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Combination of monoclonal antibodies improves immunohistochemical diagnosis of *Neospora caninum*

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ABSTRACT

Histological analysis is commonly used for a conclusive diagnosis of neosporosis. Immunohistochemistry (IHC) using monoclonal (mAb) and polyclonal (pAb) antibodies can improve diagnosis; however, the use of pAb may induce cross-reactivity with other related parasites. The aims of this study were to compare the performance of mAbs and their combinations with that of pAb in IHC and evaluate the usefulness of mAb to identify *Neospora caninum* infection in aborted bovine fetal tissues. For this purpose, mAbs targeting NcSRS2 (4.15.15) or NcGRA7 (4.11.5 and 1/24-12) and one pAb collected from a rabbit inoculated with *N. caninum* tachyzoites were tested by IHC. Artificial standardized tissue sections were prepared as positive controls using homogenized bovine brain spiked with cultured tachyzoites of *N. caninum*. The numbers of labeled parasites were counted in each positive control section. In addition, four equal proportional combinations of the mAbs were also analyzed in the IHC. Finally, the pAb and the best combination of mAbs obtained in the positive control experiments were tested with tissue sections of naturally-infected cattle. To confirm analytical specificity, mAbs and a pAb were tested with *Toxoplasma gondii* and *Besnoitia besnoiti* positive control slides and tissues sections from naturally infected cattle containing *Sarcocystis* spp. and *B. besnoiti* antigens. The mAb 4.15.15 detected 57% of the total parasites in sections while 4.11.5 and 1/24-12 were able to detect 49% and 41%, respectively. For the mAb combinations (I: 1/24-12 + 4.11.5, II: 1/24-12 + 4.15.15, III: 4.15.15 + 4.11.5, IV: 1/24-12 + 4.11.5 + 4.15.15), the detection capacity was 32.4%, 79.4%, 66.6% and 60.7% for each combination, respectively. The best mAb combination (1/24-12 and 4.15.15) and the pAb serum detected 100% (18/18) of naturally-infected animals. *Sarcocystis* spp. or *B. besnoiti* were not detected by mAb combinations in IHC, however the pAb cross-reacted with *Sarcocystis* spp. cysts. These results confirm the usefulness of mAb application in IHC to *N. caninum*.

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1. Introduction

Neospora caninum is a protozoan parasite first described in dogs (Bjerkas et al., 1984) and cattle (O'Toole and Jeffrey, 1987; Parish et al., 1987). It was named in the same decade after a retrospective examination of dog tissues (Dubey et al., 1988a), and *in vitro* isolated from canine puppies with neurological disorders (Dubey et al., 1988b). Dogs (*Canis familiaris*), coyotes (*Canis latrans*), Australian dingoes (*Canis lupus dingo*) and gray wolves (*Canis lupus*) have been identified as definitive hosts of the parasite (McAllister et al., 1998; Gondim et al., 2004; King et al., 2010; Dubey et al., 2011). Neosporosis is considered the major cause of abortion in cattle, leading to significant economic losses in animal production (Thurmond and Hietala, 1997; Trees et al., 1999; Corbellini et al., 2002; Tiwari et al., 2007).

There is a wide range of diagnostic tests to identify *N. caninum* infection in cattle; however, fetal histopathology of brain and other organs such as heart, liver and lung has been proved to be an important tool to identify the parasite or characteristic lesions caused by it (Wouda et al., 1997; Pereira-Bueno et al., 2003; Dubey and Schares, 2006; Pescador et al., 2007).

Histopathology is considered a necessary technique to diagnose neosporosis, however, identification of *N. caninum* in tissue sections stained by haematoxilin–eosin (H&E) is difficult because, even in well preserved tissues, the number of parasites in bovine fetuses is normally low (Dubey et al., 2006) and, *N. caninum*-characteristic lesions are not always related to the presence of parasites (Boger and Hattel, 2003). In some situations, autolysed tissues may impair the identification of lesions and associated parasites (Dubey and Schares, 2006).

By immunohistochemical staining (IHC) it is possible to identify immunogenic epitopes and demonstrate the number, distribution and localization of pathogens in tissue sections (Haines and West, 2005; Ramos-Vara et al., 2008). IHC targeted to *N. caninum* was first performed by Lindsay and Dubey (1989), and can improve histopathological assessment, because the identification of parasites by conventional H&E staining is not reliable (Dubey and Schares, 2006), especially when *N. caninum*-characteristic lesions are not observed (Sondgen et al., 2001; Boger and Hattel, 2003).

Both monoclonal (mAb) and polyclonal (pAb) antibodies can be employed in IHC. The former usually binds to a specific epitope, providing a greater analytical specificity, while pAb can recognize several epitopes of the same pathogen (Ramos-Vara et al., 2008). In spite of its sensitivity in IHC examinations for *N. caninum*, pAb can produce cross-reactivity with other cyst-forming parasites as *Toxoplasma gondii* (McAllister et al., 1996; Sundermann et al., 1997; van Maanen et al., 2004) and *Sarcocystis cruzi* in IHC (Peters et al., 2000).

The mAb technology has been widely used in diagnostic tests to detect *N. caninum* infection (Cole et al., 1994; Baszler et al., 1996, 2001; Bjorkman and Hemphill, 1998; Schares et al., 1999; Srinivasan et al., 2006; Aguado-Martinez et al., 2010; Sohn et al., 2011). However, the usefulness and reliability of mAbs in IHC using naturally-infected tissue were not evaluated yet.

The aims of this study were to compare the performance of murine mAbs and their combinations with that of pAb in IHC and evaluate the usefulness of mAb to identify *N. caninum* infection in aborted bovine fetal tissues.

