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ORIGINAL PAPER

Diagnosis of *Sarcocystis cruzi*, *Neospora caninum*, and *Toxoplasma gondii* infections in cattle

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Abstract The aim of the study was to diagnose *Sarcocystis* sp. infections in cattle and to detect coinfections by *Toxoplasma gondii* and/or *Neospora caninum*. Blood, diaphragm, esophagus, and myocardium from 90 beef cattle from Argentina were collected. Histopathological, immunohistochemical, polymerase chain reaction assays, and direct microscopical examination were carried out. Sarcocysts from myocardium were measured and counted. Indirect fluorescent antibody test (IFAT) for the three protozoans was performed. *Sarcocystis cruzi* sarcocysts were found in 100% of myocardium samples. Sarcocysts per gram ranged from 8 to 380 with higher values found in adult cattle ($p<0.001$). *T. gondii* and *N. caninum* were not detected by immunohistochemistry. *T. gondii* DNA was found in myocardium of 2/20 seropositive animals, while *N. caninum* DNA was not found. Antibodies against *S. cruzi* were detected in all samples, those against *N. caninum* in 73% and against *T. gondii* in 91% of the samples (IFAT titer ≥25). It is concluded that serology by IFAT is a suitable method to diagnose these protozoan infections due to its specific IgG detection; therefore, IFAT may be a useful tool to evaluate the impact of each protozoan infection in coinfected animals.

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Introduction

Sarcocystis cruzi, *Neospora caninum*, and *Toxoplasma gondii* infections are distributed worldwide in cattle. *S. cruzi* is the most prevalent of the *Sarcocystis* species infecting cattle; in many countries, over 90% of adult cattle have been found infected (Dubey et al. 1989). Occasionally, *S. cruzi* causes abortion, acute systemic illness, and poor growth (Dubey et al. 1989). Reports about *Sarcocystis* infections in Argentina are scarce, and only the presence of sarcocysts in cattle has been reported (Moriena et al. 1989). *N. caninum* is now recognized as a major cause of bovine abortions in many countries, including Argentina (Dubey 2003; Venturini et al. 1999). *T. gondii* infection is zoonotic and usually subclinical in cattle. The role of bovines in the epidemiology of toxoplasmosis is uncertain (Dubey et al. 2005).

Serological methods like indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays, detect antibodies to *S. cruzi*, *N. caninum*, and *T. gondii* antigens in single or mixed infections (Uggla et al. 1987; Dubey et al. 1996). Qualitative and quantitative diagnosis of *Sarcocystis* sp. may be accomplished by microscopical examination, but techniques like immunohistochemical staining and polymerase chain reaction (PCR) methods are necessary for the specific diagnosis of *N. caninum* and *T. gondii* infection (Dubey and Beattie 1988; Dubey and Schares 2006; Ho et al. 1997).

The aims of the present study were to diagnose *Sarcocystis* sp. infections, to detect *Sarcocystis* sp. antibodies by IFAT and to identify coinfections by *T. gondii* and/or *N. caninum* in Argentinean beef cattle.

Materials and methods

Cattle

Samples from beef cattle (n=90) were obtained in two slaughterhouses from Buenos Aires Province, Argentina. Five animals were sampled in each of the 18 visits to the slaughterhouses from June to December 2004 from 3 categories: heifers (n=30; 14–16 months old), steers (n=30; 24–30 months old), and adult cattle (n=30; 10 bulls and 20 cows, 42–112 months old).

There is approximately 50 million head of cattle in Argentina, most of them raised in extensive systems. Sample size was considered representative of the *Sarcocystis*-infected cattle population based on 90% prevalence with 99% confidence and 10% accuracy (Cannon and Roe 1982).

Samples

Fifteen milliliters of blood, 50 g of diaphragm, 10 cm of esophagus, and 100 g of myocardium (left ventricle) were collected from each animal. Refrigerated samples were transported within 6 h to the laboratory where 1 g of each fresh tissue was processed, 1 g was kept in 10% neutral buffered formalin for histopathological studies, and 1 g was stored at −20°C for molecular assays. Sera obtained from blood samples were stored at −20°C until processing.

