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Anti-*Neospora caninum* and anti-*Sarcocystis* spp. specific antibodies cross-react with *Besnoitia besnoiti* and influence the serological diagnosis of bovine besnoitiosis

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ABSTRACT

Bovine besnoitiosis control remains a challenge because the disease continues to spread and control relies solely on accurate diagnosis coupled to management measures. However, recent studies have reported that routinely used ELISAs may raise a high number of false-positive results. Herein, cross-reactions between *Besnoitia besnoiti* antigens and anti-*Neospora caninum* and/or anti-*Sarcocystis* spp.-specific antibodies were studied in an in house ELISA since *N. caninum* and *Sarcocystis* spp. are closely related parasites, and both infections are highly prevalent in cattle worldwide. The serum panel was composed of the following categories: sera from *B. besnoiti*-seronegative ($n=75$) and -seropositive cattle ($n=66$), *B. besnoiti*-based-ELISA false-positive reactors ($n=96$) together with *N. caninum* ($n=36$) and *Sarcocystis* spp. ($n=42$) -seropositive reference cattle sera. *B. besnoiti* tachyzoite based western blot (WB) results classified animals as seropositive or seronegative. Sera were analyzed for the detection of anti-*N. caninum* by WB and ELISA and anti-*Sarcocystis* spp.-specific antibodies by WB and IFAT. Those samples recognizing a *Sarcocystis* spp. 18–20 kDa antigenic region and *N. caninum* 17–18 kDa immunodominant antigen were considered to be *Sarcocystis* spp. and *N. caninum* seropositive, respectively. The category of *B. besnoiti* based-ELISA false-positive reactors showed the highest number of sera with specific anti-*Sarcocystis* spp. and anti-*N. caninum* antibodies (74%; 71/96), followed by the *N. caninum*-seropositive cattle category (52.8%; 19/36). In contrast, few *B. besnoiti*-seronegative and -seropositive cattle showed antibodies against *Sarcocystis* spp. and *N. caninum* (10.7%; 8/75 and 1.5%; 1/66), respectively). This study revealed that *B. besnoiti* false-positive ELISA results were associated not only with the presence of anti-*N. caninum* and anti-*Sarcocystis* spp. antibodies (χ^2: 78.36; $p<0.0001$; OR: 34.6; CI: 14–88) but also with high antibody levels against them using ELISA and IFAT tests, respectively ($p<0.05$; *t*-test). These results may explain why only some animals seropositive to *Sarcocystis* spp. and/or *N. caninum* are *Besnoitia* false-positive reactors. Therefore, sera meeting these requirements should be included in future validations of serological tests for bovine besnoitiosis.

1. Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan parasite that belongs to the genus *Besnoitia,* family Sarcocystidae and subfamily Toxoplasmatinae. It is the causative agent of bovine besnoitiosis, a chronic and a debilitating disease responsible for severe economic losses, mainly due to poor body condition, sterility in bulls and eventual death (reviewed by Álvarez-García et al., 2014b). In Western Europe, there is concern over the spread of bovine besnoitiosis and recent epidemiological studies have revealed high seroprevalence rates in areas where the disease is endemic (Liénard et al., 2011; Álvarez-García et al., 2014a; Gutiérrez-Expósito et al., 2014).

At present, control relies on accurate diagnosis coupled to management measures as no effective drugs or vaccines are available. For an accurate diagnosis a combination of clinical inspection and serology is necessary in order to detect both acute and chronically infected animals with or without visible tissue cysts (subclinically infected cattle) (Frey et al., 2013; García-Lunar et al., 2013a). For this purpose, a number of serological methods including IFAT, ELISA, western blot (WB) and direct agglutination tests have been devel-

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oped (Cortes et al., 2006; Fernández-García et al., 2009a, 2010; Schares et al., 2010, 2013; Waap et al., 2011). However, previously validated ELISA tests that are frequently employed in diagnosis and epidemiological studies have shown false-positive results that may significantly influence prevalence studies and control (Schares et al., 2010; Nasir et al., 2012; Gazzonis et al., 2014). Moreover, Uzeda et al. (2014) also reported the presence of Besnoitia false-positive results by IFAT. Therefore, the WB test is currently recommended as a confirmatory assay (García-Lunar et al., 2013a).