2. Materials and methods

2.1. Monoclonal and polyclonal antibodies

Murine monoclonal antibodies targeted to *N. caninum* tachyzoite and bradyzoite antigens were selected for this study. Three mAbs that react to *N. caninum* tachyzoites were used: 4.11.5, typed as IgG2 (Schares et al., 1999), 1/24-12, typed as IgG2b (Aguado-Martinez et al., 2010) and 4.15.15 typed as IgG2a (Schares et al., 2000). Both mAbs, 4.11.5 and 1/24-12 react against a 33 kDa-granule dense protein (NcGRA7) while mAb 4.15.15 (IgG2a) recognizes an epitope on a 38 kDa *N. caninum* tachyzoite surface protein (Schares et al., 1999). Hybridoma cells were maintained in serum free medium PANSERIN^{TM} 401 (PAN biotech GmbH, Germany) supplemented with 1% of a vitamin solution (Biochrom, Berlin, Germany) and non-essential amino acids solution (Biochrom, Berlin, Germany), and split twice a week. The supernatant of the culture was filtered using a 0.2 μm membrane filter (Sartorius, Göttingen, Germany) and stored at 8 °C until used.

Equal volume combinations of mAbs were prepared to evaluate whether their detection capacity could be increased. Combinations were named I to IV as: I: 1/24-12 + 4.11.5, II: 1/24-12 + 4.15.15, III: 4.15.15 + 4.11.5 (1/2 of each one) and IV: 1/24-12 + 4.11.5 + 4.15.15 (1/3 of each one).

Rabbit pAb sera obtained after inoculation with tachyzoites of *N. caninum* NC-1 (Dubey et al., 1988b), *T. gondii* (generous gift of J.P. Dubey) and *Besnoitia besnoiti* Bb-GER1 (Basso et al., 2011) were used in this study.

2.2. Parasites

N. caninum tachyzoites of the NC-1 strain (Dubey et al., 1988b) and the RH strain (Sabin, 1941) of *T. gondii* were maintained by continuous passages in VERO cells. The Portuguese Bb1Evora03 strain of *B. besnoiti* (Cortes et al., 2006) was maintained in MARC-145 cells. All parasites were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% of vitamin, non-essential amino acids, L-glutamine, penicillin–streptomycin and 5% fetal calf serum (all from Biochrom, Berlin, Germany) at 37 °C and 5% CO_2. Parasites were harvested and purified as previously described (Schares et al., 1999).

2.3. N. caninum positive controls and tissue blocks

For the quantitative assessment of immunohistochemical reactions, a standardized positive control tissue was prepared with a known number of parasites, which consisted of 2 g of bovine negative brain mixed in a tissue grinder with 10^7 tachyzoites of each parasite. A dialysis tube (diameter: 6-mm) was filled with the tachyzoite-brain mixture which was strictly homogenized to avoid the inclusion of air bubbles, closed and fixed in 10% buffered

Table 1
Tissue fragments from naturally-infected bovine fetuses with diagnosis of neosporosis which were used as positive controls.

Animal	Number of tissue fragments per animal						
	CNS[a]	Liver	Lung	Kidney	SM[b]	Heart	Total
1	1	2	4	1	2	0	10
2	2	1	0	1	2	0	6
3	0	0	1	1	0	1	3
4	2	1	1	1	1	1	7
5	2	1	2	1	1	1	8
6	1	1	1	1	0	0	4
7	1	1	1	1	0	0	4
8	1	1	1	1	0	0	4
9	0	1	1	1	0	0	3
10	1	1	1	1	0	0	4
11	0	1	1	1	1	1	5
12	1	1	1	1	0	0	4
13	0	1	1	1	0	0	3
14	1	0	0	0	0	0	1
15	0	0	0	0	1	1	2
16	1	1	1	1	0	0	4
17	1	0	0	0	0	0	1
18	0	1	1	1	0	0	3
Total	15	15	18	15	8	5	76

[a] Central nervous system.
[b] Skeletal muscle.

formalin for three days before embedded in paraffin. From this artificial control, tissue sections were prepared to be employed in IHC.

For the validation of IHC reactions in non-experimental tissues (naturally-infected fetuses), 20 paraffin tissue blocks from Brazil and 34 from Spain were analyzed. Tissues had been collected from 18 *N. caninum*-naturally infected cattle. Diagnosis of neosporosis was confirmed by histopathological evaluation (Brazilian and Spanish blocks), immunohistochemistry (Brazilian blocks) and PCR (Spanish blocks). Bovine tissue sections included brain ($n = 15$), liver ($n = 15$) lung ($n = 18$), kidney ($n = 15$), skeletal muscle ($n = 8$) and heart ($n = 5$) (Table 1). Tissue sections from naturally-infected animals were tested with pAb and with a mAb combination identified as being optimal in IHC with positive artificial control tissues. All experiments were performed in triplicates. Reactions were considered positive if at least one of three sections showed labeled structures consistent with tachyzoites or cysts. Paraffin blocks containing brain tissue sections (CNS) from three *N. caninum* naturally-infected dogs from Germany were also used in the tests.

2.4. Standardization of antigen retrieval

To choose the method of antigen retrieval (AR), three sets of four sections of standardized positive control tissues were employed in IHC with the pAb and the different mAbs (Ramos-Vara et al., 2008). The first set was used without AR, the second one with enzymatic digestion (0.2% Pepsin-HCl) and the third one with heat-induced AR (citrate buffer pH 6.0 in microwave at 480 W for 10 min). After counting the parasites in each section, the average number of parasites was calculated for each treatment.

