Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 148 (2007) 213–218
veterinary parasitology
www.elsevier.com/locate/vetpar

Humoral immune response in pregnant heifers inoculated with *Neospora caninum* tachyzoites by conjunctival route

M.G. de Yaniz [a], D.P. Moore [a,*], A.C. Odeón [b], A. Cano [b], D.B. Cano [b], M.R. Leunda [b], C.M. Campero [b]

[a] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*
[b] *Instituto Nacional de Tecnología Agropecuaria (INTA), 7620 Balcarce, Argentina*

Received 21 March 2007; received in revised form 2 May 2007; accepted 20 June 2007

Abstract

The aim of this study was to compare systemic humoral immune responses in pregnant heifers inoculated with *Neospora caninum* tachyzoites by conjunctival and intravenous routes. Twenty nine heifers separated in three experimental groups were studied: Group 1 ($n = 10$ animals) and Group 2 ($n = 9$ animals) were inoculated with 10^8 of *N. caninum* tachyzoites by conjunctival and intravenous routes at 5th month of gestation, respectively; Group 3 ($n = 10$ animals) were non-inoculated control animals. An indirect fluorescent antibody test (IFAT) and western immunoblotting (IB) were used to analyze the humoral immune response. All animals from Group 1 developed *N. caninum* specific antibody responses after conjunctival inoculation recording the highest antibody titer (mean ± SE: 160 ± 49.9) at 6th month of gestation. There were statistical differences between humoral immune responses found in Group 1 and 2 being higher in the second one at 6.5th, 8.5th and 9th months of gestation ($P < 0.05$). Interestingly, all heifers from Group 1 reverted to seronegative status at the end of gestation. No increase in antibody was detected in the uninfected control group. Same pattern of *N. caninum* antigens was recognized by sera from heifers inoculated by conjunctival route and heifers inoculated by intravenous route. Recognized antigens were 116, 92, 84, 77, 45, 40, 25–26 and 17–18 kDa. The conjunctival instillation of *N. caninum* tachyzoites in pregnant heifers induces specific systemic antibodies. Further work is needed in order to clarify the consequences of this novel experimental route of infection not only on the fetus but also on the dam.

Keywords: Bovine; Conjunctiva; Humoral immune response; *Neospora caninum*

1. Introduction

Neospora caninum is a protozoan parasite that has emerged as a major cause of reproductive failure in the cattle industry worldwide including Argentina (Dubey, 2003; Moore, 2005). Recognized stages of *N. caninum* are tachyzoite, bradyzoite and sporozoite (Dubey, 2003). Up to date dogs and coyotes have been identified as definitive hosts (McAllister et al., 1998; Gondim et al., 2004) and not only domestic animals but also several wild species are intermediate hosts (Dubey, 2003). Although control strategies were described (Thurmond and Hietala, 1995) and several approaches to develop immunogens to cattle have been also performed (Andrianarivo et al., 1999, 2000, 2005; Choromanski and Block, 2000; Moore et al., 2005), there is a need to improve such potential vaccines and to develop drugs for treatment.

Using tachyzoites or oocysts, different challenge experimental routes like intravenous (Barr et al., 1994;

* Corresponding author at: Veterinary Pathology, INTA Balcarce CC 276, (7620) Balcarce, Argentina. Tel.: +54 2266 439100 20; fax: +54 2266 43 9101.
E-mail address: pmoore@balcarce.inta.gov.ar (D.P. Moore).

0304-4017/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2007.06.030

Andrianarivo et al., 2001; Williams et al., 2000, 2003), intramuscular, (Dubey et al., 1992; Barr et al., 1994; Andrianarivo et al., 2001), subcutaneous (Dubey et al., 1992; Maley et al., 2003; Macaldowie et al., 2004), oral (Uggla et al., 1998; De Marez et al., 1999; Gondim et al., 2002; Trees et al., 2002), and intrauterine (Barr et al., 1994; Serrano et al., 2006) have been investigated, albeit the conjunctival route has not been explored yet. Exposure to *Neospora*-tachyzoites by conjunctival route is improbable in the nature; however, interesting previous works related to brucellosis in cattle have confirmed that an attenuated strain inoculated by the conjunctival route prevented the abortions caused after challenge with a pathogenic strain (Fensterbank and Plommet, 1979). More over, elevated systemic antibody titers were also developed after conjuctival inoculation of *Toxoplasma gondii* tachyzoites in Guinea pigs (Skorich et al., 1988).

