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

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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.

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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;
214 M.G. de Yaniz et al. / Veterinary Parasitology 148 (2007) 213–218

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 (Ugglà et al., 1998; De Maréz 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; Arsa-prost, 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 10⁸ 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 × 10⁸ 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 non-detergent sulphobetaine (DSB and NDSB, respectively) was carried out according to Zintl et al. (2005). Following incubation, membranes were washed and antigen-antibody reaction was visualized using 4-chloro-1-naphthol (Sigma; Sigma Chemical Co, St. Louis, USA) (Alvarez-Garcia 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.
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 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 conjunctival 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.

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