2.5. Immunohistochemistry (IHC)

Five-μm-thick paraffin embedded standardized positive control and natural tissue sections were de-waxed in Roti-histol® (Carl Roth, Karlsruhe, Germany) and Xylene for 25 min and rehydrated through graded ethanol. The endogenous peroxidase was quenched by immersion in 0.3% H_2O_2 in distilled water for 20 min. Antigen was retrieved followed by a blocking step employing a commercial fetal horse serum (Biowest, France) for the tests using pAb or mAb. The blocking serum was previously tested negative by immunoblotting (IB) with total non-reduced antigen of *N. caninum*, *T. gondii* and *B. besnoiti* essentially as described, but using an anti-horse-IgG Peroxidase conjugate (Schares et al., 1998; Azevedo et al., 2010; Schares et al., 2010). Slides were treated with hybridoma supernatants (mAbs) or pAb diluted at 1:1000 (anti-*N. caninum* and anti-*B. besnoiti* sera) and 1:8000 (anti-*T. gondii* serum) overnight at room temperature. The antigen-antibody reactions were detected using the ABC technique with a commercial kit (Vectastain, Vector Laboratories Inc., Burlingame, CA, USA) following manufacturer's instructions. The slides were treated with diaminobenzidine (DAB) diluted in an imidazole buffer (0.01 M, pH 7.1) and counterstained with Papanicolau solution. Incubations in increasing concentrations of ethanol and xylene followed, and finally the slides were mounted in Entellan (Merck, Darmstadt, Germany) and visualized under an Olympus AHBT-3 microscope (Schares et al., 1999).

To evaluate the capacity of mAbs in detecting *N. caninum* tachyzoites, at least four standardized positive control sections were analyzed with mAbs separately, with mAb combinations, and with a *N. caninum* pAb. The number of detected parasites was determined by light microscopy in each treatment.

At least three serial sections from each block containing naturally-infected bovine tissue were tested with *N. caninum* pAb serum and an optimal combination of mAbs (combination with the best IHC results in standardized positive control tissues) to compare antibody performance to detect protozoan infection.

To evaluate the analytical specificity of mAbs and pAb, IHC was performed with *T. gondii* and *B. besnoiti* artificial positive control sections with four repetitions for each tested primary antibody. Additionally, tissues from naturally-infected cattle containing bradyzoite stages of *Sarcocystis* spp. and *B. besnoiti* were analyzed. At least two serial sections from each block were tested with *N. caninum* pAb serum and mAbs combinations.

As negative controls, sections of all samples were incubated with PBS/blocking serum or cell culture medium (PANSERIN™ 401) instead of the primary antibody, and sections of bovine non-infected brain were used with pAb or mAbs.

2.6. Immunoblotting (IB)

IB was performed to identify possible cross-reactions among pAb, mAbs and antigens from *T. gondii* and *B. besnoiti*. Prior to their use in IHC, all mAbs were tested by IB employing *N. caninum* antigen.

Total antigen lysate of *N. caninum* tachyzoites, *T. gondii* tachyzoites and *B. besnoiti* (tachyzoites and bradyzoites) were prepared, electrophoresed under reduced and non-reduced conditions, and electroblotted onto Immobilon-Polyvinylidene Difluoride (PVDF) membranes as previously described (Schares et al., 1999).

The blotted strips were cut and blocked with PBS, 0.05% Tween, 2% liquid fish gelatin (Serva, Heidelberg) for 30 min. To test the reactivity of each antibody, undiluted mAb and a 1:200 dilution of pAb were incubated at room temperature for 1 h with each membrane. After five steps of PBS-T washing, strips were incubated with a peroxidase-conjugate anti-mouse and anti-rabbit IgG/IgM (whole molecule, Dianova, Germany) in a 1:500 dilution for 1 h, followed by three and two washes with PBS-T and PBS, respectively. A 4-chloro-1-naphthol substrate (Sigma–Aldrich, USA) was used to visualize reactions. A commercial protein molecular weight marker (LMW marker, Amersham, UK) run under the same conditions was used to determine the relative molecular weights of the stained antigens.

2.7. Statistical analysis

To check if the number of parasites differ between AR methods, the Kruskall–Wallis one way analysis of variance was applied, since the values were not normally distributed as confirmed by the Shapiro–Wilk test.

The number of parasites detected by mAbs in combination and pAb were normally distributed as determined by the Shapiro–Wilk test; therefore, analysis of variance with Tukey test for multiple comparisons was used (Proc GLM, SAS software).

To compare the agreement between antibody detection of mAbs and pAb in naturally-infected tissues, *Kappa* was calculated. The significant level (alpha) of 5% was used in all statistical analysis.

3. Results

The analysis of different AR protocols revealed that heat-induced AR using citrate buffer yielded the best results. The medians of parasite numbers counted in IHC on the standardized positive control slides were different among the AR methods ($P < 0.05$; Fig. 1).

The capacities of various mAbs in detecting tachyzoites in standardized positive control sections in comparison to the capacity of the *N. caninum* pAb serum are illustrated in Fig. 2. The mAb 4.15.15 detected 57% of the total number of parasites in section while 4.11.5 and 1/24-12 detected 49% and 41%, respectively.

The detection capacities of four mAb combinations (I: 1/24-12 + 4.11.5, II: 1/24-12 + 4.15.15, III: 4.15.15 + 4.11.5 and IV: 1/24-12 + 4.11.5 + 4.15.15) in comparison to the detection capacity of pAb are shown in Fig. 3. The number of parasites detected by pAb was considered as reference. Combinations I, II, III and IV detected 32.4%, 79.4%, 66.6% and 60.7% of the tachyzoites detected by pAB in standardized positive tissue sections, respectively. The mAb combination which reached the best detection capacity (II) was used in the following experiments with naturally-infected fetal sections.

