Infectious disease

Infection with different *Neospora caninum* strains causes differences in the glycosylation pattern in the uteri and placentae of *Neospora caninum*-infected heifers

Sergio G. Caspe [a, b, *], Jose L. Konrad [c, d], Dadin P. Moore [b, c], Juan M. Sala [b], Paola Della-Rosa [b], Luis M. Ortega-Mora [e], Diana R. Bacigalupe [f, g], Maria C. Venturini [f], Carlos M. Campero [a], Claudio G. Barbeito [c, f]

[a] *Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, UK*
[b] *Instituto Nacional de Tecnología Agropecuaria, Av. Rivadavia 1439, C1033AAE, Buenos Aires, Argentina*
[c] *Consejo Nacional de Investigaciones Científicas y Técnicas, Blvd. 27 de Febrero 210 Bis, S2000EZP Rosario, Sante Fe, Argentina*
[d] *Facultad de Ciencias Veterinarias, Universidad del Nordeste, Juan Bautista Cabral 2131, W3402BKG, Corrientes, Argentina*
[e] *Facultad de Ciencias Veterinarias, Universidad Complutense de Madrid, Av. Puerta de Hierro, s/n, Moncloa − Aravaca, 28040, Madrid, Spain*
[f] *Facultad de Ciencias Veterinarias, Universidad Nacional de la Plata, Avenue 7 No. 776, CP1900, La Plata, Buenos Aires, Argentina*
[g] *Departamento de Zoonosis, Municipalidad de Rauch, Rivadavia 750, B7203 Rauch, Buenos Aires, Argentina*

ARTICLE INFO

ABSTRACT

Neospora caninum is an obligate intracellular parasite that causes abortion in ruminants. Different strains produce differences in the severity of disease outcomes. These differences may cause physiological or pathological changes in cells, modifying the intercellular interactions and intracellular transport pathways that could be evidenced by identifying the terminal sugars. This study aimed to characterize the oligosaccharide pattern in the bovine placenta and uterus after infection with tachyzoites of three different strains of *N. caninum* (Nc-1, Nc-6 Argentina and Nc Spain-7) during early gestation. Fourteen heifers were inoculated intravenously on day 70 of gestation with 2×10^8 *N. caninum* tachyzoites and samples of placentae and uteri were analysed by histology and lectin histochemistry. In the infected groups, severe placentitis was associated with changes in lectin binding in the vascular endothelium by *Lens culinaris* agglutinin (LCA), *Pisum sativum* agglutinin (PSA) and *Ricinus communis* I (RCA-I) lectins, in the epithelial cells of the endometrial glands by RCA-I, *Dolichos biflorus* agglutinin (DBA), succinylated wheat germ agglutinin, peanut agglutinin (PNA), concanavalin-A (CON-A), LCA, PSA and *Phaseolus vulgaris* erythroagglutinin (PHA-e), and in the trophoblast layer by PNA, CON-A, LCA, PSA, PHA-e, soybean agglutinin, RCA-I, DBA and *Bandieraea simplicifolia* agglutinin (BSA-I). The results suggest that *N. caninum* causes changes in the glycosylation pattern in the maternofetal interface tissues and might cause abortions in early gestation due to changes in the cellular structure of the placenta.

1. Introduction

Bovine neosporosis is a reproductive disease that causes economic losses in cattle worldwide. This disease is caused by infection with the intracellular protozoan parasite *Neospora caninum*. Transplacental infection is the primary route of transmission [1,2]. Transplacental passage may cause lesions [3], resulting in various pregnancy outcomes including abortion, birth of calves with neurological signs or apparently healthy congenitally infected calves [4].

Genetic and phenotypical differences have been observed between *N. caninum* strains [5–12]. This genetic diversity may cause differences in protein expression, which could be the reason for the variability in the biological characterization, pathogenicity and in-vitro growth characteristics of these organisms [6,9,11,13–16]. Some of these proteins are related to adhesion and cell invasion of the parasite [11,17,18] and strain variability may result in variable severity in the placental lesions and pregnancy outcomes.

