Ciência Rural, Santa Maria, v.46, n.9, p.1613-1617, set, 2016 http://dx.doi.org/10.1590/0103-8478cr20151365
ISSN 1678-4596

Molecular detection of protozoa of the *Sarcocystidae* family in sheep from the State of Rio Grande do Sul, Brazil

Detecção molecular de protozoários da família *Sarcocystidae* em ovinos no Estado do Rio Grande do Sul, Brasil

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ABSTRACT

*Infections caused by protozoa belonging to the **Sarcocystidae** family have worldwide distribution and are common in ruminants, leading to considerable economic losses. This study evaluates **Sarcocystis** spp., **Toxoplasma gondii** and **Neospora caninum** infections in sheep from Southwest region of Rio Grande do Sul, Brazil. Myocardium samples of 80 sheep raised on extensive system were collected. Tissue cysts were detected by direct examination and presence of infective agents was confirmed by PCR. Macroscopic evaluation did not reveal changes, but direct microscopic examination showed cysts in 76.2% (61/80, 95% CI: 66.9 – 85.9) samples, and all cysts were morphologically similar to those caused by **Sarcocystis tenella** or **Sarcocystis arieticanis**. PCR detected **Sarcocystis** spp. DNA in 21.2% (17/80, CI: 12.3-30.2) of the tested samples and **T. gondii** DNA in 15% (12/80, CI: 7.2-22.8). Moreover, 6.2% (5/80, CI: 2.1-13.9) samples contained DNA of both protozoan. The presence of **N. caninum** nucleic acids was not observed in tested samples. However, all PCR-positive samples (23.7%-19/80, CI: 14.4-33.1) were also positive by direct examination (microscopic cysts). Thus, a high occurrence of microscopic tissue cysts was detected in sheep from southwest region of Rio Grande do Sul State. Although PCR did not show good sensitivity to identify the causative agents of these cysts, it revealed the presence of **Sarcocystis** spp. and **T. gondii** in ovine cardiac muscle samples. This may predispose the contamination of animals and humans by these protozoa.*

Key words: ***Sarcocystis** spp., **Toxoplasma gondii**, **Neospora caninum**, PCR, diagnosis.*

RESUMO

*Infecções causadas por protozoários da família **Sarcocystidae** apresentam distribuição mundial, sendo comuns em ruminantes, responsáveis por causar importantes perdas econômicas. Este estudo avaliou infecções de **Sarcocystis** spp., **Toxoplasma gondii** e **Neospora caninum** em ovinos da região sudoeste do Rio Grande do Sul, Brasil. Foram coletadas amostras de miocárdio de 80 ovinos criados em sistema extensivo. Cistos teciduais foram detectados por exame direto, com a presença dos agentes confirmada por PCR. A avaliação macroscópica não revelou alterações, porém, no exame microscópico direto, foram verificados cistos em 76,2% (61/80, 95% IC: 66,9-85,9) das amostras, sendo todos morfologicamente semelhantes ao **Sarcocystis tenella** ou **Sarcocystis arieticanis**. A PCR detectou DNA de **Sarcocystis** spp. em 21,2% (17/80, IC: 12,3-30,2) das amostras testadas e DNA de **T. gondii** em 15% (12/80, IC: 7,2-22,8). Em 6,2% (5/80, IC: 2,1-13,9), foram detectados DNA de ambos os protozoários. Todas as amostras positivas no PCR (23,7%-19/80, IC: 14,4-33,1) também foram positivas no exame direto (cistos microscópicos). Assim, uma alta ocorrência de cistos teciduais microscópicos em ovinos da região sudoeste do Rio Grande do Sul foi detectada. Apesar de a PCR não ter mostrado uma boa sensibilidade na identificação dos agentes causadores desses cistos, foi possível verificar a presença de **Sarcocystis** spp. e **T. gondii** em amostras do músculo cardíaco de ovinos. Dessa maneira, a presença destes protozoários pode predispor a contaminação de humanos e animais.*

Palavras-chave: ***Sarcocystis** spp., **Toxoplasma gondii**, **Neospora caninum**, PCR, diagnóstico.*

INTRODUCTION

Protozoa classified in ***Sarcocystidae*** family of the phylum Apicomplexa are parasites characterized by the presence of sexual phase of development in the intestine of definitive hosts with oocyst liberation, and a lifecycle stage of tissue cysts in intermediate hosts (HECKEROTH & TENTER,

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Received 09.30.15 Approved 04.11.16 Returned by the author 06.06.16
CR-2015-1365.R3

2007). Protozoa of the genera ***Toxoplasma***, ***Sarcocystis*** and ***Neospora*** are important pathogens that cause abortions and significant economic losses in production animals (TENTER, 1995; DUBEY, 2003). Parasites belonging to these families have worldwide distribution. Moreover, the genus ***Toxoplasma*** can cause zoonotic infectious disease, which is considered an important public health problem (DUBEY, 2009).