Table 2
Results of immunohistochemical analyses of tissue sections of naturally infected cattle using a polyclonal rabbit anti-*Neospora caninum* serum (pAb) or a combination of two monoclonal antibodies (mAb-Comb-II: mAb 1/24-12 + mAb 4.15.15).

No. of tissue sections	mAb-Comb-II Neg	mAb-Comb-II Pos	Total
pAb Neg	34	13	47
pAb Pos	10	19	29
Total	44	32	76

Neg = negative, Pos = positive.

The Tukey's test did not show statistically significant differences among the mAb combinations II, III and IV; however, when pAb was employed, the number of detected tachyzoites was significantly higher than in any of the mAb combinations ($P < 0.005$).

Tissue sections of all naturally-infected bovine fetuses tested positive using mAb or pAb as primary antibodies in IHC. The signal intensity was the same for both antibodies (Fig. 4). Overall, 32 (42%) of 76 tissues used in this study were considered positive in IHC with mAb combination II. Results for all tissues are described in Fig. 5 and in Table 2.

The level of agreement between the use of mAb combination and pAb in IHC, revealed by kappa value was 0.3712 ± 0.1143 (confidence interval of 95%). The observed concordance was 70%.

Cross-reactivity was not observed when mAb and pAb were tested with *T. gondii* or *B. besnoiti* tachyzoites by IHC when artificial control tissue was employed. Cross-reaction with these protozoa was detected by IB when pAb was used as primary antibody. The pAb reactions against antigens of *T. gondii* tachyzoites revealed almost seven bands (ranging from ~30 kDa until 97 kDa) and three bands (~50–70 kDa) under reducing and non-reducing conditions, respectively. Reactions were recorded with tachyzoites (four antigens between 14–66 kDa and one antigen of ~66 kDa under reducing and non-reducing conditions, respectively) and bradyzoites (four antigens among 20–66 kDa under reducing conditions) of *B. besnoiti*. Cross-reactivity was not seen in IB when mAb were employed as primary antibody. Cross-reactivity was detected by IHC when pAb was used in a heart bovine section with *Sarcocystis* spp. cysts (Fig. 6).

N. caninum in CNS of naturally infected dogs were detected by mAbs combination II and pAb (Fig. 7).

4. Discussion

In the present study three murine mAbs (1/24-12, 4.11.5 and 4.15.15) (Schares et al., 1999, 2000; Aguado-Martinez et al., 2010) and a rabbit pAb to *N. caninum* were employed in a quantitative assessment of *N. caninum* tachyzoites by IHC using standardized positive control tissues. The performance of mAb cocktails was compared with the pAb in positive control slides and the best mAb combination was then employed to detect the parasite in naturally-infected bovine aborted fetuses. Our results show that the mAb combination with optimal analytical sensitivity in standardized positive control tissues consisting of two

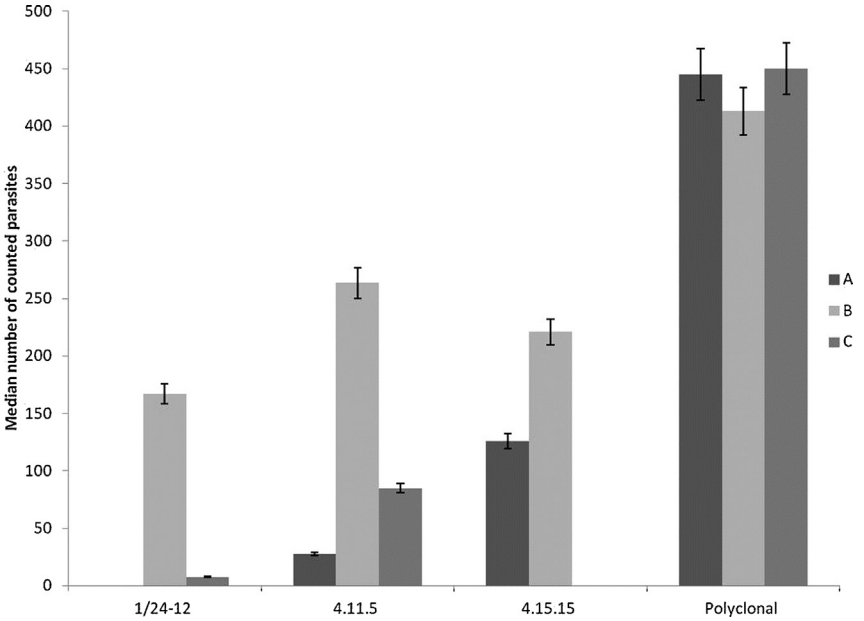

Fig. 1. Median number of *Neospora caninum* tachyzoites detected by immunohistochemistry in standardized positive control sections using monoclonal antibodies (1/24-12, 4.11.5 and 4.15.15), a polyclonal antibody, and different techniques of antigen retrieval. (A) Without antigen retrieval; (B) heat induced antigen retrieval with citrate buffer pH 6.0; (C) pepsin digestion. Whiskers represent 95% confidence intervals.

mAbs (1/24-12, 4.15.15) showed a high analytical specificity, because cross reaction with related parasites was not seen using this cocktail in IHC or the mAbs separately in IB. Furthermore, the analytical sensitivity of the combined mAbs was similar to pAb when naturally infected tissues were used in IHC.