The aim of the present study was to compare systemic humoral immune responses by using indirect fluorescent antibody test (IFAT) and immunoblotting (IB) analysis in pregnant heifers inoculated with *N. caninum* tachyzoites by conjunctival and intravenous routes.

2. Materials and methods

2.1. Animals and experimental design

Twenty-nine heifers Aberdeen Angus, Hereford and-their crossbreeds, 2 years old, located at INTA-Balcarce, Argentina were used. Herd of origin was brucellosis and tuberculosis free. All females were previously consistently seronegative for *N. caninum* by using an IFAT (Moore et al., 2005). Animals maintained good body condition (score: 6) under extensive management in native pasture during all the experiment. Previous to breeding, all animals were vaccinated with a commercially available polyvalent killed vaccine against bovine virus diarrhea virus (BVDV) and infectious bovine rhinotracheitis virus (IBRV) (San Jorge Bagó®, Argentina). Animals were synchronized with prostaglandin (PG) (Cloprostenol 75 μg; Arsaprost, ARSA SRL®, Argentina). After 48 h of the last PG injection, animals were bred during 1 month by natural mating with two *N. caninum* seronegative A. Angus bulls. After 35 days from the end of breeding time, pregnancy was confirmed by using ultrasound and rectal palpation.

Cattle were randomly assigned into three groups: Group 1 ($n = 10$ animals) and Group 2 ($n = 9$ animals) were inoculated with 10^8 of *N. caninum* tachyzoites by conjunctival and intravenous routes, respectively. Challenge was performed at 5th month of gestation. Group 3 ($n = 10$ animals) acted as sentinel group in order to detect any possible horizontal transmission. Animals were daily observed throughout the experimental period and pregnancy was monitored at 2-week intervals by rectal palpations. Calving data were recorded and calves were observed until 6th month of age. All animal usage was according to protocols from the Animal Ethics Committee at INTA-Balcarce, Argentina.

2.2. N. caninum inoculum

N. caninum tachyzoites NC-1 strain (Dubey et al., 1988; kindly supplied by Dr M.C. Venturini from La Plata Veterinary College, Argentina) were grown in VERO cells and the inoculum was prepared as it was described previously (Moore et al., 2005). Parasites were harvested when ≥80% of the monolayer was infected. Tachyzoites were isolated by passage of the cell monolayer through 21, 23, 25 and 27 gauge needles and then passed through a 5 μm filter (Millipore, MILLEX®: Bedford, USA) to remove excess cell culture debris. The tachyzoites were washed and counted adjusting 1×10^8 parasites in 150 μl and 3 ml of PBS for Group 1 and 2, respectively.

Animals inoculated by conjunctival route were appropriately immobilized; however, neither sedatives nor anesthetics were used. Seventy and five microliters were instilled between the ocular and palpebral conjunctiva using micropipette and tips. Each eye was carefully closed avoiding any loss of inoculum.

2.3. Sampling and IFAT

Blood samples were collected from a jugular vein three times at 1 month intervals before challenge and at 2-week intervals from challenge to the end of gestation. Calves were also bled at 1 month intervals from birth to 6th month of age. Unfortunately, calves were not bled before colustrum intake due to extensive herd management.

IFAT was performed as previously described (Dubey et al., 1988) using a fluorescein isothiocyanate (FITC) labelled affinity-purified rabbit anti-bovine IgG (Sigma, St. Louis, USA). *Neospora*-specific antibodies were measured using dilutions of serum from 1:200 to endpoint titre for heifers (Reichel and Drake, 1996) and 1:25 for their calves (Moore et al., 2005). Positive and negative control sera were used (VMRD, Inc., USA). Slides were examined with an epifluorescence

microscope (Nikon Fluophot, 40 × 1.3). Antibody titres were expressed as the reciprocal of the highest serum dilution that showed distinct whole parasite fluorescence.

2.4. Antigen preparation, SDS-PAGE and immunoblotting analysis

Parasite growing and purification were basically carried out as described above. Following purification, tachyzoites were pelleted by centrifugation at 600 × *g* for 10 min and stored at −80 °C.