False-positive reactions have been suggested to be due to cross-reactions with related apicomplexan parasites (Nasir et al., 2012; Gazzonis et al., 2014). Indeed, previous studies have identified shared antigens among members of the family Sarcocystidae *(Neospora caninum, Toxoplasma gondii* and *B. besnoiti)* (Zhang et al., 2011; García-Lunar et al., 2013b). In particular, *N. caninum* and *Sarcocystis* spp. are closely related parasites, and both infections are highly prevalent in cattle worldwide (Dubey et al., 1989; Dubey, 2003). Over 90% of adult cattle have been reported to be infected with *Sarcocystis cruzi* in many countries (Moré et al., 2008). However, serological cross-reactions between *Sarcocystis* spp. that affect cattle (*S. cruzi, S. hirsuta, S. hominis* and *S. rommeli*) (Dubey and Lindsay, 2006; Dubey et al., 2015) and *B. besnoiti* have never been studied. Shkap et al. (2002) determined that at low IFAT dilutions, anti-*N. caninum* sera reacted with *B. besnoiti* antigens in some individual samples. This finding may have important implications for the diagnosis as Bartels et al. (2006) reported high seroprevalence rates of *N. caninum* infection in several European countries.

The aim of the present study was to investigate the origin of *B. besnoiti* false-positive ELISA results using an appropriate sera panel. For this purpose, the association between *B. besnoiti* false-positive ELISA results obtained with an in house ELISA and the presence and level of anti-*Sarcocystis* spp. and/or *N. caninum*-specific antibodies were studied. We considered WB as a reference test for *Sarcocystis* spp. infection diagnosis. Thus, we firstly determined the pattern of *Sarcocystis* spp. cystozoites antigen recognition. The presence and level of anti-*Sarcocystis* spp. and/or -*N. caninum* antibodies were then investigated.

2. Material and methods

2.1. Experimental design and serum samples

A total of 315 sera samples were included in the present study and were classified into five different groups (1, 2, 3, 4 and 5). Groups 1, 2 and 3 were established based on the results obtained with a *B. besnoiti* in house ELISA based on a soluble tachyzoite extract (Fernández-García et al., 2010) and a *B. besnoiti* tachyzoite-based WB performed under non-reducing conditions (García-Lunar et al., 2013a). For both assays, BbSpain-1 isolate was employed (Fernández-García et al., 2009b). WB was considered as gold standard (García-Lunar et al., 2013a). Groups 1 and 2 were composed of sera from *B. besnoiti*-seronegative and -seropositive animals, respectively, according to both ELISA and WB analysis. Group 3 comprised sera from *B. besnoiti*-seronegative animals by WB with a false-positive ELISA result. Groups 4 and 5 corresponded to reference sera representative of *Sarcocystis* spp. and *N. caninum* infections, respectively.

In all groups, the presence of anti-*Sarcocystis* spp. antibodies was determined using WB and the anti-*Sarcocystis* spp. specific antibody level was determined by IFAT as described in Sections 2.3 and 2.4, respectively. The presence of anti-*N. caninum* antibodies was determined using WB, whereas ELISA was employed for determining the anti-*N. caninum* specific antibody level (Álvarez-García et al., 2002, 2003).

2.1.1. Group 1: Sera from B. besnoiti-seronegative cattle

The sera from 75 cows and heifers from dairy and beef herds located in central Spain ($n = 20$) (Fernández-García et al., 2010), Italy ($n = 18$) (Gazzonis et al., 2014), Argentina ($n = 20$) and Mexico ($n = 17$) were included in the study. A total of 56 serum samples came from herds with no history of bovine besnoitiosis, and the 75 animals showed any compatible sign of besnoitiosis. All sera were seronegative for *B. besnoiti* infection by both WB and ELISA.