Production of mAbs for diagnostic purposes has been widely reported (Cole et al., 1994; Bjorkman and Hemphill, 1998; Schares et al., 1999; Baszler et al., 2001; Srinivasan et al., 2006; Aguado-Martinez et al., 2010; Sohn et al., 2011). Because mAbs are directed against a specific epitope of a parasite antigen, the probability of cross-reaction with closely related parasites is low. In contrast, pAb consisting of antigens against a large number of epitopes can induce background signals and non-specific reactions (Ramos-Vara et al., 2008; Aguado-Martinez et al., 2010). Since *N. caninum* can share similar proteins with other Apicomplexan protozoan parasites such as *T. gondii* and *B. besnoitii*, cross-reactivity may occur in immunological tests (Sundermann et al., 1997; Nishikawa et al., 2002). Therefore, the use of mAbs can be extremely useful as a diagnostic tool to identify *N. caninum* infection.

Before performing quantitative assessment and testing antibodies in naturally-infected tissues, different techniques of AR were employed in IHC to find an optimized protocol that permitted retrieval of specific proteins from

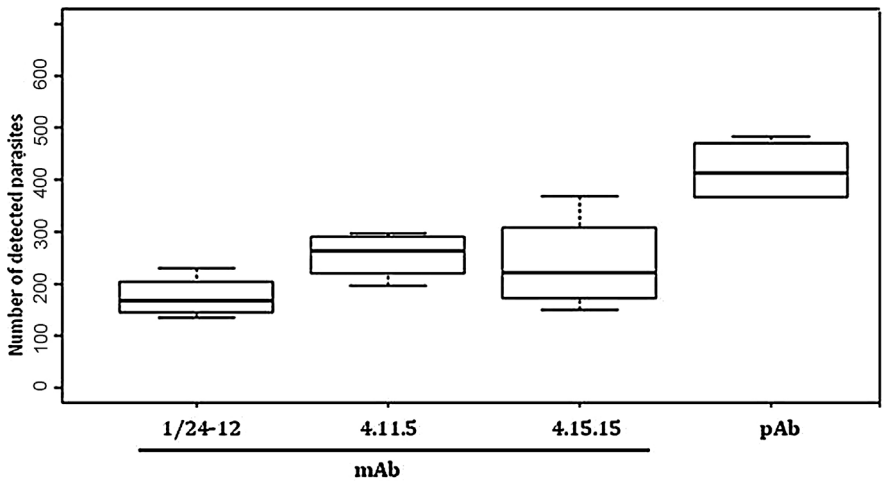

Fig. 2. Box-plot representing first and third quartiles, median, minimum and maximum values of the number of parasites detected in standardized positive control sections by IHC using monoclonal antibodies (mAb) (1/24-12, 4.11.5 and 4.15.15) and a polyclonal antibody (pAb) against *Neospora caninum* tachyzoites.

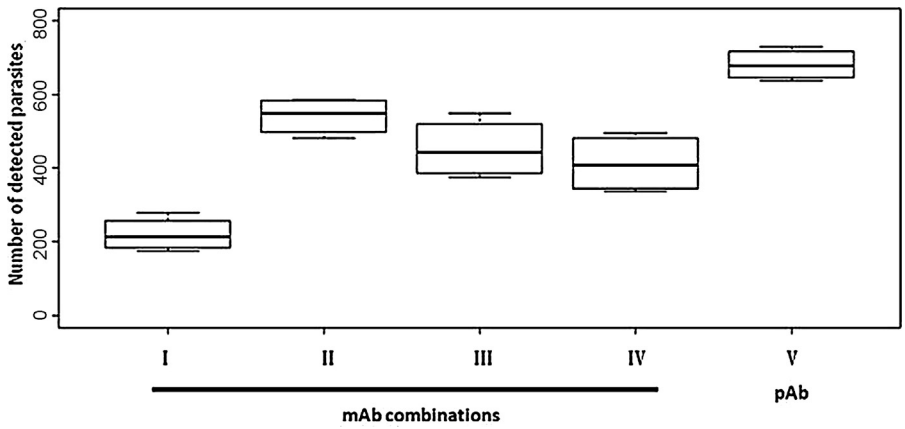

Fig. 3. Box-plot representing first and third quartiles, median, minimum and maximum values of the number of parasites detected in standardized positive control sections by IHC using four monoclonal antibody combinations (I: 1/24-12 + 4.11.5, II: 1/24-12 + 4.15.15, III: 4.15.15 + 4.11.5 and IV: 1/24-12 + 4.11.5 + 4.15.15) and polyclonal antibody against *Neospora caninum* tachyzoites.

the formalin-fixed-paraffin-embedded tissues. With this purpose, sets of positive control sections were tested by IHC without AR, with heat-induced AR and enzymatic digestion as suggested by Ramos-Vara et al. (2008).

In this study, the mAb 1/24-12 could not detect any parasite in positive control sections when IHC was performed without AR. A possible reason could be that the epitope this mAb detects on a dense granule protein (NcGRA7) was