Lectin histochemistry (LHC) is a technique that uses lectins to recognize and bind specific carbohydrates, including glycosidic linkages of polysaccharides, glycoproteins and glycolipids [19,20].

This specific binding facilitates identification of complex structures, revealing physiological or pathological changes in cells, intercellular interactions and intracellular transport pathways by identifying the terminal sugars [21,22]. This technique has been successfully used for identifying pathological changes in the reproductive tract caused by infectious agents [23—29]. The aim of this study was to use LHC to characterize changes in the pattern of carbohydrate expression in bovine placentae and uteri after infection with tachyzoites of three different strains of *N. caninum* during early gestation.

2. Materials and methods

2.1. Study design

Fourteen 2-year-old cycling Angus heifers from a Premium Health Scheme (www.argentina.gob.ar/senasa/programas-sanitarios/cadenaanimal/bovinos-y-bubalinos/bovinos-y-bubalinos-produccion-primaria/enfermedades-y-estrategias-sanitarias)-accredited brucellosis, tuberculosis, campylobacteriosis and trichomonosis-free herd were used. The heifers were seronegative for *N. caninum* and *Toxoplasma gondii* by immunofluorescence antibody test (<1:25). The animals were randomly assigned to four groups: A (n = 2; control group), B, C and D (n = 3; for each challenged group). Animals from groups B, C and D were inoculated at 65 days of gestation (dg) with 10^8 tachyzoites of *N. caninum* (Nc-1, Nc-6 Argentina or Nc Spain-7, respectively) in 2 ml of phosphate-buffered saline solution (PBS; pH 7.4) by intravenous jugular inoculation. Group A received 2 ml of PBS as a placebo.

All the heifers were synchronized using sodium cloprostenol (Cicladase DL; Syntex, www.syntexar.com) and natural mating with two bulls for 24 h. Pregnancies were confirmed by transrectal ultrasonography 35 days post breeding. Animals were observed daily and pregnancy was monitored twice weekly throughout the experimental period. Forty-eight days after inoculation the heifers were slaughtered and placenta and uterus samples were collected and processed by standard histological methods. Fresh placenta samples were also taken for polymerase chain reaction (PCR) analysis.

2.2. Gross, histological and immunohistochemical examinations

Gross and histological examinations of tissues were performed as described [30]. Samples of placentae and uteri were collected, examined grossly, fixed in 10% buffered formalin, paraffin embedded, sectioned (5 μm), mounted on glass slides (Superfrost Plus; Fisher Scientific, www.fishersci.com) and stained with haematoxylin and eosin (HE). The histopathological changes were classified on a subjective scale from 0 to 3 based on the number and severity of inflammatory lesions as described [10]. Tissues having microscopic lesions compatible with *N. caninum* infection were selected and analysed by immunohistochemistry (IHC) using an avidin—biotin complex procedure (Vectastain-Elite ABC Kit; Vector Laboratories, www.vectorlabs.com) as described [30,31]. Tissues from the uninfected control group and tissues from known positive field cases of *N. caninum* infection were included in each batch of sections.

2.3. Lectin histochemistry

LHC was performed as described [32]. Briefly, formalin-fixed samples of placentae and uteri were sectioned at 5 μm, mounted on adhesive glass slides (Superfrost Plus; Fisher Scientific) and labelled with 12 lectins using a commercial kit (Lectin Kit 1 Biotinylated [BK-1000]; Vector Laboratories). The lectins used were: concanavalin-A (CON-A), *Lens culinaris* agglutinin (LCA), *Pisum sativum* agglutinin (PSA), succinylated wheat germ agglutinin

Table 1
Name, acronym, source and major binding specificities of the 12 lectins used in the study