Ovines can be intermediate hosts for four ***Sarcocystis*** species (***S. gigantea, S. medusiformis, S. tenella*** and ***S. arieticanis***) and can form macroscopic or microscopic cysts in different tissues of production animals (DUBEY et al., 1989). These animals can be infected if they ingest sporocysts or sporulated oocysts present in food or water (DUBEY & LINDSAY, 2006). In sheep, the main clinical signs of ***Sarcocystis*** infection are anorexia, weight loss and hyperthermia. However, depending on the number of sporocysts ingested, nervous signs, premature birth, and abortion are observed (HECKEROTH & TENTER, 2007).

Toxoplasma gondii and ***Neospora caninum*** are two genera of protozoa that are biologically similar. They are responsible for causing abortion and reproductive problems in ruminants (DUBEY et al., 2007; DUBEY, 2009). Toxoplasmosis is a zoonotic disease that affects many species of animals; however, felids are the definitive hosts (FRENKEL et al., 1970; DUBEY, 2009). Canids are definitive hosts of ***N. caninum***, which is also a major cause of abortion in cattle (GONDIM, 2006; DUBEY et al., 2007). A large number of tissue cysts are found in ***T. gondii*** seropositive sheep (DUBEY & JONES, 2008). Hence, the objective of the present study was to evaluate ***Sarcocystis*** spp., ***T. gondii*** and ***N. caninum*** infections in sheep from the Southwest region of Rio Grande do Sul, Brazil.

MATERIALS AND METHODS

Myocardium samples (15g) of 80 healthy sheep (number of animals slaughtered in four collecting days), aging 8 to 36 months, of both genders, raised on extensive system, were collected from one slaughterhouse under Federal Inspection Service, from the Southwest region of Rio Grande do Sul. Samples were stored in plastic bags under refrigeration at 4°C until processing to detect tissue cysts by direct examination. Aliquots with 5g of heart tissue were scarified individually. Then, 20mL of phosphate buffered saline (PBS) was added, and the mixture was filtered and the solution collected in a Petri dish for observation under an inverted microscope (magnification 10x).

Total DNA was extracted from approximately 50mg of each tissue sample of cardiac muscle using a commercial kit (Wizard genomic DNA purification kit, Promega, Madison, WI, USA), according to the manufacturer's instructions, with modifications in the lysis step, in accordance with the methods of MORÉ et al. (2011). Following extraction, DNA concentration in each sample was estimated by measuring ultraviolet light (UV) absorbance at 260nm, and the total DNA was stored at -20°C until use. Total DNA was subjected to polymerase chain reaction (PCR) using specific primers for each one of the tested agents (Table 1). Each reaction was performed in a total volume of 25μL, containing 5X PCR buffer; 10mM dNTPs; 100ng of each primer; 2.5 units of Taq polymerase and 100ng of total DNA used as template. The PCR products were visualized by UV illumination after electrophoresis on a 1% agarose gel stained with GelRed® (Biotium Inc., CA, USA).

Three PCR protocols were applied to each sample (one for each protozoan), and the amplification products were analyzed together

Table 1 - Target gene amplified by polymerase chain reaction (PCR), with primer sequences and base pair size (bp) of PCR amplification products of ***Sarcocystis*** spp., ***Toxoplasma gondii*** and ***Neospora caninum***.

	Target gene	Primer sequence	Amplified product
Sarcocystis spp.	18s rDNA	F: CGCAAATTACCCAATCCTGA R: ATTTCTCATAAGGTGCAGGAG	700bp
Toxoplasma gondii	B1	F: CGCTGCAGGGAGGAAGACGAAAGTTG R: CGCTGCAGACACAGTGCATCTGGATT	529bp
Neospora caninum	Nc5	F: CCCAGTGCGTCCAATCCTGTAAC R: CTCGCCAGTCAACCTACGTCTTCT	338bp

by gel electrophoresis. ***Sarcocystis*** spp. PCR was performed according to YANG et al. (2002), under the following conditions: initial denaturation for 4min at 95°C, followed by 40 cycles of denaturation for 40sec at 94°C, annealing for 30sec at 59°C and extension for 60sec at 72°C; with a final extension for 6min at 72°C. For ***T. gondii***, PCR was performed essentially as described by HOMAN et al. (2000), with: initial denaturation for 2min at 95°C, followed by 35 cycles of denaturation for 60sec at 94°C, annealing for 60sec at 65°C, and extension for 60sec at 72°C; with a final extension for 10min at 72°C. For ***N. caninum***, PCR was performed according to MÜLLER et al. (1996), under the following conditions: initial denaturation for 2min at 95°C, followed by 40 cycles of denaturation for 60sec at 95°C, annealing for 60sec at 60°C and extension for 60sec at 72°C; with a final extension for 2min at 72°C. Total DNA extracted from a sample of sheep cardiac muscle infected with ***Sarcocystis*** spp. was used as a positive control. For ***T. gondii*** and ***N. caninum***, DNA obtained from tachyzoites of RH and NC-1 strains, respectively, were used.

All data were analyzed using SAS software (SAS Institute Inc., Cary, NC). Results were analyzed by Chi-Square test, at a significance level of $P<0.05$.

