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Short communication

Prevalence of *Sarcocystis* spp. in Argentinean cattle

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ABSTRACT

Sarcocystis cruzi, *S. hirsuta* and *S. hominis* are apicomplexan parasites that affect cattle worldwide with variable prevalence. The aim of the present study was to evaluate the prevalence of *Sarcocystis* spp. in Argentinean cattle comparing microscopic fresh examination and molecular methods. Blood, myocardium and loin samples were collected in five slaughterhouses from a total of 380 bovines. Origin of animals was representative of the major beef cattle production area of Argentina. Samples were analyzed by fresh microscopical examination, transmission electron microscopy (TEM), IFAT and PCR-RFLP. Thin walled sarcocysts corresponding with *S. cruzi* were found in 99.5% of heart samples. Sarcocysts were detected in 73.1% of loin samples; 71.5% had *S. cruzi* cysts and 23.1% had thick walled sarcocysts (*S. hirsuta* or *S. hominis*). TEM observation revealed the presence of characteristic *S. hominis* and *S. hirsuta* cyst walls in 7 and 1 loin samples respectively. Using IFAT, 379/380 animals had titers 25 or higher, showing a full agreement with fresh examination. Amplification products were detected in 35.5% (135/380) of loin samples; however *Sarcocystis* species could only be determined by RFLP in 29 samples. Agreement between fresh examination and PCR was low (Kappa value = 0.262). This is the first report of *S. hominis* and *S. hirsuta* in Argentina. Further studies are needed to improve the sensitivity of molecular methods for species identification, especially for differentiation of *S. cruzi* and *S. hirsuta* from the zoonotic species *S. hominis*. The results of the present study and others focusing on sensitivity and specificity of *Sarcocystis* spp. diagnostic methods should contribute to improve food safety.

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1. Introduction

Sarcocystis spp. infections affect cattle worldwide and are frequently asymptomatic. *Sarcocystis cruzi*, *S. hirsuta* and *S. hominis* have canids, felids and humans as definitive hosts respectively, and can affect bovines as intermediate hosts producing muscle cysts (sarcocysts). *S. cruzi* has the highest prevalence; over 90% of adult cattle have been found infected in many countries including Argentina, being cardiac muscle the most affected tissue (Böttner et al., 1987; Dubey et al., 1989; Fukuyo et al., 2002; Moré et al., 2008). Prevalence of *S. hirsuta* and *S. hominis* is variable in different parts of the world, being oesophagus and other muscles, but not myocardium, the most affected (Dubey et al., 1989); however, epidemiological reports have been scarce in the last few years. Although *Sarcocystis* species are generally considered non-virulent for cattle, *S. hominis* infection was occasionally associated to eosinophilic myositis (Wouda et al., 2006) and condemnation of beef due to presence of *S. hirsuta* macroscopic

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cysts was reported (Dubey et al., 1990). The relevance of *S. hominis* infection lies in its zoonotic character (Fayer, 2004).

Differential diagnosis of the three *Sarcocystis* species affecting cattle based on morphological features of the sarcocyst walls can be achieved by microscopy and electron microscopy (Dubey et al., 1989). These methods are specific but time consuming, limiting their application in a high number of samples. Diagnostic methods used in epidemiologic studies include artificial digestion, histopathology and/or fresh examination of tissue samples. Artificial digestion is one of the most sensitive diagnostic methods; it allows detection of bradyzoites released from sarcocysts, but not species identification. Histopathological and fresh examinations allow differentiation between thick- ($\geq$3 μm) and thin-walled (<1 μm) cysts, but not species differentiation within the thick-walled cysts (*S. hirsuta* and *S. hominis*). However, the sensitivity of histopathological examination is lower than that of fresh examination, due to the smaller volume of sample that can be processed (Dubey et al., 1989).

Molecular diagnosis appears to be an important tool for epidemiologic research in sarcocystosis. Several studies used polymerase chain reaction (PCR) for diagnosis of *Sarcocystis* infections, with special emphasis in species identification using sequencing and/or restriction fragment length polymorphism (RFLP) analysis of the 18S rDNA (Fischer and Odening, 1998; Li et al., 2002; Yang et al., 2002). However, since reports comparing PCR with other diagnostic methods in large scale studies are lacking, the sensitivity of these molecular methods is unknown.

