirulent) and VEG - type III (virulent) were used in inoculation and reinoculation with oocysts of *T. gondii*, respectively. The oocysts *T. gondii* (ME49 and VEG strains) used in the challenges of the animals were kindly provided by Prof. João Luis Garcia (UEL, Brazil).

On day zero (D0), immediately prior to inoculation with *T. gondii* oocysts, non-pregnant females were randomly allocated to experimental groups and transferred to 5 bays as follows (Tab. 1): GI, GII, GIII (reinoculation at 40 - initial, 80 - intermediate and 120 - final - days of gestation, respectively), GIV (inoculation - positive control) and GV (no inoculation - negative control).

Table 1. Experimental design of goats that were non-inoculated, inoculated and reinoculated orally with *Toxoplasma gondii* oocysts

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of goats</th>
<th>Experimental inoculation (ME49 strain)</th>
<th>Experimental reinoculation (VEG strain)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oocysts</td>
<td>Titer IgG (IFAT ≥64) <em>T. gondii</em></td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>2.5 x 10³</td>
<td>negative</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>2.5 x 10³</td>
<td>negative</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>2.5 x 10³</td>
<td>negative</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>2.5 x 10³</td>
<td>negative</td>
</tr>
<tr>
<td>V</td>
<td>5</td>
<td>SPS</td>
<td>negative</td>
</tr>
</tbody>
</table>

SPS: Sterile physiological solution.

Twenty non-pregnant females (groups I, II, III and IV) were orally inoculated with 2.5 x 10³ *T. gondii* oocysts ME49 strain. The 5 remaining females were kept as negative controls (group GV).

The experimental reinfections were performed at different stages of gestation: 40 (GI), 80 (GII) and 120 (GIII) days of gestation with 2.5 x 10³ *T. gondii* VEG oocysts, which were given orally to 15 pregnant females that had been previously infected (ME49 strain) and had IgG titers IFAT<1,024

The remaining pregnant females comprised the positive (GIV - infected) and negative (GV - uninfected) control groups.

On days 0 (prior to inoculation), 3, 6, 9, 15, 21 and every 7 days post-inoculation (DPI), the animals underwent clinical tests (heart and respiratory rates and rectal temperature), and blood samples (10mL/goat) were collected by jugular venipuncture using vacuum tubes without an anticoagulant.

For the experimental reinfections, the females underwent clinical tests and blood sampling at 3 days post-reinoculation (DPR) and then weekly until the end of gestation.

Serum anti-*Toxoplasma* IgG antibodies were measured by the IFAT (Camargo, 1964). The samples were considered positive when the IgG titers were ≥64 (Figueiredo et al., 2001). The slides used for the IFAT were prepared with antigens from the RH strain (Sabin, 1941).

The IFAT (Conrad et al., 1993), buffered acidified plate antigen test (Alton et al. 1988) and microscopy serum agglutination test (Cole et
al., 1973) were performed for the diagnosis of *N. caninum*, *Brucella* and *Leptospira*, respectively.

Hormone treatment was used for the induction and synchronization of ovulation (Maia, 1997) to allow for mating to occur at a fixed time for all the inoculated females of groups I, II, III and IV (titers IFAT-IgG <1,024) and the uninoculated females with *T. gondii* (GV). Before and after natural mating the males were examined for the presence of antibodies anti-*T. gondii*, *N. caninum*, *Brucella* and *Leptospira*.

Thirty-five days after mating, transabdominal ultrasounds were used to confirm pregnancies in the does. Ultrasound examinations were then performed every 15 days until the end of pregnancy. All deliveries were monitored by a veterinarian. After kidding, blood samples were collected from the goats and their offspring (heart blood) to perform the IFAT. No offspring of the uninfected, infected and reinfected goats with *T. gondii* (GV). Before and after natural mating the males were examined for the presence of antibodies anti-*T. gondii*, *N. caninum*, *Brucella* and *Leptospira*.

The reproductive parameters evaluated were as follows (Tab. 2): gestation period (days) and number (prolificacy) and gender of offspring. The clinical disorders were during parturition of the goats (dystocia) and in the offspring (body malformation, stillbirth and weakness).

