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

R.S. Uzêda[a], G. Schares[b], L.M. Ortega-Mora[c], C.R. Madruga[a], A. Aguado-Martinez[c], L.G. Corbellini[d], D. Driemeier[e], L.F.P. Gondim[a,*]

[a] *Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia, Av. Ademar de Barros 500, CEP 40170-110 Salvador, Bahia, Brazil*
[b] *Institute of Epidemiology, Friedrich-Loeer-Institut-Federal Research Institute for Animal Health, Seestrasse 55, 16868 Wusterhausen, Germany*
[c] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, E-28040 Madrid, Spain*
[d] *Faculdade de Medicina Veterinária, Departamento de Medicina Veterinária Preventiva, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, CEP 91540-000 Porto Alegre, Rio Grande do Sul, Brazil*
[e] *Faculdade de Medicina Veterinária, Departamento de Patologia Clínica Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, CEP 91540-000 Porto Alegre, Rio Grande do Sul, Brazil*

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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*.

* Corresponding author. Tel.: +55 71 3283 6759; fax: +55 71 3283 6730.
E-mail address: pita@ufba.br (L.F.P. Gondim).

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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