Serological tests for *S. cruzi, N. caninum*, and *T. gondii*

Sera were tested by the IFAT for the detection of antibodies to *S. cruzi*, *N. caninum*, and *T. gondii*. For *S. cruzi* antibody detection, bradyzoites from naturally infected bovine hearts were used as antigen. One hundred grams of minced myocardium were mixed with 400 ml of digestion solution (2.5 g pepsin [0.7 U-FIP/mg] and 10 ml HCl in 1 l of water solution) and put in a magnetic stirrer for 20 min at 37°C (Lunde and Fayer 1977, modified). The suspension was filtered through 300, 150, and 53 μm sieves into 50 ml centrifuge tubes and centrifuged at 500×g for 5 min. Supernatants and the upper layer of the sediment were discarded; the pellet was washed three times with phosphate-buffered saline (PBS) and diluted in PBS to a final concentration of 4 million bradyzoites/ml. Bradyzoites were fixed onto 12-well IFAT slides and stored at −20°C. For *N. caninum* and *T. gondii* serology, cell culture-derived tachyzoites of NC-1 (Dubey et al. 1988) and RH strains (Sabin 1941), respectively, were used as antigens.

Sera were diluted twofold in PBS starting at 1:25 dilution. A rabbit antibovine IgG fluorescein isothiocyanate conjugate (Sigma Bio Sciences, St. Louis, USA) was used. Bovine positive and negative control sera were used in each analysis. Fetal serum was used as negative control. Complete peripheral fluorescence of merozoites was considered positive. IFAT titers to *S. cruzi*, *N. caninum*, and *T. gondii* were compared by the correlation test.

Cattle sera were also examined for *T. gondii* antibodies using the modified agglutination test (MAT) as described by Desmonts and Remington (1980) with serum samples diluted as described above.

Microscopical studies

Fresh samples

One gram of fresh myocardium, esophagus, and diaphragm samples from each of the 90 animals were dissected under a stereomicroscope for sarcocysts, and those from the myocardium were counted. Differences in the amount of sarcocysts among cattle categories were evaluated by the goodness of fit test (Epi 6).

Length and wall thickness of 129 sarcocysts from the myocardium (50 from 10 heifers, 27 from 8 steers, and 52 from 12 adults) were measured with a calibrated eyepiece. Differences of cyst length among cattle categories were evaluated by the ANOVA test.

Histopathology and immunohistochemistry

Thirty heart samples that had the greatest counts of sarcocysts or serological titers ≥200 to *S. cruzi* antigen were selected and embedded in paraffin. For histopathological studies, 5 μm sections were stained with hematoxylin and eosin.

Twenty heart samples from animals with positive IFAT antibody titers for *N. caninum* (25 to 800) and *T. gondii* (25 to 100) were processed by immunohistochemical staining for *N. caninum* and *T. gondii* with the LSAB+System HRP using a commercial kit according to the manufacturer's instructions (Dako Cytomation, Carpinteria, USA).

Polymerase chain reaction

From each of the 20 samples selected for immunohistochemical staining, 25 to 50 μg of heart was processed for PCR. DNA was isolated using a commercial DNA-extraction kit (DNeasy® Tissue Kit, QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. The amplification was performed with the specific primer pair Np 6/Np21 (Müller et al. 1996; Yamage et al. 1996; Kaufmann et al. 1996) and B22/B23 (Bretágne et al. 1993) for *N. caninum* and *T. gondii*, respectively. For *N. caninum* DNA detection, 1 μl of genomic DNA from each sample was added to tubes with a PCR master mix containing 2.5 μl of 10x PCR buffer (Fermentas, Hanover, USA); 200 μM each of dATP, dTTP, dGTP, and dCTP; 0.15 μl of *Taq* DNA polymerase (5 U/μl Fermentas); and 0.4 μM from each primer in a final volume