From the average prolificacy indexes (total, viable and unviable) determined for uninfected, infected and reinfected goats with *T. gondii* (Tab. 2) we estimated: total production of kilograms of live weight (kg total LW) of goats for slaughter (1 kid = 4 months of age and 20kg body weight), gross revenue and loss of gross revenue in U.S. dollars (US$), designed for a herd of 1,000 matrices (Tab. 3).

**RESULTS**

The infected females with *T. gondii* ME49 oocysts (groups I, II, III and IV) showed alterations in rectal temperature and developed humoral anti-*Toxoplasma* IgG response when compared to the non-infected goats (GV). Notably, no females exhibited antibodies against *N. caninum*, *Brucella* and *Leptospira* throughout the experimental period.

Hyperthermia was observed at 3 and 9 DPI in all groups infected with *T. gondii* and a peak (40.2°C) in GIV at 9 DPI. At 21 DPI, the females infected with *T. gondii* seroconverted (IgG-IFAT≥64) reaching mean titers as high as 16,384 (GII, GIII and GIV) that persisted for more than 90 days. The IgG titers (IFAT<1,024) stabilized at 119 DPI in all of the females infected with *T. gondii*, and on this day the reproductive management was initiated in all goats. The bucks used in the present study were not infected with *T. gondii*, *N. caninum*, *Brucella* and *Leptospira* after natural mounting.

In the reinfected females with *T. gondii* VEG oocysts (groups I, II and III) there was a relevant increase in anti-*Toxoplasma* IgG titers (IFAT≥1,024), at different stages of pregnancy (40, 80 and 120 days of gestation), being at 28 (GI), 7 (GII) and 3 (GIII) DPR. In the GIV goats the IgG titers remained below 1,024 throughout the gestation period.

There was a variation in the rates of prolificacy between uninfected females, infected and reinfected by *T. gondii* (Tab. 2). The unviable prolificacy indexes were 0.8 (GI), 1.2 (GII) and 0.2 (GIII) observed only in the reinfected groups with *T. gondii*.

Clinical disorders were only observed during the kidding (dystocia) of females reinfected with *T. gondii* (groups I, II and III) and in their offspring (body malformation, stillbirth and weakness), being affected as follows (Table 1): GI four offspring (57.1%), GII six offspring (75.0%) and GIII only one offspring (16.7%). Several offspring of the infected goats and all the offspring of the reinfected goats exhibited IgG antibodies IFAT ≥64 (Tab. 2). All kidding was monitored by a veterinarian.
Goats reinfected…

Table 2. Reproductive parameters and clinical disorders in non-infected, infected and reinfected goats with *Toxoplasma gondii* and their offspring

<table>
<thead>
<tr>
<th>Group* (n=5)</th>
<th>mean gestation period (days)</th>
<th>number of offspring (prolificity)</th>
<th>offspring gender</th>
<th>Titulation IFAT anti-<em>T. gondii</em> (IgG &gt;64)</th>
<th>Clinical disorders</th>
<th>Affected offspring (%)</th>
<th>Prolificacy indexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>146.0</td>
<td>7 (1.4±0.5)</td>
<td>M 2</td>
<td>+5</td>
<td>I1M, 1M/2F</td>
<td>4 (57.1)</td>
<td>0.6</td>
</tr>
<tr>
<td>II</td>
<td>144.0</td>
<td>8 (1.6±0.5)</td>
<td>4 4</td>
<td>+5</td>
<td>2M/3F</td>
<td>6 (75.0)</td>
<td>0.4</td>
</tr>
<tr>
<td>III</td>
<td>143.0</td>
<td>6 (1.2±0.4)</td>
<td>2 2</td>
<td>+5</td>
<td>1M</td>
<td>1 (16.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>IV</td>
<td>149.3</td>
<td>6 (1.2±0.4)</td>
<td>2 4</td>
<td>+5</td>
<td>(3)+1F</td>
<td>normal</td>
<td>nule</td>
</tr>
<tr>
<td>V</td>
<td>149.0</td>
<td>10 (2.0±1.0)</td>
<td>4 6</td>
<td>-5</td>
<td>normal</td>
<td>normal</td>
<td>nule</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>37 (1.5±0.3)</td>
<td>19 18</td>
<td>+20(5) 24(13)</td>
<td>3/37</td>
<td>11/37 (29.7)</td>
<td>-</td>
<td>1.04±0.6 0.4±0.5</td>
</tr>
</tbody>
</table>

*Reinoculation (VEG strain): 40 (GI), 80 (GII) and 120 (GIII) days of gestation; inoculation (ME49 strain) - pregnant positive control (GIV); and no inoculation - pregnant negative control (GV) with *T. gondii* oocysts.