Lectin	Acronym	Source	Binding specificity
Concanavalin-A	CON-A	Jack-bean	α-D-Man > α-D-Glc
Lens culinaris agglutinin	LCA	Lentil	α-Man
Pisum sativum agglutinin	PSA	Peas	α-Man, α-Glc
Succinylated wheat germ agglutinin	SWGA	Wheat	β1-4-D- GlcNac
Phaseolus vulgaris erythroagglutinin	PHA-e	Common bean	Bisected complex N-linked sequences
Soybean agglutinin	SBA	Soybean	α-D-GalNAc and α/β-Gal
Ulex europaeus-I	UEA-I	Common gorse	α-L-Fuc
Wheat germ agglutinin	WGA	Wheat	α-D-GlcNAc > Neu- Nac
Peanut agglutinin	PNA	Peanut	Galβ1,3GalNAcβ1 > Galβ1,4GalNAcβ1
Dolichos biflorus agglutinin	DBA	Horse gram	α-D-GalNAc
Ricinus communis-I	RCA-I	Castor bean	α/β-D-Gal
Gliffonia (Bandeiraea) *simplicifolia* agglutinin-I	BSA-I	African bean	α1-D-Gal

(SWGA), *Phaseolus vulgaris* erythroagglutinin (PHA-e), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA-I), wheat germ agglutinin (WGA), peanut agglutinin (PNA), *Dolichos biflorus* agglutinin (DBA), *Ricinus communis* I (RCA I) and *Bandieraea simplicifolia* agglutinin (BSA-I) with different specificities (Table 1).

Briefly, after dewaxing and rehydrating using a slide stainer (Shandon Varistain; Fisher Scientific), sections were treated with hydrogen peroxide 3% (Merck Life Sciences Ltd., www.merckgroup.com) (v/v) in methanol at room temperature (RT) for 30 min to inactivate endogenous peroxidase activity. After rinsing three times in PBS (pH 7.2), non-specific binding was blocked with 20% normal goat serum (Fisher Scientific) in PBS at RT for 30 min. The sections were then incubated for 1 h at RT with biotinylated lectins. The optimal lectin concentration was 30 mg/ml in PBS for all lectins, except PNA, which was used at a concentration of 10 mg/ml. The slides were incubated with an avidin—biotin—peroxidase complex (Vectastain ABC Kit; Vector Laboratories) for 45 min and the horseradish peroxidase was activated by incubation for 1—2 min with 3,3′-diaminobenzidine (DAB Kit; Dako, www.agilent.com). Specimens were rinsed in distilled water, dehydrated with graded ethanol solutions, cleared in xylene and mounted in Fisher Permount Mounting Medium (Fisher Scientific). Placentae from group A were used as technique controls. The intensity of lectin binding was scored from 0 (none) to 3 (very intense) as described [26].

The same formalin-fixed paraffin-wax tissue blocks were sectioned at 5 μm, mounted on Superfrost slides (Fisher Scientific) and labelled with an anti-*N. caninum* hyperimmune polyclonal rabbit antiserum (kindly provided by Dr. M. Anderson, University of California, Davis, USA) diluted 1:200 as described [30,31]. IHC was performed as described [31]. Briefly, a second set of paraffin sections were mounted on positively charged glass slides (Superfrost Plus) and dehydrated, enzymatically treated with 0.4% pepsin (Sigma-Aldrich, www.sigmaaldridge.com) for antigen retrieval, treated using a freshly made solution of 3% hydrogen peroxide (Perhydrol; Merck Life Science UK Ltd., www.merckmillipore.com) in methanol (v/v) for 30 min to inactivate endogenous peroxidase activity, washed three time with PBS and incubated with 20% normal goat serum (Sigma-Aldrich) to block non-specific immunoglobulin binding. After washing twice with PBS, a goat anti-rabbit IgG conjugate (Envision™ + System Horseradish Peroxidase-labelled Polymer; Dako), diluted 1:20 (v/v) was applied

Table 2
Histopathological lesions and PCR and immunohistochemistry results in uterus and placenta