RESULTS

Ovine cardiac muscle did not show any macroscopic changes, but microscopic analysis revealed cysts in 76.2% (61/80, 95% CI: 66.9-85.9) of the samples. These cysts showed similar morphology as ***S. tenella*** or ***S. arieticanis***, ranging in size between 400-900µm and walls between 0.5-3µm (HECKEROTH & TENTER, 1999). PCR results detected ***Sarcocystis*** spp. DNA in 21.2% (17/80, CI: 12.3-30.2) and ***T. gondii*** DNA 15% (12/80, CI: 7.2-22.8) of the tested samples respectively. Moreover, 6.2% (5/80, CI: 2.1-13.9) of the samples showed DNA from both protozoa (Figure 1). However, ***N. caninum*** nucleic acid was not reported in any of the animal samples tested. All PCR-positive samples, 23.7% (19/80, CI: 14.4-33.1) were also positive by direct examination (detection of microscopic cysts).

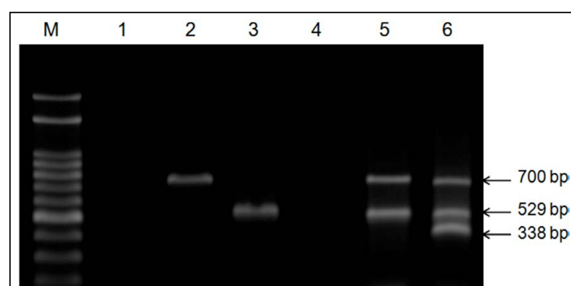

Figure 1 - Protozoa DNA detection by PCR in myocardium samples from ovines. ***Sarcocystis*** spp. (700bp); ***Toxoplasma gondii*** (529bp); and ***Neospora caninum*** (338bp). *Lane M*: Molecular weight marker (100bp); *lane 1*: negative control; *lanes 2, 3, 4, 5*: myocardium samples; *lane 6*: positive control. To lanes 5 and 6 were added amplified products of uniplex-PCR for better results visualization.

DISCUSSION

Studies of other authors have shown that rate of infection in ovines with tissue cysts of ***Sarcocystis*** spp. is usually high, reaching almost 100% of the examined sheep (THORNTON, 1972; LATIF et al., 1999). Occurrence of microscopic cysts in cardiac muscle of various animal species, infected with ***Sarcocystis*** spp., is more frequent than a possible presence of macroscopic cysts in the same tissue (LATIF et al., 1999). There are four ***Sarcocystis*** species that are reported in sheep, two of them transmitted by canids and form microscopic cysts (***S. tenella*** and ***S. arieticanis***), and the other two transmitted by felines (***S. gigantea*** and ***S. medusiformis***) and form macroscopic cysts (DUBEY & LINDSAY, 2006; TITILINCU et al., 2008).

Molecular diagnostic techniques are being used to determine the specific causative agents of tissue cysts (MORÉ et al., 2008; HAMIDINEJAT et al., 2014). PCR is highly specific, but in some cases, it is difficult to perform DNA extraction and to achieve the desired concentrations of samples, which limits the sensitivity of the technique (ALFONSO et al., 2009). Moreover, cysts are randomly distributed in the host tissues, and their concentration is lowered depending on the volume or the number of samples to be extracted (PIERGILI, 2004). In the present study, PCR assay showed lower sensitivity than direct examination (23.7% versus 76.2%, respectively). This may be because the sample volume used for nucleic acids extraction (50mg) was insufficient to detect parasitic DNA, as the concentration of tissue cysts is random, or because PCR assay presents a reduced ability to detect positive samples. PCR sensitivity can be improved by using the nested-PCR technique (XIANG et al., 2009), or the real-time PCR technique, which can also be used to detect different types of parasitic infections (BELL & RANFORD-CARTWRIGHT, 2002).

In the present study, ***T. gondii*** DNA was reported in 15% of myocardium samples. The fact that

the DNA was detected even though the protozoan was not isolated from tissues is important, as the infection by ***T. gondii*** spreads through the consumption of raw or undercooked tissues (LUNDÉN & UGGLA, 1992). ***T. gondii*** seropositive sheep usually present cysts that are reported in various edible tissues of these animals (DUBEY & KIRKBRIDE, 1989; LUNDÉN & UGGLA, 1992). Risk of infection in humans is a factor to be considered, since cases have been reported of individuals being infected through consumption of meat or meat products (TENTER et al., 2000). ***N. caninum*** DNA was not detected in the tested samples. However, infection in sheep is rare, and only a few cases of abortion and congenital diseases have been reported for this species (CORBELLINI et al., 2001; DUBEY, 2003). It is possible that this low frequency of detection is related to a higher concentration of cysts in the brain of infected animals with ***N. caninum*** (DUBEY & LINDSAY, 2006).

CONCLUSION

Results from this study showed a high occurrence of microscopic tissue cysts in the cardiac muscle of ovines from southwest Rio Grande do Sul. Although the PCR assay presented low sensitivity to identify the causative agents of these cysts, it was determined the presence of ***Sarcocystis*** spp. and ***T. gondii*** in myocardial samples from sheep, which can be a risk for animal and human contamination.

ACKNOWLEDGEMENTS

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support and for scholarship to the first, second and fourth authors.

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