Extraction of parasite proteins using detergent and non-detergent sulphobetaine (DSB and NDSB, respectively) was carried out according to Zintl et al. (2005). Briefly, the pellets were re-suspended in PBS containing 1% SB (w/v) (Sigma, St. Louis, MO, USA) and 1 M NDSB201 (FLUKA, Sigma-Aldrich, Germany) to a final concentration of approximately 1×10^8 tachyzoites per ml. The parasite suspensions were incubated for 30 min at 37 °C. Subsequently, the preparations were centrifuged at 12,000 × *g* at 4 °C for 30 min and the supernatant collected. A protein concentration was determined using the BCA protein assay kit (Pierce Labs., Rockford, IL, USA). For protein precipitation, the preparation was mixed with acetone (1:4), vortexed vigorously and incubated for 1 h at −80 °C. Subsequently, the precipitated protein was collected by centrifugation at 12,000 × *g* at 4 °C for 20 min. The precipitated protein was then rehydrated with loading buffer containing 4% (v/v) β-mercaptoethanol and 2% (p/v) sodium hydrogen carbonate (Bio-Rad, USA) at concentration of 2 mg/ml for *N. caninum* protein and 0.25 mg/ml cells VERO and immediately boiled for 5 min at 95 °C. These samples were stored at −20 °C (Okeoma et al., 2004).

Electrophoresis was carried out on 12% polyacrylamide gels (Invitrogen NuPage electrophoresis system). Twenty micrograms of *N. caninum* protein were loaded per lane. Molecular weight (MW) standard (Bio-Rad, USA) was also loaded to monitor antigen separation, electrophoretic transfer and estimation of the MWs of the different antigens recognized by the serum samples. Gels were transferred onto nitrocellulose membranes (Millipore, USA). Membranes were incubated with a blocking buffer containing 5% non-fat milk powder diluted with PBS containing 1% Tween-20 (PBS-T). Nitrocellulose membranes were cut in strips, which were exposed to a pooled bovine serum samples from heifers belonging to Group 1, 2 and 3. Bovine sera were diluted with the blocking buffer at 1:50. The strips were incubated for 3 h with mild shaking at room temperature (RT). Then, membranes were washed three times with PBS-T and incubated with an anti-bovine IgG HRP-conjugated rabbit antibody (Sigma, St. Louis, MO, USA) used at dilution of 1:200 in blocking solution for 1 h 30 min at RT (Staubli et al., 2006). Following incubation, membranes were washed and antigen-antibody reaction was visualized using 4-chloro-1-naphthol (Sigma; Sigma Chemical Co, St. Louis, USA) (Alvarez-García et al., 2002).

2.5. Statistical analysis

Serum antibody responses (IFAT-titres) were compared between groups by using PROC-MIXED SAS for one-way repeated measures analysis of variance (ANOVA) with treatment as the grouping factor and time as the repeated measures factor (Littell et al., 1998). Data were log10-transformed before statistical analyses. Post hoc Tukey's pair-wise comparisons were performed when significant differences between treatment groups were detected. All statistical analyses were considered significant at the $P < 0.05$ level.

3. Results

All heifers were pregnant after mating. Neither deleterious clinical symptoms nor abortions were observed in any animals. Twenty seven healthy calves were delivered at the end of the gestation period. Unfortunately, two calves (one from Group 1 and one from Group 2) died due to dystocia.

3.1. N. caninum specific antibody titers by IFAT

All animals from Group 1 developed *N. caninum* specific antibody responses after conjunctival inoculation. Although some of them were seropositive by a short period of time (data not shown), a significant statistical interaction was recorded between treatment and time ($P < 0.05$). IFAT-titres were increased gradually after challenge until 6th month of gestation where the highest antibody titer was recorded (mean ± SE: 160 ± 49.9) (Fig. 1). Titres decreased to the end of gestation with a second peak at 7.5th month (120 ± 44.2). No heifers showed titers at the end of gestation (Fig. 1).

Heifers from Group 2 also developed *N. caninum* specific antibodies. A significant statistical interaction was recorded between treatment and time, and maximum titer (622 ± 195) was reached at 6.5th month of gestation (Fig. 1). Heifers in Group 2 remained seropositive at the end of gestation. There were also statistical differences between humoral immune responses found in Group 1

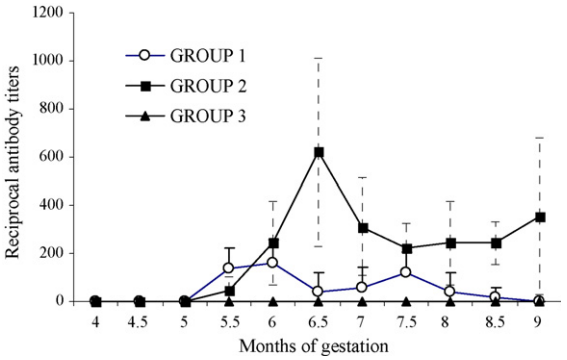

Fig. 1. *N. caninum*-antibody titers by IFAT in heifers inoculated by conjunctival and intravenous routes.

and 2 being higher in the second one at 6.5th, 8.5th and 9th months of gestation ($P < 0.05$). Finally, no increase in antibody was detected in the uninfected controls (Group 3) throughout the experiment.