Group	Animal ID	Inoculum	HE	PCR[a]	IHC[a]
A	1	PBS	−	- (0/5)	- (0/5)
	2	PBS	−	- (0/5)	- (0/5)
B	1	10^8 Nc-1 tachyzoites	++	+ (4/5)	+ (1/5)
	2	10^8 Nc-1 tachyzoites	+	+ (4/5)	- (0/5)
	3	10^8 Nc-1 tachyzoites	++	+ (5/5)	+ (2/5)
C	1	10^8 Nc-6 Argentina tachyzoites	+++	+ (5/5)	+ (2/5)
	2	10^8 Nc-6 Argentina tachyzoites	+++	+ (5/5)	+ (3/5)
	3	10^8 Nc-6 Argentina tachyzoites	+++	+ (5/5)	+ (3/5)
D	1	10^8 Nc Spain-7 tachyzoites	+++	+ (5/5)	+ (2/5)
	2	10^8 Nc Spain-7 tachyzoites	+++	+ (5/5)	+ (3/5)
	3	10^8 Nc Spain-7 tachyzoites	+++	+ (5/5)	+ (4/5)

HE, haematoxylin and eosin; PCR, polymerase chain reaction; IHC, immunohisto-chemistry, PBS, phosphate-buffered saline.
Severity of histopathological lesions: -, absent; +, mild; ++, moderate; +++, severe.
[a] Five samples taken from each animal; +, positive; -, negative; no. of positive animals in parentheses.

for 30 min according to the manufacturer's instructions. After washing again with PBS, the slides were incubated with an aminoethyl-carbazole substrate (Dako North America Inc., www.agilent.com) before washing in tap water, counterstaining with haematoxylin (Haematoxylin Solution, Mayer's; Merck Life Science UK Ltd.) and mounting with Histomount (Fisher Scientific). Placental sections from group A were included as negative control tissues in each batch of sections. Positive control tissue consisted of formalin-fixed brain tissue from mice experimentally inoculated with the NC-1 strain of *N. caninum.*

2.4. Statistical analysis

Differences in intensity of labelling for each lectin in the treatment group were assessed using an analysis of variance with post-hoc comparisons calculated using Tukey's HSD method with a 95% confidence interval. All analyses were performed on the R system for statistical computing [33] using the lme4 [34] and glmmTMB packages for model fitting [35], the car and emmeans packages for significance testing and comparisons [36,37] and the performance and DHARMa packages for model diagnostics [38,39].

