Short communication

Immunohistochemical identification of Toxoplasma gondii in tissues from Modified Agglutination Test positive sheep

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A B S T R A C T

Toxoplasma gondii is a zoonotic agent of great importance in veterinary and public health. The aim of this study was to identify T. gondii by IHC (immunohistochemistry) in different sheep tissues and to determine if an association exists between the results obtained by this method and those obtained by the Modified Agglutination Test (MAT). Tissue specimens of twenty-six sheep seropositive for T. gondii were selected for histopathological evaluation. The presence of T. gondii was investigated in brain, liver and heart samples by IHC and a possible anti-T. gondii antibody cross reactions with other parasites. McNemar’s, Chi-square and Fisher’s Exact Tests were applied for the statistical analysis of the results. The analysed tissues showed at least one of the following histopathological changes: mild-to-moderate congestion, focal polymorphonuclear inflammatory infiltrate and multifocal or focal mononuclear inflammatory infiltrate. Sarcocystis spp. were identified in the histological sections from both the heart and diaphragm tissues of 88.5% (23/26) of the animals. A total of 46.2% (12/26) of the T. gondii seropositive sheep was also positive for T. gondii by IHC in at least one organ (brain, liver or heart). The liver IHC-positivity for T. gondii was statistically equivalent to the global individual IHC-positivity, according to McNemar’s test. In addition, IHC allowed the detection of T. gondii in infected animals regardless of the titration observed in the MAT. The statistical difference observed between the three organs when comparing the low titration group, suggested that the heart might be the most suitable organ to detect T. gondii infection by IHC. The IHC results in this study revealed that almost half of MAT positive animals could serve as potential sources of infection for humans because bradyzoites were identified in different tissues, regardless of the MAT titration.

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1. Introduction

Toxoplasma gondii is one of the most studied parasites because of its impact on veterinary and public health (Tenter et al., 2000). Seropositivity in humans has been reported in more than 80 countries (Dubey, 2010) and the prevalence ranges from 4% in Korea (Ryu et al., 1996) to 92% in the Mato Grosso State of Brazil (Figueiró-Filho et al., 2005). The major route of toxoplasmosis transmission to human is the consumption of contaminated food, especially undercooked meat containing bradyzoites cysts (Villena et al., 2012). T. gondii infection occurs in sheep world wide, but the prevalence depends on the region (Dubey, 2010). In Brazil,
the sheep seroprevalence of antibodies against this parasite has been evaluated by many studies, and the State of Paraná had the highest prevalence of *T. gondii* in sheep in the country 51.5% (158/305) (Romanelli et al., 2007).

The MAT has been used for the detection of antibodies against *T. gondii* in many animal species, including sheep (Raeghi et al., 2011; Villena et al., 2012). The animals present positive MAT titres for *T. gondii*, suggesting that they are an important source of toxoplasmosis for humans (Alvarado-Esquivel et al., 2012).

Histopathological examination by IHC is widely employed in the diagnosis of *T. gondii* infection (Pereira-Bueno et al., 2004). Nevertheless, there have been few studies to date describing the immunohistochemical detection of *T. gondii* in sheep (Pereira-Bueno et al., 2004; Motta et al., 2008). Considering that the consumption of ovine meat occurs in different countries around the world, the aim of this study was to identify *T. gondii* by IHC in different sheep tissues and to determine if an association exists between the results obtained by this method and those obtained by the MAT.

2. Materials and methods

2.1. Animal ethics approval

This study was approved by the Ethics Committee of Animal Use (CEUA) from the Universidade Federal Fluminense (UFF) under protocol number 00111/09.

2.2. Samples

Tissue samples were collected from 26 seropositive sheep with different titres for *T. gondii* by MAT, after the slaughter of the animals. These sheep belonged to a larger group of 287 animals that had been previously tested for the parasite by MAT in spite of the titres that they presented. At the time of the study, only these 26 sheep were allowed by the owners to be slaughtered. The samples were submitted to histopathological evaluation and identification of the parasite by IHC.