The aims of the present study were to evaluate the prevalence of *Sarcocystis* spp. in Argentinean cattle using several diagnostic methods and to compare microscopic fresh examination with PCR-RFLP for *Sarcocystis* spp. identification in loin samples.

2. Materials and methods

Tissue samples were collected in five slaughterhouses from a total of 380 bovines (192 cows, 103 steers, 72 heifers and 13 bulls). Origin of animals according to Argentinean provinces was as follows: 143 from Buenos Aires, 60 from Santa Fé, 50 from Córdoba, 37 from La Pampa, 30 from Corrientes, 20 from San Luis, 20 from Entre Ríos and 20 from Río Negro. Fifty milliliters of blood, 100 g of myocardium (vertex) and 100 g of loin (psoas muscle) were collected from each animal. Loin was chosen for its importance in beef commercialization and the likeliness to detect and differentiate the 3 *Sarcocystis* spp. Serum samples were stored at −20 °C until analysis. Muscles were aliquoted for fresh examination (10 g) and molecular studies (1 g).

Ten grams of each muscle were minced in a meat grinder adding 40 ml of PBS. The homogenate was filtered using a strainer with gauze, collected in a 50 ml tube, and centrifuged at 600 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 20 ml of PBS and put in a Petri dish for observation in a stereomicroscope (Nikon). Sarcocysts detected were classified as tick or thin walled. Several sarcocysts (3–15) were collected with a Pasteur pipette from each of 19 loin and 5 heart samples, fixed in 2% glutaraldehyde during 2 h, washed 3 times with PBS and submitted to the Electron microscopy Central Service from Faculty of Veterinary Sciences, National University of La Plata, Argentina. Samples were processed by standard techniques with a vacuum protocol and observed in a JEM 1200 EX II (JEOL) transmission electron microscope.

Antibodies to *Sarcocystis* spp. were detected by indirect fluorescence antibody tests (IFAT) using *S. cruzi* bradyzoites as antigen according to the technique described previously (Moré et al., 2008). Serum samples were analyzed at 1:25, 1:200 and 1:800 dilutions.

DNA was extracted from 50 mg of loin samples using a commercial kit (Wizard genomic DNA purification, Promega) according to manufacturer's instructions. The lysis step was carried out at 55 °C overnight. PCR was performed essentially as described by Yang et al. (2002) to amplify a ∼700 bp fragment from the 18 s rDNA gene. Primers used were SarcoF 5′-CGCAAATTACCCAATCCTGA-3′ and SarcoR 5′-ATTTCTCATAAGGTGCAGGAG-3′, in a final volume of 25 μl using standard conditions. Amplification was done in a termocycler (PCR Sprint, THERMO Electron Corporation) as follows: initial denaturation of 95 °C for 4 min; 40 cycles of 94 °C for 40 s, 59 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 6 min. PCR products were observed in 1.5% agarose gels stained with SYBR safe and observed in a blue light transilluminator (Invitrogen). DNA from 50 mg of cardiac muscle containing *S. cruzi* was used as positive control.

Positive samples by PCR were cut with restriction enzymes *Bcl* I y *Rsa* I (Fermentas, Hanover, USA). Briefly, 5 μl of each amplicon were mixed with 5 U of each enzyme separately, using 2 μl of buffer Tango 10X (Fermentas) in a final volume of 20 μl. Incubation was done at 37 °C for 12 h for *Rsa* I and 55 °C for 1 h for *Bcl* I. Restriction fragments were observed in 2% agarose gels using the above mentioned staining procedure. According to Yang et al. (2002) *S. cruzi* fragments are cut only with *Rsa* I (1 restriction site), *S. hirsuta* are cut with both enzymes (one restriction site for each) and *S. hominis* remains uncut with both enzymes. Agreement between fresh examination and PCR results in loin samples were estimated using the Kappa value of Win Episcope 2.0 software and the confidence intervals (CI95%) were estimated using Statistica 7 software.