Fresh tissue samples were analysed using a nested-PCR technique for the internal transcribed spacer I (ITS1) region of *N. caninum* as described [40]. Briefly, DNA was obtained from the tissue samples by extraction with a commercial kit (DNeasy Blood and Tissue Kit; Qiagen, www.qiagen.com) according to the manufacturer's recommendations. The reaction was performed on 50 μl volume, containing reaction buffer (GoTaq Reaction Buffer; Promega, www.promega.com), 0.2 mM of each dNTP, 0.25 μl of Taq polymerase (GoTaq G2 DNA Polymerase; Promega) and Nuclease-Free Water to 50 μl. The primers were used at 0.2 μM for both the primary (NN1 forward: 5′-TCAACCTTTGAATCCCAA -3′ and NN2 reverse: 5′- CGAGCCAAGACATCCATT -3′) and secondary amplification primers (NP1 forward: 5′- TACTACTCCCTGTGAGTTG -3′ and NP2 reverse: 5′- TCTCTTCCCTCAAACGCT -3′). Aliquots of the amplification product obtained in the PCR assay were analysed by agarose gel (2%) electrophoresis and the products were stained with ethidium bromide and visualized under ultraviolet light. DNA equivalent to 10^3 tachyzoites was employed in each amplification as a positive control.
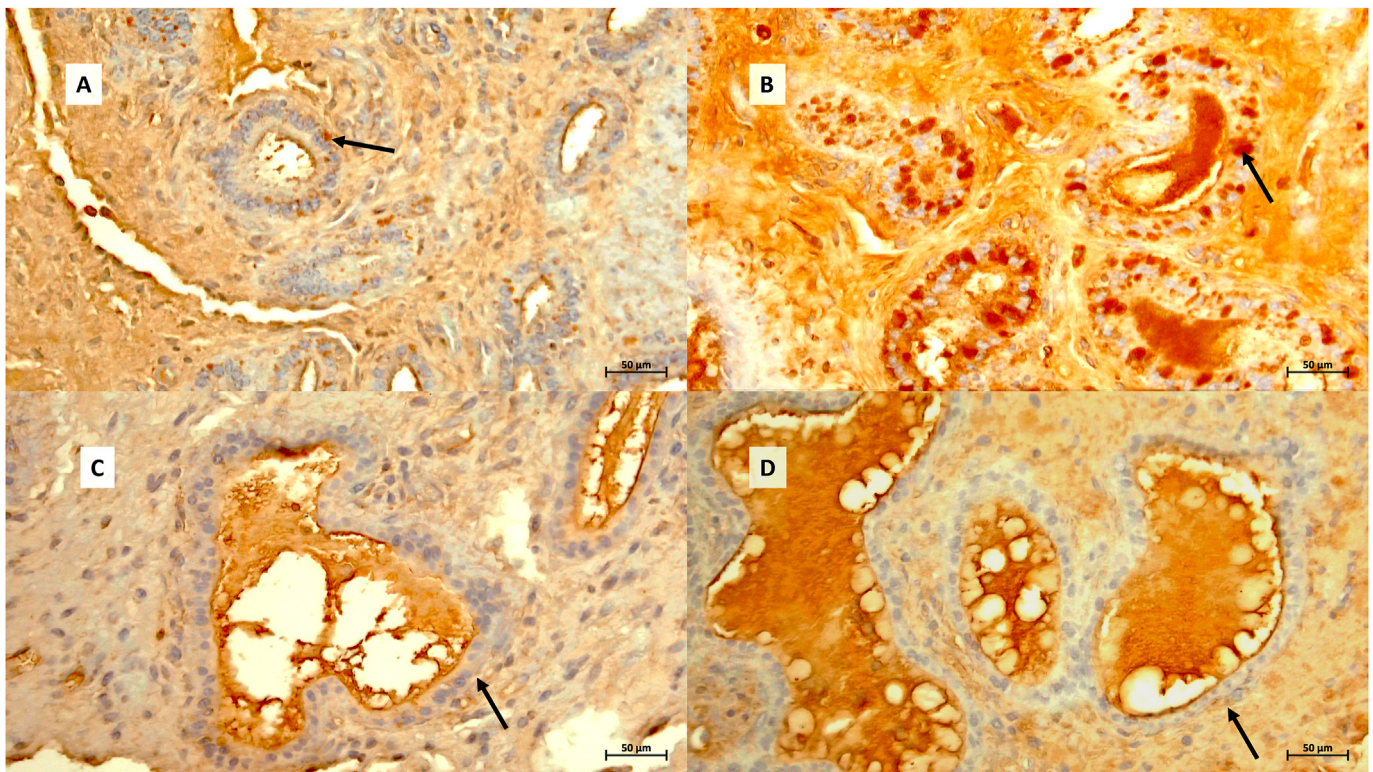