2.1.2. Group 2: Sera from B. besnoiti-seropositive cattle

A total of 66 samples came from cows and heifers from Spanish beef herds with a previous history of bovine besnoitiosis. The herds were located in areas were bovine besnoitiosis is endemic (Gutiérrez-Expósito et al., 2015) and epidemic (Fernández-García et al., 2010). No data regarding clinical signs were available for any of the animals sampled. However, all sera were seropositive for *B. besnoiti* infection by both WB and ELISA.

2.1.3. Group 3: Sera from B. besnoiti-seronegative cattle with a false-positive ELISA result

A total of 96 sera from adult dairy and beef cattle from Argentina ($n = 6$) and Italy ($n = 22$) (Gazzonis et al., 2014) and from dairy cattle from Mexico ($n = 16$) and Spain ($n = 52$) were included in the study. No animals showed any compatible sign of besnoitiosis, and the animals came from herds with no history of bovine besnoitiosis. All sera were positive for *B. besnoiti* infection by ELISA but negative by WB.

2.1.4. Group 4: Sera from S. cruzi-seropositive cattle

Sera from 42 Argentinean heifers and cows naturally infected with *S. cruzi* that showed microscopic thin-walled tissue cysts in myocardium and with IFAT titers $\geq$1:100 (Moré et al., 2008) were included in the study. All sera were seronegative to *B. besnoiti* by both WB and ELISA and seronegative to *N. caninum* by both WB and ELISA (Álvarez-García et al., 2002, 2003). All samples were employed to describe the pattern of *Sarcocystis* spp. cystozoite antigens by WB.

2.1.5. Group 5: Sera from N. caninum-seropositive cattle

Sera from 36 dairy cattle present in a herd with a history of *N. caninum*-associated abortions were analyzed. The herd comprised 200 cows and had an intra-herd seroprevalence of 85% and a 9.2% annual abortion rate. All samples were seronegative for *B. besnoiti* by both WB and ELISA and seropositive for *N. caninum* by ELISA and WB.

2.2. Parasites

Sarcocystis spp. cystozoites were obtained from naturally infected bovine hearts and were purified according to a previously described procedure (Moré et al., 2011). Briefly, 100 g of minced myocardium were mixed with 400 ml of digestion solution (2.5% pepsin, 1% HCl) and were placed in a magnetic stirrer for 20 min at 37 °C. The suspension was filtered through 300, 150, and 53 μm sieves into 50 ml centrifuge tubes and centrifuged at 500 × *g* for 5 min and it was centrifuged at 500 × *g* for 5 min. The supernatant was removed and the pellet was re-suspended in 30 ml PBS, 13.5 ml of isotonic Percoll® (GE Healthcare) and 1.5 ml saline solution (1.5 M NaCl) and centrifuged (4,000 × *g*, 10 min) (Pertoft et al., 1980). Supernatants and the upper layer of the sediment were discarded; the pellet was washed three times with PBS. Pellets with zoites were frozen at −80 °C until use for WB or resuspended in PBS to a final concentration of approximately 5×10^5 cystozoites per ml and formalin-fixed for their use in IFAT (Moré et al., 2008; Fernández-García et al., 2009a).

Tachyzoites from the Nc-1 isolate of *N. caninum* (Dubey et al., 1988) were grown in a Marc-145 cell monolayer with DMEM (Thermo Fisher Scientific) supplemented with 2% fetal calf serum (Thermo Fisher Scientific) and were purified following a previously described procedure (Pérez-Zaballos et al., 2005). Tachyzoites were pelleted by centrifugation at 1,350 *xg* 15 min and stored at −80 °C until used for ELISA and WB.

2.3. SDS-PAGE and WB

A total of 2×10^7 tachyzoites under reducing conditions and 10^7 cystozoites under non-reducing conditions were employed for *N. caninum* and *Sarcocystis* spp. electrophoresis, respectively. Membrane coating and WB were performed following a previously described method (Álvarez-García et al., 2002) in a 15% polyacrylamide gel. Images from WB membranes were obtained using a GS-800 Scanner (Bio-Rad Laboratories, CA, USA) and were analyzed with Quantity One quantification software v. 4.0 (Bio-Rad Laboratories, CA, USA).