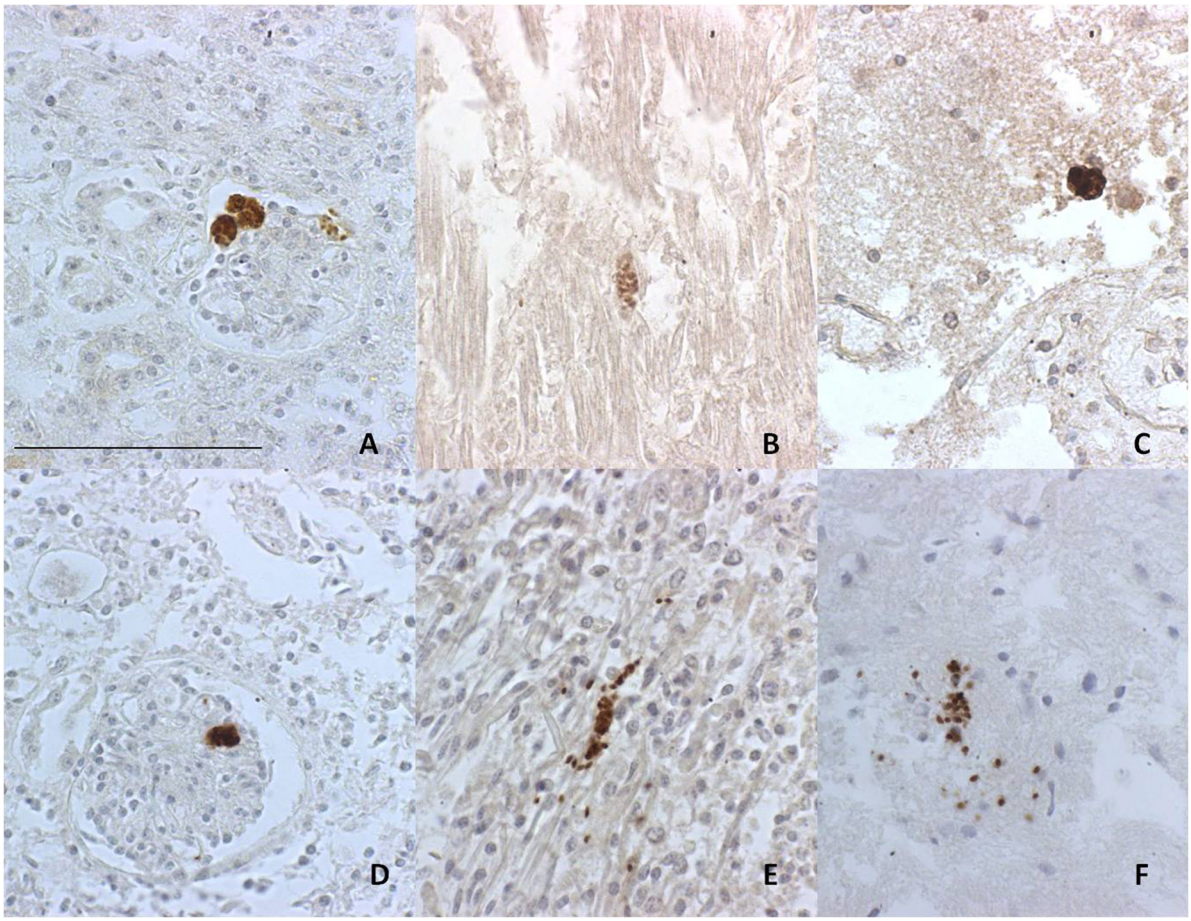

Fig. 4. IHC detection of *Neospora caninum* in naturally-infected bovine fetuses using an anti-*Neospora caninum* polyclonal antibody (pAb) (A-C), and a combination of two *N. caninum* specific monoclonal antibodies (mAb) (mAb 4.15.15 + mAb 1/24-12) (D-F). (A and D) Kidney; (B and E) Heart; (C and F) Brain. Bar: 50 μm. *Note*: Background reactions were generally more evident in sections tested with pAb than in those with mAb.

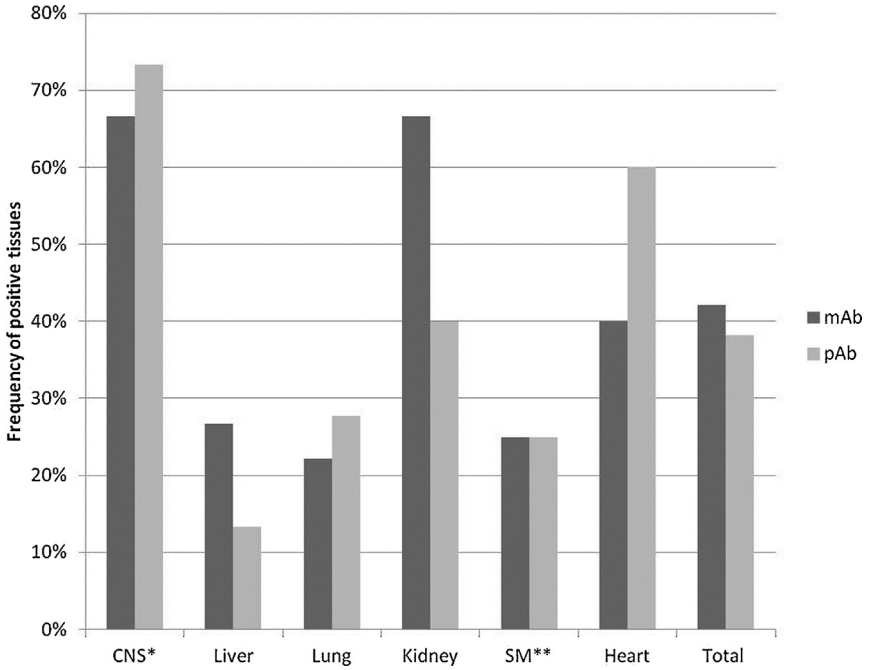

Fig. 5. Proportions of tissue sections from natural cases of bovine neosporosis with positive IHC reactions using a monoclonal antibody (mAb) combination (mAb 4.15.15 + mAb 1/24-12) and anti-*Neospora caninum* polyclonal antibody (pAb). *Central nervous system and **skeletal muscle.