2.3. Serological test

The serological analysis was performed with the MAT according to Dubey and Desmonts (1987). All samples with agglutinating activity at a dilution of 1:25 were considered positive (Sousa et al., 2009). These serum samples were subsequently titrated against reacting antigens using serial two-fold dilutions up to 1:3200.

2.4. Histopathology

Tissue specimens from liver, heart, brain, diaphragm, kidney and lung were collected from 26 *T. gondii*-seropositive sheep and fixed in neutral-buffered, 10% formalin. These specimens were routinely processed in paraffin for light microscopy and histological sections were produced for both haematoxylin–eosin (H&E) and IHC staining.

2.5. Immunohistochemistry

The presence of *T. gondii* tissue cysts was investigated in IHC-stained sections of the brain, heart and liver of 26 seropositive sheep. The histological sections were deparaffinised and hydrated, and the endogenous peroxidase was blocked with a 3% hydrogen peroxide solution. The sections were incubated in a 96 °C water bath for 30 min for antigen recovery. The nonspecific binding was blocked by incubating the sections in a solution of milk and 10% bovine serum albumin for 30 min. Subsequently, the sections were incubated for 30 min with primary rabbit anti-*T. gondii* antibody (Neomarkers, Fremont, CA, USA) diluted 1:200. The sections were treated with DAKO LSAB DAKO Corp. Carpinteria, CA, USA) as recommended by the manufacturer. Diaminobenzidine (DAB; DAKO Corporation, Carpinteria, CA, USA) was used as the chromogen to reveal the life cycle stages of the parasite, and all samples were counterstained with Harris haematoxylin. Histological sections of human brain positive for *T. gondii* were used as positive controls for the IHC technique as recommended by the manufacturer, and the primary antibody was omitted for negative controls. The samples were considered positive when bradyzoite pseudocysts were stained in brown by DAB. The animal was considered positive by IHC when at least one of the evaluated organs was positive. In addition, the positivity of the test was analysed in two ways: by the global animal status (the animal as a whole) as well as by the individual organ status (each separate organ of the animal). The tissue sections were also evaluated in order to search for possible anti-*T. gondii* antibody cross reactions with other parasites.

2.6. Statistical analysis

McNemar’s test was used to compare the results obtained by IHC. The tissue samples from the liver, heart and brain of the evaluated animals (the individual organ status) were compared to the global animal statuses.

A Fisher’s Exact Test was used to determine the association between the IHC positive and negative results in the different organs (liver, heart and brain) and the different *T. gondii* titres obtained by the MAT in all 26 seropositive animals. The animals were separated into two groups based on their titres: 1:25 to 1:50 and 1:100 to 1:3200. Fisher’s Exact Test was used for the comparative analyses between the two titration groups (1:25 to 1:50 versus 1:100 to 1:3200) and the immunohistochemical detection of *T. gondii* (positive or negative) in the samples from the brain, liver and heart, in order to identify the most suitable organ to detect infected animals even presenting low titres.

In addition, the Chi-square test was used to compare the animals that tested positive by IHC with their respective titres obtained by the MAT. This test was used to determine if there was an association between the titration at which the animal was seropositive for *T. gondii* and positive by IHC.

The Statistical Package for Social Science (SPSS) version 12.0 software was used. Differences where *P* < 0.05 were considered significant.
Table 1
Detection of anti-Toxoplasma gondii antibodies by the Modified Agglutination Test in 26 ovine serum samples and presence of cists of T. gondii in immunohistochemistry stained sections of the brain, liver and heart tissue sheep from Rio de Janeiro, Brazil.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Titres MAT</th>
<th>Presence of T. gondii cists in IHC stained sections</th>
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<td>Liver</td>
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<td>26</td>
<td>1:25</td>
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+ : positive; − : negative.