2.4. Sarcocystis *spp. IFAT*

Sera were analyzed by *Sarcocystis* spp. cystozoite IFAT in double serial dilutions starting at 1:100 following a previously described method (Moré et al., 2008; Fernández-García et al., 2009a). Peripheral but not apical fluorescence was considered specific.

2.5. N. caninum *in-house ELISA*

ELISA was performed following a previously described procedure (Álvarez-García et al., 2003). The optical density (OD) of each serum sample was converted into a relative index percent (RIPC) using the following formula: RIPC = [(OD405 sample—OD405 negative control)/(OD405 positive control–OD405 negative control)] × 100. Samples with RIPC < 6.2 were considered negative; samples with RIPC = 6.2–8.2 were considered doubtful, and samples with RIPC > 8.2 were considered positive.

2.6. Analysis of data

To determine the pattern of recognition of *Sarcocystis* spp. cystozoite antigens by WB, molecular weights of antigens were compared with the molecular weight marker and were calculated for each sample analyzed using Quantity One Software (Bio-Rad). The frequency of recognition was expressed as the percentage of animals detecting each antigen. An antigen was considered to be an immunodominant antigen (IDA) when it was recognized by more than 50% of samples analyzed.

The association between *B. besnoiti* ELISA false-positive results and the presence of anti-*N. caninum* and -*Sarcocystis* spp. antibodies by WB was estimated by using Chi-square test and by estimating the odds ratio value (OR). A Student's *t*-test was used to compare differences in the anti-*Sarcocystis* spp. or -*N. caninum* antibody levels estimated by ELISA and IFAT, respectively, between sera with *B. besnoiti* ELISA false-positive results and sera with no false-positive results. For this purpose, *Sarcocystis* spp. IFAT titers were expressed as $\log_2$ of the reciprocal of the last serum dilution that completely recognized the surface of the cystozoite. *P* values < 0.05 were considered statistically significant. Test agreement (expressed as Kappa-values) was calculated using WinEpiscope 2.0 (http://www.winepi.net/).

3. Results and discussion

Given the re-emergence of bovine besnoitiosis in Europe and the high prevalence rates recently reported in endemic areas, the implementation of control measures is urgently needed (reviewed by Álvarez-García et al., 2014b). In this scenario, serological diagnosis is crucial for detecting subclinical infected animals that may remain parasite carriers (reviewed by Álvarez-García et al., 2014b). However, recent studies have reported that routinely used commercial and in house ELISAs may produce between 18% and 30% false-positive results (Nasir et al., 2012; Gazzonis et al., 2014). Moreover, *Besnoitia*-positive IFAT results did not yield conclusive results by WB when Brazilian cattle were tested (Uzeda et al., 2014).

Thus, in the present study, the origin of false seropositive reactions in an in house ELISA was investigated for the first time. An appropriate panel of sera was employed and was composed of three categories that represented *B. besnoiti*-seronegative cattle with either an ELISA-negative or -positive result (false-positive reactors) and seropositive cattle. Since *N. caninum* and *Sarcocystis* spp. infections are highly prevalent in cattle worldwide, we included two additional categories representative of seropositive cattle to both closely related protozoa. On the other hand, cross-reactions with *T. gondii* were not studied herein since in previous studies of development and validation of *B. besnoiti* serological assays cross-reactions between anti-*T. gondii* specific antibodies and *B. besnoiti* antigens were hardly observed (Fernández-García et al., 2010; Schares et al., 2011, 2013; García-Lunar et al., 2013a). In contrast, optical density values close to the cut off points were commonly observed in *B. besnoiti* ELISAs with sera positive to *N. caninum* infection (Schares et al., 2011, 2013; García-Lunar et al., 2013a). Moreover, there are a few seroprevalence studies of *T. gondii* in cattle and the relevance of this parasitic infection in cattle remains to be further clarified. To classify sera as *Sarcocystis* spp.-seropositive or -seronegative in our study, WB was used as the reference technique as in previous studies with other closely related protozoa (Álvarez-García et al., 2002; García-Lunar et al., 2013a; Howe et al., 2014), whereas IFAT was employed for determining specific antibody levels.