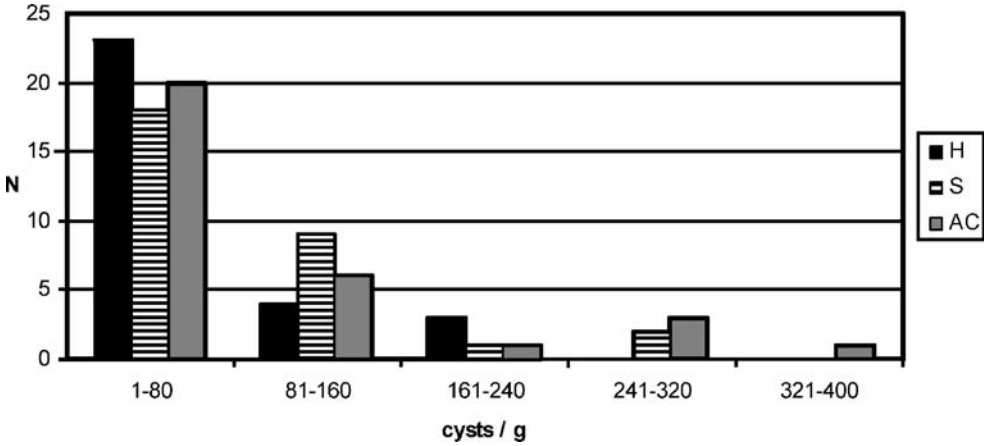

Fig. 1 Distribution of sarcocysts per gram of myocardium for each category of cattle. *H* heifers, *S* steers, *AC* adult cattle

of 25 μl. The reaction was accomplished in a thermal cycler (PCR Sprint Thermo Electron Corporation) with an initial denaturation step at 94°C for 5 min, followed by a 35-cycle program with denaturation (94°C; 30 s), annealing (55°C; 30 s), primer extension (72°C; 60 s), and a final extension at 72°C for 7 min. For *T. gondii*, the same procedure was followed with the exception that the annealing step was performed at 60°C for 30 s. Amplicons were visualized after electrophoresis in a 6% polyacrylamide gel and stained with silver staining (Harlow and Lane 1988). DNA isolated from *T. gondii* RH strain or *N. caninum* NC-1 strain were used as positive controls and replaced by nuclease-free water for the negative control.

Results

Microscopical studies

Fresh samples

Sarcocysts were found in 100% of the myocardium, 71% of the esophagus, and 28% of the diaphragm samples. The characteristics of all observed sarcocysts corresponded to *S. cruzi*: they had thin walls (<1 μm thick) and slender villar protrusions (6–13 μm long). Sarcocysts were 153 to 782 μm long with mean cyst length differing by category (301 μm for heifers, 387 μm for steers, and 442 μm for adult cattle; $p<0.01$).

The number of *S. cruzi* sarcocysts per gram of myocardium ranged from 8 to 380. Figure 1 shows the distribution of sarcocysts per gram of myocardium for each animal category. The total and mean amounts of sarcocysts counted for each category are shown in Table 1. Differences were observed in total amounts of sarcocysts between heifers and cows ($p<0.001$), steers and bulls ($p<0.001$), and males and females ($p<0.001$).

Histopathology and immunohistochemistry

Thin-walled sarcocysts were found in 28 of 30 myocardium sections, while thick-walled sarcocysts were not found. Lesions were not observed. *N. caninum* and *T. gondii* tissue cysts or tachyzoites were neither observed in hematoxylin and eosin nor in immunohistochemically stained sections.

Serological tests

Antibodies against *S. cruzi* antigen were detected in all samples, those against *N. caninum* in 73% and against *T. gondii* in 91% of the samples (IFAT titer ≥25). Figure 2 shows the distribution of animals and antibody titers to *S. cruzi*, *N. caninum*, and *T. gondii* antigen. Table 2 shows the frequency of diagnosis of single and mixed infections by IFAT. By MAT, 13 animals were positive to *T. gondii* antibodies with a titer of 25.