After colostrum intake, only calves from Group 2 were seropositive at the 1st month of age. All calves were seronegative from 2nd to 6th month of age.

3.2. Patterns of N. caninum antigen recognition

Same pattern of *N. caninum* signal antigens was recognized by sera from heifers inoculated by conjunctival route and heifers inoculated by intravenous route. Recognized antigens were 116, 92, 84, 77, 45, 40, 25–26 and 17–18 kDa (Fig. 2). No bands were observed when sera from heifers in the uninfected control group were tested.

4. Discussion

The development of a specific systemic humoral immune response after the instillation of tachyzoites of *N. caninum* on the bovine conjunctiva is coincident with previous work on toxoplasmosis, where the inoculation of tachyzoites in guinea pigs developed specific antibody titers (Skorich et al., 1988). Not only a similar quantitative immune response was observed between Group 1 and 2 at the 5.5th, 6th, 7th, 7.5th and 8th month but also the same pattern of antigen recognition was observed by western immunoblot. Coincident with previous work, bands of 116, 77, 45, 40, 26–25 and 18–17 kDa were characterized by using IB (Alvarez-García et al., 2002; Okeoma et al., 2004).

It is interesting to notice that all heifers from Group 1 reverted to the seronegative status at the end of gestation in contrast to heifers from Group 2, which remained seropositive. The fact that only transitory titers were

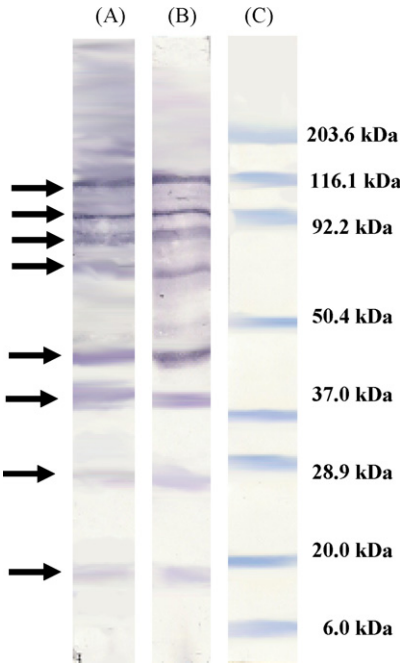

Fig. 2. Banding pattern of *N. caninum* tachyzoites with: (A) pool of sera from heifers inoculated by intravenous route; (B) pool of sera from heifers inoculated by conjunctival route and (C) Prestained MW.

developed after conjunctival inoculation could be relevant to distinguish vaccinated from non-vaccinated cattle. Nevertheless, further research should be performed in order to characterize the cellular immune response and to know whether protective mechanisms against abortion are developed after *N. caninum* exposure by conjunctival route. More over, it is also needed to establish whether cysts derived from tachyzoites inoculated by conjunctival route may develop.

It was previously reported that *T. gondii* tachyzoites invaded epithelial cells, goblet cells and macrophages in the conjunctiva (Skorich et al., 1988). Although such events were not studied in the present work; moderate mixed inflammatory reactions characterized by lymphocytes, histiocytes, and focal eosinophil infiltrates in the sclera close to the iridocorneal angle were observed by us in a preliminary work performed in two cows experimentally inoculated with *N. caninum* (unpublished data).