3. Results

3.1. Serological test

The serological results are presented in Table 1. The most frequent titres obtained were 1:50 (34.6%), followed by 1:25 (19.2%), 1:400 (15.4%), 1:3200 (11.5%), 1:100 (7.7%), 1:200 (7.7%), and 1:800 (3.8%).

3.2. Histopathological evaluation

The histopathological changes in the brain, liver and heart consisted of mild-to-moderate congestion, focal polymorphonuclear inflammatory infiltrate and multifocal or focal mononuclear inflammatory infiltrate. Hepatic vacuolar degeneration, portal fibrosis and necrosis were also observed. The lungs presented thickening of the alveolar septa, atelectasis and pulmonary emphysema. T. gondii cysts were not observed in the H&E-stained histological sections. However, Sarcocystis spp. were identified in the histological sections from both the heart and diaphragm tissues of 88.5% (23/26) of the animals. These cysts were round in shape, variable in size and were widely dispersed throughout the tissues. No significant histopathological changes were found in the other evaluated organs.

3.3. Immunohistochemistry

A total of 46.2% (12/26) of the animals evaluated were positive for T. gondii by IHC in at least one organ distributed as follows: 15.4% (4/26) had parasites only in the liver, 15.4% (4/12) in heart and liver, 7.7% (2/26) in brain and liver, 3.8% (1/26) only in the heart and 3.8% (1/26) in brain and heart (Table 1).

The three types of tissue (liver, heart and brain) demonstrated immunoreactivity to anti-T. gondii antibody, as shown in Fig. 1A–C. Small round cysts and pseudocysts containing bradyzoites were observed (Fig. 1A–C).

The intensity of the reaction was lower than that of the positive control due to the low number of cysts, despite the characteristic round shape. In liver, heart and brain, the immunostained parasites were found around the blood vessels and, in some cases, inside of them and in the parenchymatous cells.

McNemar’s test was used to compare the global animal status obtained by IHC and the individual organ status obtained by IHC reactions in the different organs. The liver IHC positivity for T. gondii was statistically equivalent ($P = 0.500$) to the global individual IHC positivity, according to McNemar’s test. However, this was not observed for the heart ($P = 0.031$) or brain ($P = 0.002$).

3.4. Evaluation of immunohistochemical cross-reactivity

Histological sections of heart tissue from nine sheep in which Sarcocystis spp. had been detected through histopathological examination were subjected to immunohistochemical analysis using primary rabbit anti-T. gondii antibody. These sections showed no positive reaction.

3.5. Immunohistochemistry and MAT comparison

Fisher’s Exact Test was used to compare the presence of immunostained T. gondii in the specimens of sheep brain, liver and heart with the titres detected by the MAT. There were no significant statistical differences between positive and negative samples (by IHC) when comparing samples of brain, liver and heart with MAT titres of 1:100 up to 1:3200. Statistical differences were only observed between the three organs when comparing the low titration group. The heart was the organ that showed most suitable to detect T. gondii infection by IHC in samples with low MAT titres (1:25 or 1:50 ($P = 0.046$)). No significant differences were found in the analysis of the brain and liver specimens ($P = 0.230$ and $P = 0.444$, respectively).

Regarding the 12 IHC-positive animals, the Chi-square test showed no statistical difference between the MAT titrations ($P = 0.065$). Immunohistochemistry was able to detect infected animals regardless of the titres observed by the MAT.

4. Discussion

Positive T. gondii immunoreactions were observed in the brain, liver and heart tissue from T. gondii-seropositive sheep, in accordance with other studies, in which structures morphologically consistent with cysts.
and tachyzoites were immunostained in brain, heart and also lungs of sheep (Motta et al., 2008; Benavides et al., 2011). In contrast, Rosa et al. (2001) did not detect cysts or tachyzoites of *T. gondii* in tissues of goats evaluated by IHC. The disparity of these results may be related to the different stages of animal infection and to the individual physical and immunological statuses of the animals; furthermore, random parasite distribution may be a factor (Rosa et al., 2001).