No significant correlation was found between IFAT titers to *N. caninum* and *T. gondii* antigen ($r=0.11$), titers to *S. cruzi*

Table 1 Mean , range, and total counts of sarcocysts/g in each cattle category

Cattle			Sarcocysts		
Categories		*n*	Total	Mean	Range
Heifers		30	1,866	62.2	10–228
Steers		30	2,448	81.6	14–188
Adult cattle	Bulls	10	1,134	113.4	8–380
	Cows	20	1,550	77.5	24–290
Total		90	6,998	77.7	8–380

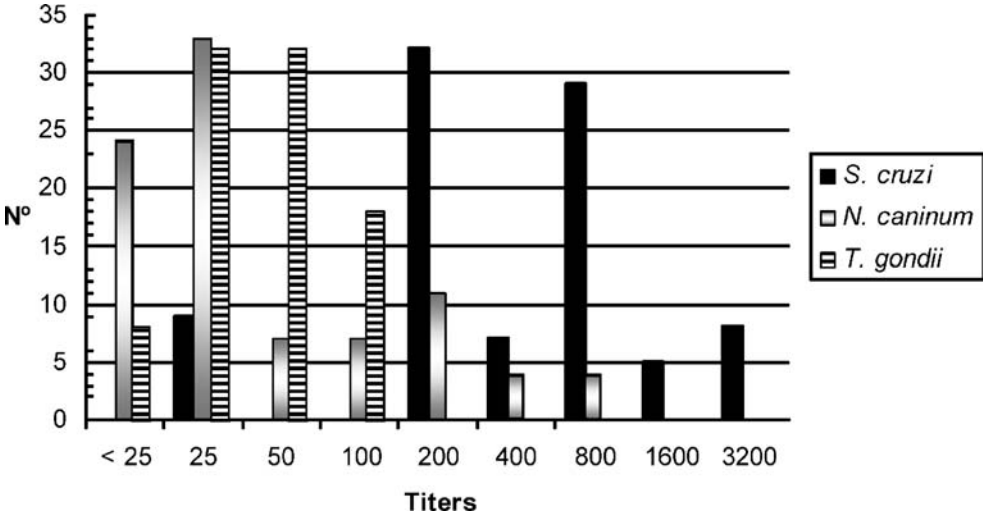

Fig. 2 Distribution of IFAT antibody titers to *S. cruzi*, *N. caninum*, and *T. gondii*. *No* number of animals

and *N. caninum* antigen (r=0.15), and titers to *S. cruzi* and *T. gondii antigen* (r=0.13).

Polymerase chain reaction

After electrophoresis, 115 bp amplicons corresponding to *T. gondii* were detected in DNA samples from a 9-year-old cow and a 2-year-old steer, both of them positive to *T. gondii* by IFAT (titer of 100). By MAT, the cow had a titer of 25 and the castrated male was negative. The corresponding 328 bp amplicon for *N. caninum* was not detected in any of the samples.

Discussion

All the sarcocysts observed corresponded to *S. cruzi*. A thin sarcocyst wall of around 1 μm characterizes *S. cruzi* and may be observed by fresh microscopical examination; a thick radially striated, 2.5 to 9.0 μm sarcocyst wall characterizes *S. hominis* and *S. hirsuta* (Dubey et al. 1989).

The frequency of *S. cruzi* detection by fresh examination was high, between 28% and 100% in the different tissues. These findings are in agreement with studies from other countries, which reported high prevalence of sarcocystosis by *S. cruzi*, myocardium being the most frequently infected tissue (Dubey et al. 1989). Previously, Moriena et al. (1989) found sarcocysts in tissues of cattle from north-eastern Argentina in 79 of 100 animals, but the *Sarcocystis* species was not reported. To our knowledge, this is the first characterization at the species level for *S. cruzi* in Argentina.