The possibility of vertical transmission in pregnant cattle inoculated with *N. caninum* tachyzoites by conjunctival route could be lower compared with the inoculation of parasites by intravenous route because *N. caninum* tachyzoites have to penetrate the conjunctival mucosa triggering immune mechanisms associated with

the mucosal system. Nevertheless, the consequences of this experimental route of infection on transplacental transmission could not be determined in this work because all calves, including those from Group 2 (inoculated by intravenous route) remained uninfected. Such event differs from previous articles (Barr et al., 1994; Andrianarivo et al., 2001; Williams et al., 2000, 2003) where transplacental infection was always recorded. The lack of evidence of vertical transmission in the second group in this experiment might be associated with a loss of virulence in the NC-1 strain (Bartley et al., 2006), which has been passed approximately 100 times onto VERO cells in our laboratories. Interestingly, previous works related to brucellosis in cattle have confirmed that an attenuated strain inoculated by the conjunctival route prevented the abortions caused after challenge with a pathogenic strain (Fensterbank and Plommet, 1979).

Finally, this novel route of infection has not been extensively evaluated in bovine neosporosis yet (Dubey et al., 2006). Although, this work demonstrates that the conjuntival instillation of *N. caninum* tachyzoites in pregnant heifers induces specific systemic antibodies, the immunological and pathological effects after conjunctival inoculation remain to be determined not only in the foetus but also in the dam.

Acknowledgements

The authors thank to Professor L. Ortega-Mora, Dr G. Alvarez-García and Dr J. Regidor from Complutense University, Madrid, Spain for technical assistance in the immunoblotting and to M.M. McAllister from University of Illinois, USA for critical suggestions in the preparation of this article.

References

Alvarez-García, G., Pereira-Bueno, J., Gomez-Bautista, M., Ortega-Mora, L.M., 2002. Pattern of recognition of *Neospora caninum* tachyzoite antigens by naturally infected pregnant cattle and aborted foetuses. Vet. Parasitol. 107, 15–27.

Andrianarivo, A.G., Choromanski, L., McDonough, S.P., Packhan, A.E., Conrad, P.A., 1999. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. Int. J. Parasitol. 29, 1613–1625.

Andrianarivo, A.G., Rowe, J.D., Barr, B.C., Anderson, M.L., Packham, A.E., Sverlow, K.W., Choromanski, L., Loui, C., Grace, A., Conrad, P.A., 2000. A POLYGEN^{TM}-adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent foetal infection in pregnant cattle following i.v./i. m. experimental tachyzoite challenge. Int. J. Parasitol. 30, 985–990.

Andrianarivo, A.G., Barr, B.C., Anderson, M.L., Rowe, J.D., Packhan, A.E., Sverlow, K.W., 2001. Immune responses in pregnant cattle and bovine fetuses following experimental infection with *Neospora caninum*. Parasitol. Res. 87, 817–825.

Andrianarivo, A.G., Anderson, M.L., Rowe, J.D., Gardner, I.A., Reynolds, J.P., Choromanski, L., Conrad, P.A., 2005. Immune responses during pregnancy in heifers naturally infected with *Neospora caninum* with and without immunization. Parasitol. Res. 96, 24–31.

Barr, B.C., Rowe, J.D., Sverlow, K.W., Bondurant, R.H., Ardans, A.A., Oliver, M.N., Conrad, P.A., 1994. Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. J. Vet. Diag. Invest. 6, 207–215.

Bartley, P.M., Wright, S., Sales, J., Chianini, F., Buxton, D., Innes, E.A., 2006. Long-term passage of tachyzoites in tissue culture can attenuate virulence of *Neospora caninum* in vivo. Parasitology 133, 421–432.

Choromanski, L., Block, W., 2000. Humoral immune responses and safety of experimental formulations of inactivated *Neospora*-vaccines. Parasitol. Res. 86, 851–853.

De Marez, T., Liddell, S., Dubey, J.P., Jenkins, M.C., Gasbarre, L., 1999. Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular immune responses. Int. J. Parasitol. 29, 1647–1657.

Dubey, J.P., 2003. Neosporosis in cattle. J. Parasitol. 89, 42–56.

Dubey, J.P., Hattel, A.L., Linsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193, 1259–1263.

Dubey, J.P., Lindsay, D.S., Anderson, M.L., Davis, S.W., Shen, S.K., 1992. Induced transplacental transmission of *Neospora caninum* in cattle. J. Am. Vet. Med. Assoc. 201, 709–713.

Dubey, J.P., Buxton, D., Wouda, W., 2006. Pathogenesis of bovine neosporosis. J. Comp. Pathol. 134, 267–289.

Fensterbank, R., Plommet, M., 1979. Vaccination against bovine brucellosis with a low dose of strain 19 administered by the conjunctival route IV. Comparison between two methods of vaccination. Ann. Rech. Vét. 10, 131–139.