Among a total of 26 *S. cruzi* cystozoite antigens recognized, 8 antigens were intensively recognized by reference sera from *S. cruzi* naturally infected cattle, which were located in 3 main antigenic regions: 55 kDa, 23–29 kDa (composed of 28.9, 27.2, 25.5, 23.9 kDa bands) and 18–20 kDa (composed of 20.8, 19.3, 18.4 kDa bands) (Fig. 1A). A serum was considered to be *Sarcocystis* spp. seropositive when at least the 18–20 kDa antigenic area was intensively recognized because it was detected in 100% of the infected animals (Fig. 1). A previous study carried out by Granstrom et al. (1990) described the immunodominant antigens of *S. cruzi* cystozoites and 20 bands, ranging from approximately 22–250 kDa, were consistently detected. However, these results are hardly comparable because 10% acrylamide gels were employed for the assays and antigens below 22 kDa were not visualized. Whether the antigens described in the present study are shared antigens among other species of *Sarcocystis* needs future clarification.

It is unclear whether WB or IFAT should be considered the gold standard for the detection of *Sarcocystis* spp.-specific antibodies. There have been scant studies on the serological diagnosis of *Sarcocystis* spp. infection in cattle. Moré et al. (2008) have described IFAT at low dilutions to be a suitable method for diagnosing *S. cruzi*-infected cattle. Cross-reactivity at low IFAT titers, however, has been widely described among other Sarcocystidae parasites (Shkap et al., 2002; Schares et al., 2010). In this study, a better agreement between IFAT and WB was obtained with a cut-off titer of 1:200 (77% sensitivity (Se); 79% specificity (Sp); $k = 0.5$) compared with a cut off titer of 1:100 (98% Se; 37.7% Sp; $k = 0.4$)". The use of *Sarcocystis* spp. cystozoite based WB as the gold standard in this work may have resulted in a substantial loss of Se. However, this approach as well as the restrictive WB seropositive criterion employed here may have resulted in high Sp. Unfortunately, due to the low number of reference sera from *S. cruzi* seropositive and seronegative cattle, the Se and Sp values of these tests could not be determined. Simi-