IFAT = indirect immunofluorescence antibody test. M = male, F = female. (+) positive and (-) negative.

None of the goats received anthelmintic treatment during the experiment and ultrasound examinations revealed no fetal changes or embryonic losses.

Estimated losses of production (in kg LW) and gross revenue (US$) due to unviable offspring were determined only for groups reinfected with *T. gondii*, such losses were (Tab. 3): GI - 3200kg LW and U.S.$ 10,577.60 (80 days gestation) and GII - 3840kg LW and U.S.$ 12,693.12 (120 days gestation).

Congenital toxoplasmosis in goats reinfected with *T. gondii* resulted in the loss of 26.5% of gross revenue, being GI (57.1%) and GII (60.0%) holders of the highest percentages of economic losses (Tab. 3).

Table 3. Prolificacy indexes of non-infected, infected and reinfected goats with *Toxoplasma gondii*, production of goats and loss of gross revenue, during a year, designed for a herd of 1,000 matrices

<table>
<thead>
<tr>
<th>Goats (n=200) / T. gondii</th>
<th>Indexes of prolificacy*</th>
<th>kg total LW - slaughter**</th>
<th>Gross revenue (US$)</th>
<th>Gross revenue viable - US$ (%)</th>
<th>Loss of gross revenue - US$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>viable</td>
<td>unviable</td>
<td>total</td>
<td>viable</td>
</tr>
<tr>
<td>Non-infected</td>
<td>2.0</td>
<td>2.0</td>
<td>0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td></td>
<td></td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>1.2</td>
<td>1.2</td>
<td>0</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td></td>
<td></td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Reinfected at 40 days of gestation</td>
<td>1.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>120</td>
<td>160</td>
<td>5,600</td>
<td>2,400</td>
</tr>
<tr>
<td>Reinfected at 80 days of gestation</td>
<td>1.6</td>
<td>0.4</td>
<td>1.2</td>
<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>128</td>
<td>192</td>
<td>2,400</td>
<td>2,560</td>
</tr>
<tr>
<td>Reinfected at 120 days of gestation</td>
<td>1.2</td>
<td>1.0</td>
<td>0.2</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>200</td>
<td>400</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td><strong>Total (%)</strong></td>
<td>1,480</td>
<td>1,088</td>
<td>392</td>
<td>29,600</td>
<td>21,760</td>
</tr>
</tbody>
</table>

*Table 1. **LW = live weight (1 kid for slaughter = 4 month old and 20 kg LW, adapted Fonseca and Bruschi, 2009). R$ 4,10/kg.LW (Agrolink, 2015) = 1 (one) US Dollar $ 3,3055 (Bovespa, 2015).

| US$/kg.LW x kg total LW (viable + unviable). | US$/kg.LW x kg total LW (viable). | US$/kg.LW x kg total LW (unviable). | [Gross revenue viable (US$ x 100/ gross revenue (US$)) and (Loss of gross revenue (US$) x 100/ gross revenue (US$))]. | [Gross revenue viable total (US$ x 100/ gross revenue total (US$)) and (Loss of gross revenue total (US$) x 100/ gross revenue total (US$))]. |
DISCUSSION

Production losses of goats for slaughter and loss gross revenues were estimated only on the prolificacy indexes unviable of reinfected goats with *T. gondii*, designed in one herd over a period of one year of production. If all positive animals for *T. gondii* were discarded and costs with the supply of good food and diagnostic tests (IFAT and ultrasound) were included, the economic losses would be much greater. According to Barros *et al.* (2010), the annual cost of a goat can be R$ 124.00 and Freyre *et al.* (1999) showed that the annual losses with toxoplasmosis in sheep flocks in Uruguay ranged from US$ 1,4 a 4,7 million.