Fig. 1. *Neospora caninum* infection, uteri, cattle. PSA labelling in endometrial glands from *N. caninum*-infected and uninfected control heifers. (**A**) Uninfected heifer. Mild labelling in supranuclear cytoplasm of acinar cells of uterine glands (arrow). (**B**) Nc-1 *N. caninum* strain-inoculated heifer. Intense labelling in supranuclear cytoplasm of acinar cells of uterine glands (arrow) and secretion. (**C**) Nc-6 Argentina *N. caninum* strain-inoculated heifer. Absence of labelling in supranuclear cytoplasm of acinar cells of uterine glands (arrow) and intense labelling of secretion. (**D**) Nc Spain-7 *N. caninum* strain-inoculated heifer. Absence of labelling in supranuclear cytoplasm of acinar cells of uterine glands (arrow) and intense labelling of secretion.
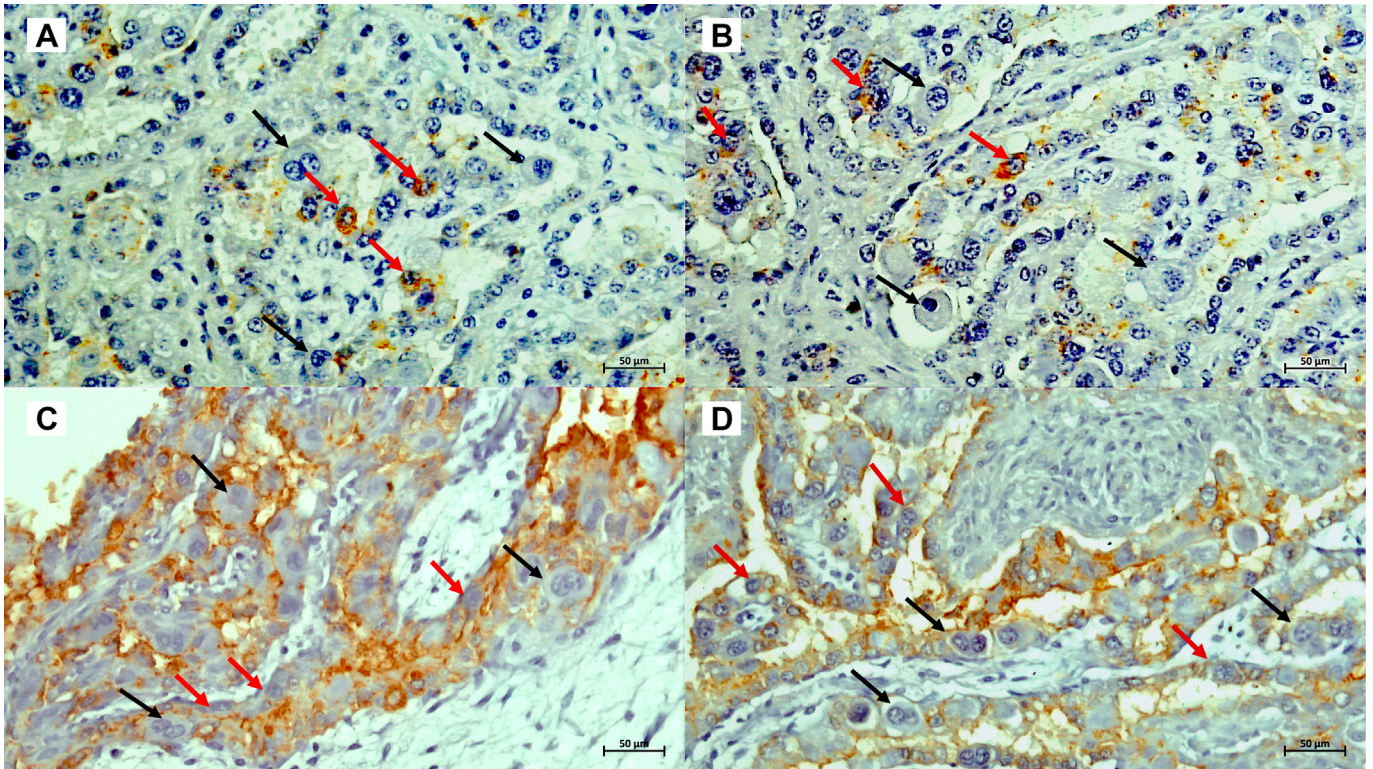