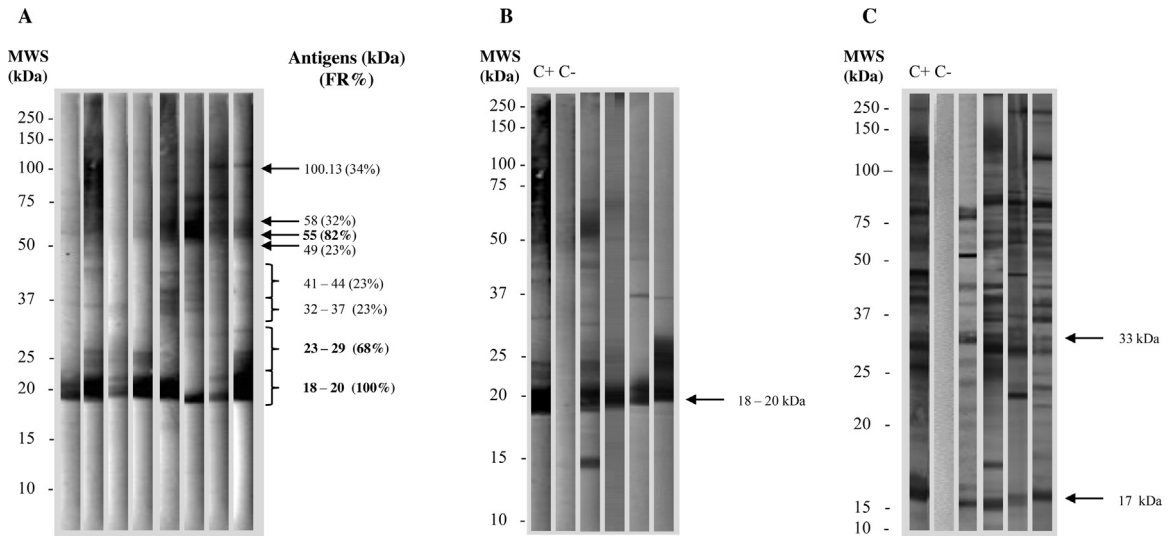

Fig. 1. Pattern of recognition of *Sarcocystis* spp.-cystozoite and *N. caninum*-tachyzoite antigens by a panel of reference sera. A: The pattern of recognition of *Sarcocystis* spp. cystozoite antigens by sera from naturally infected cattle (group 4). Antigens are shown along the right side, and the frequency of antigen recognition is shown in parenthesis (FR%). Immunodominant antigens (IDAS) are shown in bold. B: The pattern of recognition of *Sarcocystis* spp. cystozoite antigens by positive (C+) and negative (C−) control serum samples and by sera showing a *B. besnoiti* false-positive ELISA result (group 3) and seropositive for *Sarcocystis* spp. infection. The 18–20 kDa antigenic region is shown with an arrow. C: The pattern of recognition of *N. caninum* tachyzoite antigens by positive (C+) and negative (C−) control serum samples and sera showing a *B. besnoiti* false-positive ELISA result and that are seropositive for *N. caninum* infection. The 17 and 33 kDa *N. caninum* IDAS are shown with an arrow.

larly, a moderate agreement between IFAT, WB and a modified WB were observed by Duarte et al. (2003) for the diagnosis of *S. neurona* infection in equids. In this study performed by Duarte et al. (2003), the results of the operating characteristic (ROC) analysis determined that the overall accuracy of IFAT was higher than that of the WBs analyzed. In this sense, the existence of cross-reactivity with other species of *Sarcocystis* using WB has been described and its Sp depends on a careful interpretation of immunoreactive bands and/or modification of assay conditions (Arias et al., 2012).

Specific antibodies against *Sarcocystis* spp. and *N. caninum* were found in all categories examined (Fig. 1B–C, Table 1). However, 31.7% of the analyzed samples showed anti-*Sarcocystis* spp.-specific antibodies, whereas only 8.9% were seropositive for *N. caninum*. Notably, the category of *B. besnoiti* ELISA false-positive reactors showed the highest number of sera with specific antibodies directed against both *Sarcocystis* spp. and *N. caninum* (74%), followed by the *N. caninum*-seropositive animal category (52.8%) (Table 1). In contrast, few *B. besnoiti*-seronegative and -seropositive animals (categories 1 and 2, respectively) showed specific antibodies to both *N. caninum* and *Sarcocystis* spp. (10.7% and 1.5%, respectively).

The existence of *B. besnoiti* false-positive ELISA results was significantly associated with either *N. caninum* and/or *Sarcocystis* spp. seropositivity (93.8%; 90/96). The highest association was found between *Besnoitia* false-positive ELISA results and seropositive results to both *Sarcocystis* spp. and *N. caninum* infections (χ^2: 78.36; $p < 0.0001$; OR = 34.65; 95% CI: 13.6–88.5). There was also a significant association with the presence of anti-*Sarcocystis* spp. antibodies, regardless of the *N. caninum* result (χ^2: 29.90; $p < 0.0001$; OR = 5.56; 95% CI: 2.9–10.6). However, seropositivity to *Sarcocystis* spp. only was not associated with a *B. besnoiti* false-positive ELISA result (χ^2: 0.91; $p = 0.34$; OR = 1.86; 95% CI: 0.67–5.2). This finding may be related to the restrictive WB seropositive criterion employed, resulting in a substantial loss of Se as mentioned above (Duarte et al., 2003). Regarding *N. caninum* infection, *B. besnoiti* false-positive ELISA results were also associated with seropositive *N. caninum* results, regardless of *Sarcocystis* spp. results ($\chi 2$: 93.72; $p < 0.0001$; OR = 15.03; 95% CI: 8.2–27.5). Moreover, only *N. caninum* seropositive results were also associated with *B. besnoiti* false-positive ELISA results ($\chi 2$: 5.35; $p = 0.02$; OR = 4.56; 95% CI: 1.4–15). This result is in accordance with the findings of Schares et al. (2011), who previously observed the presence of ELISA cross-reactions between *B. besnoiti* and anti-*N. caninum* antibodies while evaluating the Sp of a commercial ELISA test for the diagnosis of bovine besnoitiosis.