more masked than other epitopes which were detected when the mAbs 4.11.5 (also directed against NcGRA7) and 4.15.15 (directed against NcSRS2) were employed. It is known that some epitopes may become masked during the fixation process of tissues due the formation of intra and inter-molecular links modifying the protein structure and thus reducing antibody binding (Shi et al., 2001; Ramos-Vara et al., 2008; D'Amico et al., 2009). When enzymatic treatment with pepsin was used as AR, a little enhancement was observed with the NcGRA7 specific mAbs 1/24-12 and 4.11.5. On the other hand, a dramatic reduction of detected parasites was observed with the mAb 4.15.15, specific for an epitope on the surface of *N. caninum* tachyzoites. It is well known that enzymatic digestion can destroy some epitopes during AR, as reviewed by Ramos-Vara et al. (2008), and D'Amico et al. (2009) and these epitopes are probably susceptible to enzymatic destruction. Heat-induced AR revealed the highest parasite detection capacity among the employed mAbs, which made it the chosen technique for the subsequent tests. Detection of parasites was not blocked when pAb was used with the different ways of AR, probably because a pAb usually binds to a large number of different antigens; however, during epitopes unmasking process, some cross-reacting antigens may also become exposed which may cause false positive results (reviewed by D'Amico et al. (2009)).

In the present study different combinations of mAb against *N. caninum* were employed. Parasites were quantified by IHC using standardized positive control sections prepared with homogenized bovine brain and cultured

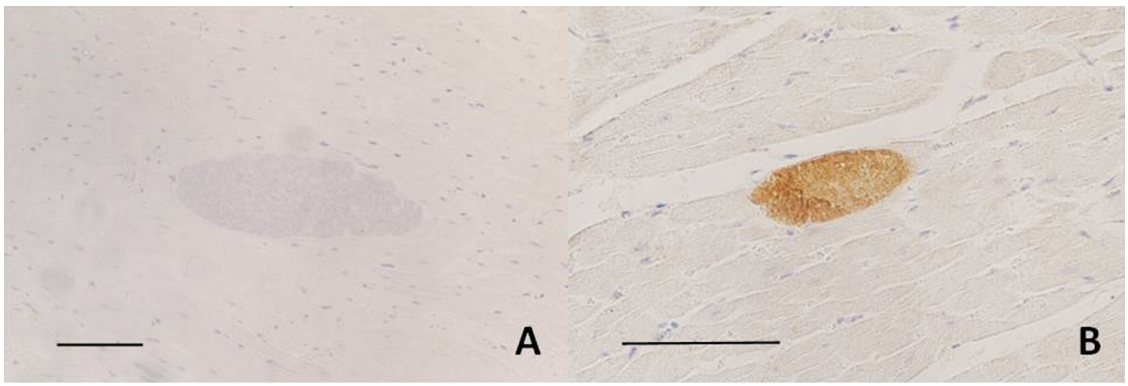

Fig. 6. Immunohistochemistry using (A) the monoclonal antibody (mAb) combination II (mAb 1/24-12 + mAb 4.15.15) and (B) polyclonal antibody (pAb) against *Neospora caninum* to analyze a heart tissue section of a bovine naturally-infected with *Sarcocystis* spp. Note the cross-reactivity when pAb was employed (B). Bar: 100 μm.

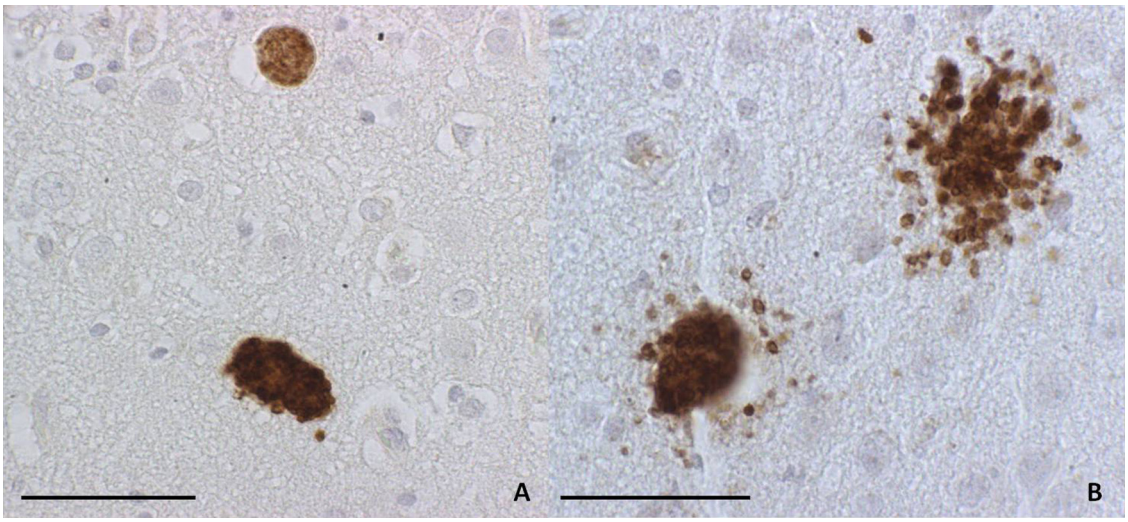

Fig. 7. Immunohistochemistry using (A) the monoclonal antibody mAb combination II (mAb 1/24-12 + mAb 4.15.15) and (B) polyclonal antibody against *Neospora caninum* in a brain section of a dog naturally infected with *N. caninum*. Bar: 50 μm.

tachyzoites; the mAb combination with the best performance in the quantitative test was compared with pAb to *N. caninum* by IHC with tissues from naturally-infected cattle. To the author's knowledge, this is the first study that aimed at the application of different mAbs to detect *N. caninum* infection by IHC.