The identification of *T. gondii* in sheep tissues by IHC allowed the comparison of the global animal infection status and the individual organ status in terms of the presence of parasites in the liver, heart and brain. This comparative analysis showed that immunohistochemical positivity for *T. gondii* in the liver was statistically equivalent to the global individual immunohistochemical positivity. The histopathological findings found in the liver in this study were observed by other authors (Munhoz et al., 2002; Pereira-Bueno et al., 2004; Motta et al., 2008).

A histopathological analysis of conventional H&E-stained sections did not allow the detection of *T. gondii* in the examined organs in the present study. The same results were described by Silva and Langoni (2001) in sheep and Rosa et al. (2001) in goats. Due to the inability of H&E staining to detect this parasite, IHC is a particularly important tool for the detection of *T. gondii* in animal tissues. It reveals the parasites both in animals with no apparent infection by conventional histopathology and in those with low blood titres of *T. gondii*-specific antibodies.

The immunohistochemical identification of *T. gondii* in sheep tissue allowed the identification of infected animals regardless of the animals level of infection. The statistical difference observed between the three organs when comparing the low titration group (1:25 and 1:50) by Fisher’s Exact Test suggested that the heart may be the best organ to detect *T. gondii* infection by IHC in animals with low titration. Villena et al. (2012) demonstrated that cardiac fluids might be a relevant matrix for toxoplasmosis survey
in sheep meat. They found a significant correlation between increasing MAT titres on cardiac fluids and the probability of isolating live parasites from the heart.

In the present study, the low titres of 1:25 and 1:50 could be both considered as possible cut-off values for MAT detection of anti-\textit{T. gondii} antibodies in sheep. In comparison, Sousa et al. (2009) considered the cut-off value 1:25. On the other hand, Dubey et al. (2008) suggested the MAT cut-off value 1:50 to test sheep serum for evidence of exposure to \textit{T. gondii}. Nevertheless, in accordance with Villena et al. (2012), more studies using serological tests with improved accuracy are needed to detect the presence of the parasite in meat destined for human consumption.

Immunostaining of \textit{T. gondii} in the sheep tissues confirmed the infection status of the animals evaluated in the present study. These results confirm the existence of a potential risk for human infection through the ingestion of parasites from ovine meat, as has been described by other studies (Halos et al., 2010; Alvarado-Esquivel et al., 2011; Dubey et al., 2011; Villena et al., 2012).

The primary rabbit anti-\textit{T. gondii} antibody used in the present study has been tested with efficacy in sheep tissue by other authors (Motta et al., 2008). Although cross-reactivity between these two parasites in serological diagnosis has not been described as a major concern (Ugga et al., 1987; Dubey et al., 1996), we evaluated the immunohistochemical cross-reactivity between \textit{T. gondii} and \textit{Sarcocystis} spp. There was no reactivity in the IHC test for \textit{T. gondii}, even though a high number of \textit{Sarcocystis} spp. was present in the conventional H&E-stained histopathological sections of the heart. This data demonstrate the efficiency of this primary antibody.

The IHC results in this study revealed that almost half of the animals positive by the MAT were possible sources of infection for humans because bradyzoites were identified in different tissues, regardless of MAT titration. However, with regard to the presence of \textit{T. gondii} tissue cysts, there was a significant difference between the animals that had high titres and those with low titres for \textit{T. gondii} in MAT. In animals that had high titres for \textit{T. gondii}, cysts were found in the three evaluated organs – liver, heart and brain, whereas in animals with low titres, the cysts were observed only in the heart. This result suggests, that the heart is the organ of choice for the detection of bradyzoites by IHC in animals with low titres. Therefore, the IHC test was able to identify the dissemination of \textit{T. gondii} as a zoonotic agent in the RJ State, suggesting that the consumption of ovine meat and organs may present an important source of infection for humans. This could partially explain the high prevalence of human toxoplasmosis in this region of Brazil.

References


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