Reports about the count of sarcocysts per gram in cattle tissues were not found in the literature. In the present study, dissection of fresh myocardium allowed the quantification and species identification of sarcocysts. Sarcocysts per gram of myocardium ranged from 8 to 380. The higher proportion of sarcocysts detected in adult cattle suggests that sarcocyst number may increase with age, which could be due to greater opportunities of older animals of consuming sporocysts and/or to the lack of immunological control of reinfections.

Males had proportionally more sarcocysts per gram of myocardium than females. Savini et al. (1992) found a higher prevalence in bulls than in females. They suggested that bulls pastured in close proximity to farm buildings may have increased risk of infection because the interspecies contact is greater in these areas. Similar management practices are applied in Argentina and may be related with higher cyst counts found in males, but differences of immune response between sexes cannot be discarded.

In addition, sarcocysts found in adult cattle were longer than those from young cattle, which indicate that cysts growth continues over time. Sarcocyst counts and measurement by sex and animal category provided novel information on the dynamics of *S. cruzi* infection.

N. caninum was detected in heart tissue from cattle fetuses by immunohistochemistry and PCR techniques (Dubey and Schares 2006) and by PCR in 1 of 6 experimentally infected cows (Ho et al. 1997). However, we were unable to detect *N. caninum* by immunohistochemistry or PCR in the myocardium of 20 naturally seropositive animals, which may be due to absence of the parasite in the examined portions or in their hearts.

T. gondii DNA was found in myocardium samples from 2 of 20 seropositive animals, but *T. gondii* organisms were not

Table 2 Frequency of diagnosis by IFAT of single and mixed infections in different animal categories

	Sc	Sc+Nc	Sc+Tg	Sc+Nc+Tg
Heifers	4	3	10	13
Steers	1	0	4	25
Adult cattle	0	0	5	25

Sc: *S. cruzi*, *Nc*: *N. caninum*, *Tg*: *T. gondii*

detected by immunohistochemical staining. This is the first report of *T. gondii* DNA detection in Argentinean cattle. The significance of this finding remains to be investigated because isolation was not performed, and therefore, the potential risk of beef as *T. gondii* infection source could not be assessed. Detection of *T. gondii* DNA has been reported previously in 3% adult cows, 2% young bulls, 6% heifers, and 1% calves in Switzerland (Wyss et al. 2000).

Antibodies to *S. cruzi* antigen were detected in all animals with IFAT titers from 25 to 3,200. Titers of 25 are indicative of infection as they could be confirmed with the parasitological findings. To our knowledge, this is the first serological study of *Sarcocystis* sp. infections in Argentinean cattle. The absence of significant correlation between antibody titers to *S. cruzi*, *N. caninum*, and *T. gondii* antigens suggests that IgG titers ≥25 are specific and animals with positive titers for more than one parasite are coinfected. Further supporting these findings, Uggla et al. (1987) did not detect serological cross reaction in calves experimentally infected with *S. cruzi* using *T. gondii* antigen for IFAT at 1:10 dilution, and Dubey et al. (1996) did not detect cross reactivity by IFAT among *S. cruzi*, *N. caninum*, and *T. gondii* in experimentally infected cattle.

Infection by the three protozoans was found in 70% of the animals, by *T. gondii* and *S. cruzi* in 21%, by *N. caninum* and *S. cruzi* in 3.4%, and only by *S. cruzi* in 5.6% of animals. More studies are needed to determine if the high coinfection rate also occurs in the larger Argentinean cattle population and determine the potential implications of coinfection with these parasites on beef production.

In conclusion, serology by IFAT is a suitable method to diagnose *S. cruzi*, *T. gondii*, and *N. caninum* infections in cattle due to its specific IgG detection; therefore, IFAT may be a useful tool to evaluate the impact of each protozoan infection in coinfected animals. In addition, myocardium samples for PCR and IHQ seem to be less appropriate to diagnose *N. caninum* and *T. gondii* infections in adult cattle.

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