Specific antibody levels against *Sarcocystis* spp. and *N. caninum* were also measured by IFAT and ELISA, respectively (Table 2). Sera showing discordant results between serological techniques (IFAT and WB for *Sarcocystis* spp., and ELISA and WB for *N. caninum)* were not included in the study and were more numerous for *Sarcocystis* spp. infection diagnosis ($n = 76$). On the other hand, only 20 samples showed discordant results for *N. caninum* infection. In general terms, *Sarcocystis* spp.-seropositive samples showed low IFAT antibody levels because approximately 90% sera showed IFAT titers equal to or lower than 1:200 (groups 1, 2, 4 and 5). Interestingly, those sera with a *B. besnoiti* false-positive ELISA result (group 3) showed higher antibody levels (more than 27% of the samples had *Sarcocystis* spp. IFAT titers equal or higher than 1:400). Our results contrast with those reported by Moré et al. (2008), where most Argentine cattle showed anti-*S. cruzi* antibody levels equal to or higher than 1:200. However, differences in the performance of the assays may explain these discrepancies. Regarding *N. caninum* infection, almost 50% of sera showing a *B. besnoiti* false-positive ELISA result had RIPC values higher than 40%, whereas between 80 and 100% of sera from groups 1, 2 and 4 proved to be seronegative. As expected, significantly higher specific antibody levels against *Sarcocystis* spp. and *N. caninum* corresponded to sera from group 3 (*B. besnoiti* seronegative animals with a positive ELISA result) ($p < 0.05$ *t*-test). These results showed that sera with high antibody levels anti-*N. caninum* and anti-*Sarcocystis* spp. are more likely to show *B. besnoiti* false-positive ELISA results. Our results agree with those reported in the study carried out by Shkap et al. (2002), in which cross-reactivity at low IFAT dilutions (1:16 and 1:64) was found only between two sera showing high anti-*N. caninum* antibody levels (1:3,200) and *B. besnoiti* antigen. Several *B. besnoiti* antigens responsible for these cross-reactions have recently been identified by 2-DE SDS-PAGE. Up to 25 *B. besnoiti*-cross-reactive antigens were recognized by a pool of sera from *N.*

Table 1
Detection of anti-*Sarcocystis* spp. and/or anti-*N. caninum* antibodies by WB.

Panel of reference sera	N° of sera	Seropositive to *Sarcocystis* spp. (%)	Seropositive to *N. caninum* (%)	Seropositive to *N. caninum* and *Sarcocystis* spp. (%)	Seronegative to *N. caninum* and *Sarcocystis* spp.(%)
Sera from *B. besnoiti*-seronegative cattle	75	20 (26.7%)	4 (5.3%)	8 (10.7%)	43 (57.3%)
Sera from *B. besnoiti*-seropositive cattle	66	26 (39.4%)	1 (1.5%)	1 (1.5%)	38 (57.6%)
Sera from *B. besnoiti*-seronegative cattle with a false-positive ELISA result	96	12 (12.5%)	7 (7.3%)	71 (74%)	6 (6.2%)
Sera from *S. cruzi*-seropositive cattle	42	42 (100%)	0	0	0
Sera from *N. caninum*-seropositive cattle	36	0	17 (47.2%)	19 (52.8%)	0
Total	315	100 (31.7%)	28 (8.9%)	99 (31.4%)	88 (27.9%)

Table 2
Distribution of anti-*Sarcocystis* spp. and anti-*N. caninum* specific antibodies according to IFAT titers and ELISA relative index percent (RIPC) values, respectively.