3. Results

Thin walled sarcocysts corresponding with *S. cruzi* were found in 99.5% (CI95%: 100–98.5; 378/380) of heart samples; one of the negative animals had sarcocysts in loin. Sarcocysts were detected in 73.1% (CI95%: 77.6–68.6; 278/380) of loin samples; 71.5% had *S. cruzi* cysts (CI95%: 75.7–67.3; 272/380) and 23.1% (CI95%: 27.3–18.9; 88/380) had thick walled sarcocysts (*S. hirsuta* or *S. hominis*). Eighty-two of the 88 samples with thick wall sarcocysts also had *S. cruzi* cysts. Thick wall sarcocysts were detected in loin samples from 19.4% (CI95%: 23.9–14.9; 14/72) of heifers, 26.6% (CI95%: 29.6–23.4; 51/192) of cows, 16.5% of steers (CI95%: 20.7–12.3; 17/103) and 46.1% (CI95%: 58.1–34.1; 6/13) of bulls. Rio Negro province was the only location where animals were negative for thick wall sarcocysts.

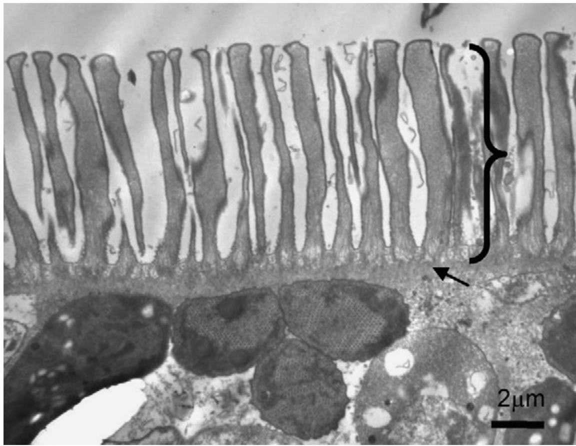

Fig. 1. Transmission electron microscopy image of a thick-walled sarcocyst showing long and palisade-like protrusions (key) supported by an electrodense layer (arrow). These protrusions are characteristic of *S. hominis*.

Electron microscope observation revealed the presence of characteristic *S. cruzi* cyst wall in 4 heart and 4 loin samples. Characteristic cyst walls for *S. hominis* (Fig. 1) and *S. hirsuta* (Fig. 2) were detected in 7 and 1 loin samples, respectively. Eight samples (1 from heart and 7 from loin) were discarded during processing since sarcocysts were not observed on semi-thin sections. Antibodies against *Sarcocystis* spp. were detected in 99.7% (CI95%: 100–98.7; 379/380) of serum samples; the negative animal was also negative in fresh examination showing a full agreement between test. Distribution of titers was as follows: 100 animals with titer 25; 206 animals with titer 200 and 73 animals with titer 800. Amplification products were detected in 35.5% (CI95%: 40.3–30.7; 135/380) of samples which were further analyzed by RFLP. The restriction enzyme pattern corresponding with *S. cruzi* was found in 16 samples. Fragments uncut with both enzymes (*S. hominis*) were detected in 13 samples; 8 of these also showed the *S. cruzi* pattern. The restriction pattern of *S. hirsuta* was not found in any sample. All remaining positive samples (106/135; 78,5%, CI95%: 85.5–71.5;) showed inconclusive results by RFLP. The sensitivity of PCR was lower than fresh examination since 151 positive samples by fresh examination were negative by PCR. Therefore, the Kappa value was 0.262 (CI95%: 0.187–0.337).

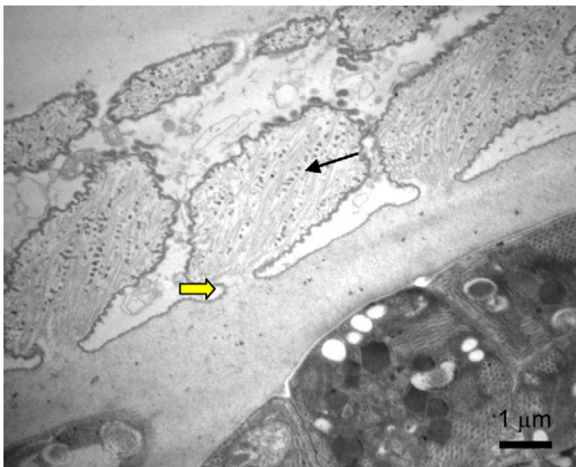

Fig. 2. Transmission electron microscopy image of a thick walled sarcocyst with tongue-like protrusions, containing microfilaments and dense granules. Each protrusion is narrow in its base (yellow arrow), wider in the middle portion and tapered at the end. These protrusions are characteristics of *S. hirsuta*.