Fig. 2. *Neospora caninum* infection, placentae, cattle. PNA labelling in trophoblast layer from *N. caninum*-infected and uninfected control heifers. (**A**) Uninfected heifer. Intense labelling in mononuclear trophoblast cells (MNCs; red arrows) but no labelling in binucleated trophoblast cells (BNCs; black arrows) or glycocalyx. (**B**) Nc-1 *N. caninum* strain-inoculated heifer. Very intense labelling in MNCs (red arrows) but no labelling in BNCs (black arrows) or glycocalyx. (**C**) Nc-6 Argentina *N. caninum*-strain inoculated heifer. Intense labelling in glycocalyx but no labelling in BNCs (black arrows) or MNCs (red arrows). (**D**) Nc Spain-7 *N. caninum* strain-inoculated heifer. Very intense labelling in glycocalyx but no labelling in BNCs (black arrows) or MNCs (red arrows).
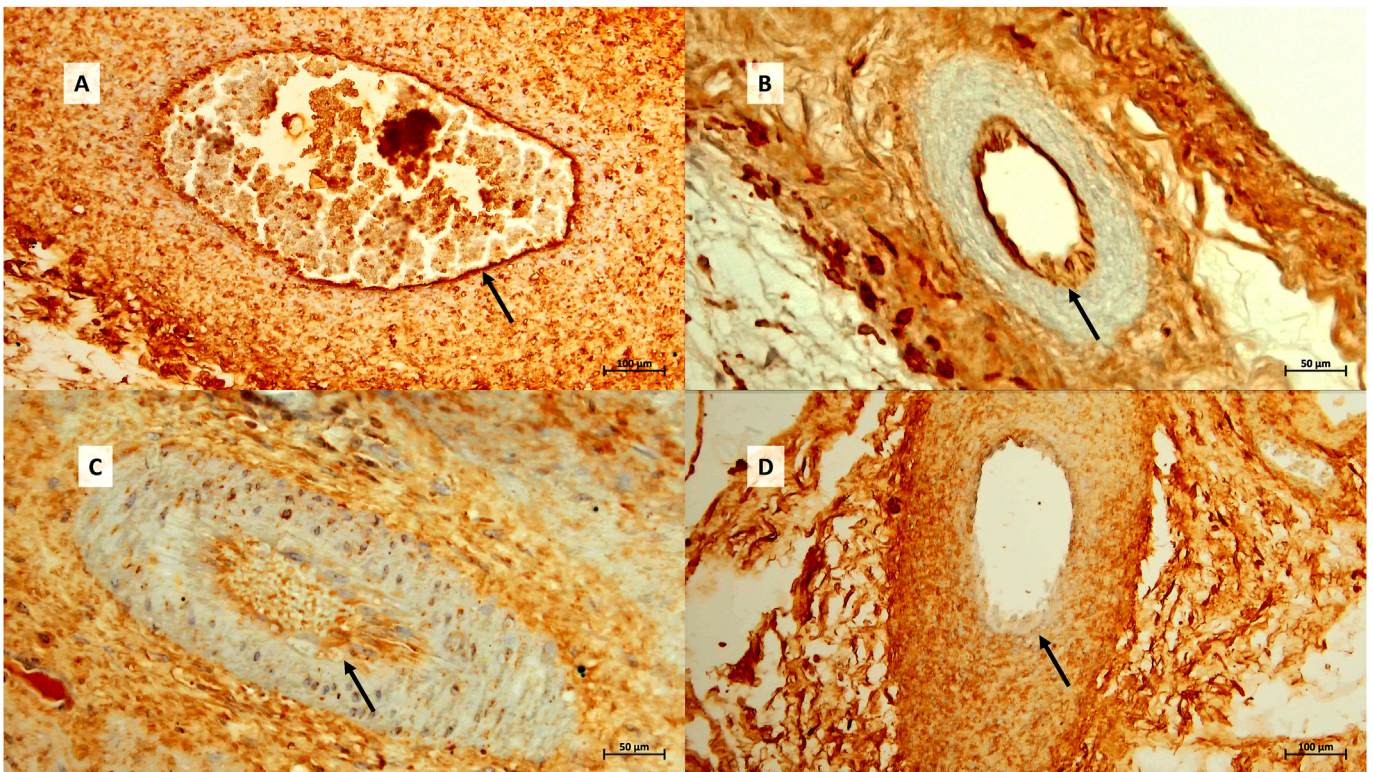