	Sarcocystis spp. IFAT titres (%)					Total
Panel of reference sera[a]	<1:100	1:100	1:200	1:400	1:800	
Sera from *B. besnoiti*-seronegative cattle	24 (46.2%)	7 (13.5%)	15 (28.8%)	6 (11.5%)	0	52
Sera from *B. besnoiti*-seropositive cattle	0	5 (20%)	16 (64%)	4 (16%)	0	25
Sera from *B. besnoiti*-seronegative cattle with a false-positive ELISA result	5 (5.7%)	15 (17.2%)	41 (47.1%)	24 (27.6%)	2 (2.3%)	87
Sera from *S. cruzi*-seropositive cattle	0	14 (33.3%)	24 (57.1%)	4 (9.5%)	0	42
Sera from *N. caninum*-seropositive cattle	14 (42.4%)	2 (6.1%)	14 (42.4%)	3 (9.1%)	0	33
	N. caninum ELISA RIPCS (%)					Total
Panel of reference sera[b]	<6.2	>8.2 ≤20	>20 ≤40	>40 ≤100	>100	
Sera from *B. besnoiti*-seronegative cattle	60 (83.3%)	4 (5.6%)	3 (4.2%)	5 (6.9%)	0	72
Sera from *B. besnoiti*-seropositive cattle	61 (100%)	0	0	0	0	61
Sera from *B. besnoiti*-seronegative cattle with a false-positive ELISA result	7 (8.3%)	8 (9.5%)	23 (27.4%)	41 (48.8%)	5 (6%)	84
Sera from *S. cruzi*-seropositive cattle	42 (100%)	0	0	0	0	42
Sera from *N. caninum*-seropositive cattle	0	5 (13.9%)	14 (38.9%)	15 (41.7%)	2 (5.6%)	36

[a] Sera samples showing discordant results between *Sarcocystis* spp. cystozoite based IFAT and WB under non reducing conditions were not included in the statistical analysis.
[b] Sera samples showing doubtful ELISA results (≥6.2 ≤8.2) and sera showing discordant results between *N. caninum*-tachyzoite-based-ELISA and WB were not included in the statistical analysis.

caninum-infected cattle and some of them were identified to be highly conserved enzymes involved in metabolism, such as heat shock protein 60 and 90, fructose 1-6 bisphosphatase aldolase, enolase and actin (García-Lunar et al., 2013b). When Schares et al. (2013) removed cross-reacting antigens with *N. caninum* during the development of an enriched membrane extract for ELISA, this new assay showed higher Se and Sp values using a serum panel that included 20 sera with a *B. besnoiti* false-positive and doubtful ELISA result (Nasir et al., 2012). In the closely related protozoa *T. gondii* and *N. caninum*, several antigens located on the surface of tachyzoites also improved the Se and Sp of the serological assays (Schares et al., 1999; Montoya and Liesenfeld, 2004; Dubey and Schares, 2006; Petersen and Liesenfeld, 2007).

4. Conclusion

In conclusion, this study revealed that sera with high antibody levels anti-*N. caninum* and anti-*Sarcocystis* spp. are more likely to show *B. besnoiti* false-positive in house ELISA results. This finding explains why only some animals co-infected with either *Sarcocystis* spp. and/or *N. caninum* may be *Besnoitia* false-positive reactors, taking into account that *Sarcocystis* spp. infection is present in almost 100% of cattle and that *N. caninum* infection is also highly prevalent depending on the region or country studied (Reichel et al., 2013). As the *B. besnoiti* in house ELISA employs a soluble tachyzoite extract, similar results could be obtained with other ELISAs based on similar antigenic extracts that already showed false positive results (Nasir et al., 2012). Thus, based on the results obtained herein, a higher number of sera from cattle infected with *Sarcocystis* spp. and or *N. caninum* should be included in the validation of serological assays to diagnose *B. besnoiti* infection. However, further comparative validation studies are essential for updating the diagnostic characteristics of all the available ELISA assays. Finally, the impact that *B. besnoiti* seropositivity may have on the diagnosis of bovine neosporosis should be the subject of further research.

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