The above draws attention to two aspects regarding the occurrence of caprine toxoplasmosis in Brazil and in the world. First, we need studies that account for the economic losses of toxoplasmosis in the field since there is a high prevalence of antibodies for *T. gondii* infection in goat herds (Varaschin *et al.*, 2011; Garcia *et al.*, 2012, Sharif *et al.*, 2015), which does not exclude the occurrence of infected animals with *T. gondii*. Second, the results of our study showed the possibility of toxoplasmic reinfection and transplacental infection in pregnant goats chronically infected with *T. gondii* with unviable offspring. It is noteworthy that reinfected females with *T. gondii* did not show pyrexia and abortion, common clinical signs of *T. gondii* infection (Dubey *et al.*, 1980; Dubey, 1988; Abouzeid *et al.*, 2010) and ultrasound examination did not reveal any change to the fetus during pregnancy, showing an ineffective exam in the prenatal diagnosis of toxoplasmosis.

Although the total prolificacy indexes determined for females reinfected with *T. gondii* were considered normal for the goats (Gordon, 1997), and the average of prolificacy of the group reinfected on 80 days of gestation was high and next females negative for *T. gondii*, there was a relevant amount of birth offspring economically unviable in all groups reinfected with *T. gondii* in different stages of pregnancy (40, 80 and 120 days of gestation).

The toxoplasmic reinfection (VEG strain) in pregnant goats chronically infected with *T. gondii* (ME49 strain) occurred with increasing titers IgG (IFAT), indicative of acute toxoplasmosis (Dubey and Kirkbride, 1989) and congenital disease. This demonstrated that the chronic infection with *T. gondii* did not prevent reinfection following acute disease with different strains of the parasite (Araújo *et al.*, 1997; Silva *et al.*, 2014), with violation of immunity (Dao *et al.*, 2001) the strain of low virulence against another strain of higher virulence (Dzitko *et al.*, 2006). It is known that the VEG strain is more virulent compared to the ME49 strain (Howe and Sibley, 1995).

The reinfected females with *T. gondii* generated apparently healthy and defective offspring in all stages of gestation (40, 80, and 120 days of gestation). This indicates that the passage of oocysts *T. gondii* via placenta was not limited only to one of the strains and those fetuses who came in contact with the most virulent strain (VEG strain) developed congenital toxoplasmosis. Furthermore, the reproductive management in all goats was initiated when the infected females with *T. gondii* displayed IgG titers that indicated chronic infection (Dubey and Kirkbride, 1989). This served to rule out any chance of the animals experiencing acute infection (ME49 strain) at the time of fertilization and experimental reinoculation with *T. gondii* (VEG strain). Also, no animals received anthelmintic, which excluded the risk of teratogenicity by this product.

The transplacental infection by *T. gondii* of the reinfected goats with *T. gondii* was confirmed by the presence of IgG antibodies in all their offspring, by IFAT considered standard technique for the diagnosis of toxoplasmosis (Camargo, 1964). This was reinforced by the fact that the placentas of ruminants, of the synepitheliochorial type, do not allow the passage of maternal antibodies to the fetus (Wooding, 1992; Agerholm *et al.*, 2006; Broaddus *et al.*, 2009) and the offspring did not receive colostrum, preventing the passive transfer of immunoglobulins (O’Brien and Sherman, 1993).

It is worth mentioning that the goats belonging to the infected group with *T. gondii* ME49 strain (GIV) showed chronic toxoplasmosis throughout pregnancy, confirmed by the presence of lower titers of IgG-IFAT (Dubey and Kirkbride, 1989), with the birth of all normal offspring, though...
some had antibodies IgG anti-\textit{T. gondii} (IFAT). This showed that congenital transmission by \textit{T. gondii} seriously studied in infected goats (Dubey \textit{et al.} 1980; Abouzeid \textit{et al.} 2010) can occur without reacutization of toxoplasmosis.

In this study it was found that toxoplastic reinfection during pregnancy causes clinical disorders in goats chronically infected with \textit{T. gondii} and their offspring with the birth of unviable animals and loss of gross revenue, at different stages of pregnancy (40, 80 and 120 days of gestation), with the largest estimates of these losses being in the initial and intermediate stages of pregnancy. On the other hand, it is important to evidence that both infected and reinfected females with \textit{T. gondii} that no had unviable offspring and neither had loss of gross revenue, this did not prevent offspring from exhibiting IgG antibodies. This fact is relevant beyond the economic aspects of toxoplasmosis. There is the issue of public health in relation to commercialization and ingestion of products from these apparently healthy animals.

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REFERENCES