Gondim, L.F.P., Gao, L., McAllister, M.M., 2002. Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. J. Parasitol. 88, 1159–1163.

Gondim, F.P., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004. Coyotes (*Canis latrans*) are definitive host of *Neospora caninum*. Int. J. Parasitol. 34, 159–161.

Littell, R.C., Henry, P.R., Ammermen, C.B., 1998. Statistical analysis of repeated measures data using SAS procedures. J. Anim. Sci. 46, 1216–1231.

Macaldowie, C., Maley, S.W., Wright, S., Bartley, P., Esteban-Redondo, I., Buxton, D., Innes, E.A., 2004. Placental pathology associated with fetal death in cattle inoculated with *Neospora caninum* by two different routes in early pregnancy. J. Comp. Pathol. 131, 142–156.

Maley, S.W., Buxton, D., Rae, A.G., Wright, S.E., Schock, A., Bartley, P.M., Esteban-Redondo, I., Swales, C., Hamilton, C.M., Sales, J., Innes, E.A., 2003. The pathogenesis of Neosporosis in pregnant cattle: inoculation at mid-gestation. J. Comp. Path. 129, 186–195.

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., Mcguire, A.M., 1998. Dogs are definitive host of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1478.

Moore, D.P., 2005. Neosporosis in South America. Vet. Parasitol. 127, 87–97.

Moore, D.P., Leunda, M.R., Zamorano, P.I., Odeón, A.C., Romera, S.A., Cano, A., De Yaniz, M.G., Venturini, M.C., Campero,

C.M., 2005. Immune response to *Neospora caninum* in naturally infected heifers and heifers vaccinated with inactivaded antigen during the second trimestre of gestation. Vet. Parasitol. 130, 29–39.

Okeoma, C.M., Williamson, N.B., Pomroy, W.E., Stowell, K.M., 2004. Recognition patterns of *Neospora caninum* tachyzoite antigens by bovine IgG at different IFAT-titres. Parasite Immunol. 26, 177–185.

Reichel, M.P., Drake, J.M., 1996. The diagnosis of *Neospora* abortions in cattle. NZ Vet. J. 44, 151–154.

Serrano, E., Ferre, I., Osoro, K., Aduriz, G., Mateos-Sanz, A., Martinez, A., Atxaerandio, R., Hidago, C.O., Ortega-Mora, L.M., 2006. Intrauterine *Neospora caninum* inoculation of heifers. Vet. Parasitol. 135, 197–203.

Skorich, D.N., Chiappino, M.L., Nichols, B.A., 1988. Invasion of the Guinea pigs conjunctiva by *Toxoplasma gondii*. Invest. Ophthalmol. Vis. Sci. 29, 1871–1880.

Staubli, D., Nunez, S., Sager, H., Schares, G., Gottstein, B., 2006. *Neospora caninum* immunoblotting improves serodiagnosis of neosporosis. Parasitol. Res. 99, 648–658.

Thurmond, M.C., Hietala, S., 1995. Strategies to control *Neospora* infection in cattle. Bovine Pract. 29, 60–63.

Trees, A.J., McAllister, M.M., Guy, C.S., McGarry, J.W., Smith, R.F., Williams, D.J., 2002. *Neospora caninum*: oocyst challenge of pregnant cows. Vet. Parasitol. 109, 147–154.

Uggla, A., Stenlund, S., Holmdahl, O.J., Jakubek, E.B., Thebo, P., Kindahl, H., Björkman, C., 1998. Oral *Neospora caninum* inoculation of neonatal calves. Int. J. Parasitol. 28, 1467–1472.

Williams, D.J.L., Guy, G.S., McGarry, J.W., Guy, F., Tasker, L., Smith, R.F., Maceachenr, K., Cripps, P.J., Kelly, D.F., Trees, A.J., 2000. *Neospora caninum*-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival. Parasitology 121, 347–358.

Williams, D.J.L., Guy, C.S., Smith, R.F., Guy, F., McGarry, J.W., McKay, J.S., Trees, A.J., 2003. First demonstration of protective immunity against foetopathy in cattle whit latent *Neospora caninum* infection. Int. J. Parasitol. 33, 1059–1065.

Zintl, A., Pennington, S.R., Mulcahy, G., 2005. Comparison of different methods for the solubilisation of *Neospora caninum* (Phylum *Apicomplexa*) antigen. Vet. Parasitol. 135, 205–213.