4. Discussion

The prevalence of *S. cruzi* reported in this study is in agreement with previous reports in Argentina and other countries (Dubey et al., 1989; Moré et al., 2008). These results suggest close contact between hosts and great efficiency of the parasite to pass from canids to bovines and vice versa. Taking into account the high prevalence of *S. cruzi* reported by studies from several countries, most beef consumed worldwide could be considered infected with this parasite (Böttner et al., 1987; Fukuyo et al., 2002; Moré et al., 2008; Vercruysse et al., 1989; Woldemeskel and Gebreab, 1996). As previously reported, a positive IFAT result reflects *Sarcocystis* spp. infection, even with titer 25 (Moré et al., 2008). Reports of prevalence of *S. hirsuta* and *S. hominis* have been scarce in the last 10 years; however previous studies report a variable prevalence, up to 80%, of thick-walled sarcocysts (Böttner et al., 1987; Dubey et al., 1989; Vercruysse et al., 1989). Furthermore, the diagnostic method applied in some reports of low prevalence (Ono and Ohsumi, 1999) should be taken into consideration, since histological methods have shown low sensitivity (Dubey et al., 1989) which could result in lower estimation of the real prevalence. Fresh examination applied in the present study allowed identification of thick and thin-walled sarcocysts with high sensitivity, detecting a thick-walled sarcocyst prevalence of 23% in loin samples with a wide distribution in the main beef cattle provinces of Argentina. Using TEM we confirmed thick-walled sarcocysts as *S. hominis* and *S. hirsuta*. This is the first report of *S. hominis* and *S. hirsuta* in Argentina, with lower prevalence than reported in other countries (Böttner et al., 1987; Dubey et al., 1989; Vercruysse et al., 1989).

Unfortunately, PCR and RFLP methods showed lower sensitivity than fresh examination (35.5% versus 73.1% respectively), and most positive samples were inconclusive on RFLP species identification, resulting in low agreement between fresh examination and PCR (Kappa value = 0.262). This could be due to sample volume used for DNA extraction (50 mg) that yielded insufficient parasite DNA, or the PCR technique used, which was effective when a high amount of parasites, like isolate strains or purified bradizoites or sporocysts, was used (Yang et al., 2002).

The sensitivity of PCR can be improved using a nested PCR technique, and the identification of different *Sarcocystis* species can be performed by subsequent RFLP analysis as proposed by Xiang et al. (2009). In order to improve sensitivity and specificity in molecular diagnosis from tissue samples, magnetic capture techniques (Opsteegh et al., 2010) and real time PCR were developed for *Toxoplasma gondii* and other parasitic infections (Bell and

Ranford-Cartwright, 2002), and could potentially be used for *Sarcocystis* spp. as well.

Rejection of beef from Brazil and Argentina due to *Sarcocystis* spp. infection was reported in the last 5 years by the rapid alert system for food and feed (RASFF) adopted by the European Community (http://ec.europa.eu/food/food/rapidalert/reports/). The diagnostic methods applied and the validity of these rejections need to be discussed considering the high prevalence of *S. cruzi* infections in cattle worldwide and the unfeasibility of obtaining *S. cruzi*-free beef, that would make the withdrawing of infected meat from commercialization impractical. In these cases, a differentiation of *S. cruzi* and *S. hirsuta* from the zoonotic species *S. hominis* would be of high practical importance.

Future control measures should emphasize awareness in farmers and all the beef production chain to prevent these infections, especially by *S. hominis*. This requires the help of veterinary practitioners and a consensus message from scientists. International regulations for beef commercialization and diagnostic methods applied for *Sarcocystis* infections need to be further discussed. The results of the present study and others focusing on sensitivity and specificity of *Sarcocystis* spp. diagnostic methods should encourage the dialogue on these topics in order to improve food safety.

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