All antibodies used in this study were capable of efficiently recognize *N. caninum* antigens. MAb 1/24-12 and 4.11.5 labeled a band of 33 kDa in tachyzoites by IB, and 4.15.15 bound a 38 kDa protein band. These results confirm previous reports of others (Aguado-Martinez et al., 2010). Previous results also showed that the NcGRA7 antigen recognized by mAbs 1/24-12 and 4.11.5 were not only present in tachyzoites but also bradyzoites which makes this antigen an ideal candidate as a diagnostic target for IHC (Aguado-Martinez et al., 2010).

This study demonstrated that mAb combinations increased mAb detection capacity. Up to 79.4% of parasites were detected by combination II (4.15.15 and 1/24-12) relative to the number of parasites detected by pAb. Probably, a synergic effect was observed since each mAb binds to a separate antigen; mAb 4.15.15 can bind to a surface antigen (p38) (Schares et al., 2000) and 1/24-12 can recognize a dense granule protein of 33 kDa (GRA-7) (Aguado-Martinez et al., 2010).

The quantitative test with standardized positive control tissues revealed that the pAb capacity of detection (680 parasites in average score) is slightly higher than combination II of 4.15.15 and 1/24-12 (540 parasites in average score). Nevertheless this difference was statistically significant ($p = 0.045$).

When tissue sections of natural *N. caninum* infections were employed in this study, 42% (32/76) and 38% (29/76) were positive by IHC using mAb combination II and pAb, respectively. The number of *N. caninum* organisms detected in naturally infected cattle by IHC is expected to be low due to the focal distribution of lesions caused by *N. caninum* and the low number of parasites commonly observed in these tissues (van Maanen et al., 2004). Here we could notice that under natural conditions the mAb combination presented a slightly higher sensitivity than pAb although this difference was statistically not significant.

It is well documented that the number of parasites in *N. caninum* naturally-infected bovine fetuses is low. After evaluation of tissues from 80 bovine fetuses, Wouda et al. (1997) observed *N. caninum* tachyzoites in 85% of the brains, 14% of the hearts, and 26% of the livers. From the total of analyzed brains, cysts were found in only 21%. These findings show how sparse are lesions and distribution of parasites in aborted bovine fetuses. The immune response of the host probably has a role in this scenario. The response targeted to kill the host cell, may destroy the parasites (reviewed by Dubey et al., 2006), what results in a low number of parasite stages in the tissues; this can explain why we could find more parasites in artificial blocks (tachyzoite numbers are known) than in naturally infected tissues.

The agreement between mAb and pAb application was assessed by kappa value. The rate of 0.37 represents a fair agreement between the two tests (Landis and Koch, 1977; Viera and Garrett, 2005); however, the observed agreement was 70%. This difference between kappa value and level of concordance may be due the low number of parasites commonly found in bovine aborted fetus (van Maanen et al., 2004), once kappa value may be affected by low prevalence, rare findings and in case of rare diseases. In some situations, kappa may not be reliable to evaluate rare observations, even when high a level of concordance is observed (Viera and Garrett, 2005).

After transmission from an infected cow to its fetus, the parasites can be spread and cause characteristic lesions in several tissues such as heart, skeletal muscle, lung, liver and kidney, with a predilection by CNS (Anderson et al., 1991; Barr et al., 1991; Wouda et al., 1997; Corbellini et al., 2002). Our IHC findings corroborate with literature reports, once 11/15 (73%) and 10/15 (67%) of CNS sections were positive using pAb and mAb, respectively. The parasite is more frequently described in the brain than in other organs

(Dubey and Schares, 2006). In our study, parasite detection in heart (60%) and kidney (67%) were high using pAb and mAb, respectively, what reinforces the importance of these tissues in IHC examination.

In this study, mAb had a good performance in IHC, once it did not cross-react with tachyzoites of *T. gondii* and *B. besnoiti* tested by IHC and IB, or with cysts of *B. besnoiti* and *Sarcocystis* spp. from naturally infected cattle by IHC. Cross-reactivity was detected when pAb were tested by IHC in tissue sections containing *Sarcocystis* spp., and by IB using *T. gondii* (tachyzoites) and *B. besnoiti* (bradyzoites) as antigens.

It is known that Apicomplexan parasites share common proteins (Howe and Sibley, 1999; Zhang et al., 2011). Several studies have described antigens that can be identified by both *N. caninum* and *T. gondii* specific antisera, as protein disulfide isomerase (PDI), heat-shock protein 70 (HSP70), ribosomal protein 1 (RP1) (Liao et al., 2005) and a 42-kDa rhoptry protein (Ahn et al., 2001). Furthermore, antiserum against *T. gondii* bradyzoite (BAG5) has shown cross-reactivity with the same antigen of *N. caninum* (McAllister et al., 1996) and *Sarcocystis cruzi* bradyzoites (Peters et al., 2001). In IHC these common antigens (as the cross-reactive antigens found in this study in IB) can be exposed with different antigen retrieval methods, as an enzyme treatment, and be labeled by pAb (van Maanen et al., 2004). Several studies have shown cross-reactivity between pAb and *T. gondii* in IHC (McAllister et al., 1996). Sundermann et al. (1997) described a commercial pAb antibody specific for *T. gondii* that was not efficient in labeling all bradyzoites of this parasite in tissue sections and strongly cross-reacted with *N. caninum* tachyzoites.

Our study demonstrated a good performance of mAb in IHC compared to pAb in terms of cross-reactive prevention. Further studies may focus on combinations of mAbs targeted to *N. caninum*-related coccidia, such as *T. gondii*, *Besnoitia* spp., and *Sarcocystis* spp. which should improve the efficiency of IHC in clinical and epidemiological investigations.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.07.008.

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