Fig. 3. *Neospora caninum* infection, placentae, cattle. PSA labelling in endothelium from *N. caninum*-infected and uninfected control heifers. (**A**) Uninfected heifer. Intense labelling in endothelial cells (arrow). (**B**) Nc-1 *N. caninum*-strain inoculated heifer. Mild labelling in endothelial cells (arrow). (**C**) Nc-6 Argentina *N. caninum*-strain inoculated heifer. Weak labelling in endothelial cells (arrow). (**D**) Nc Spain-7 *N. caninum*-strain inoculated heifer. No labelling in endothelial cells (arrow).
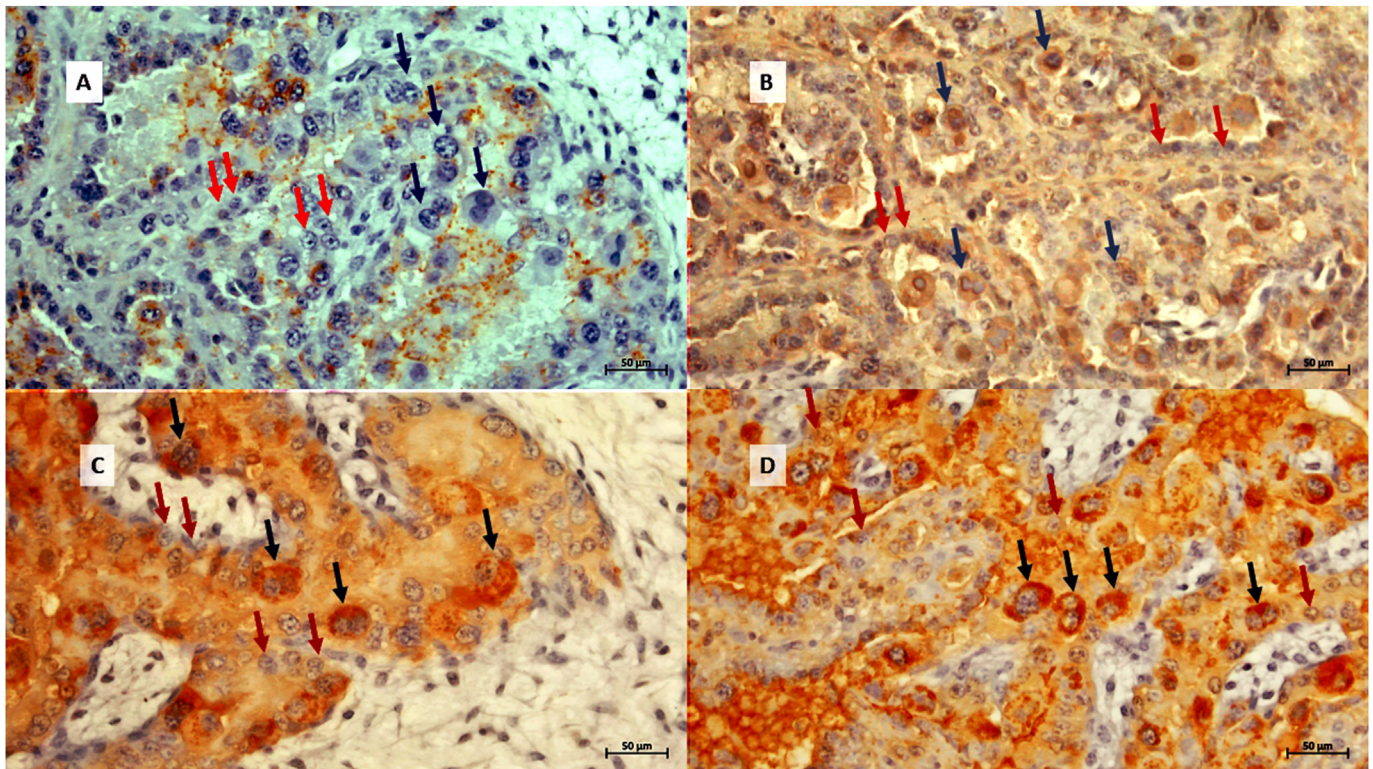
Fig. 4. *Neospora caninum* infection, placentae, cattle. RCA-I labelling in trophoblast layer from *N. caninum*-infected and uninfected heifers. (**A**) Uninfected heifer. Mild labelling in glycocalyx and weak labelling in mononuclear trophoblast cells (MNCs; red arrows) but no labelling in binucleated trophoblast cells (BNCs; black arrows), (**B**) Nc-1 *N. caninum* strain-inoculated heifer. Mild labelling in BNCs (black arrows) and glycocalyx and weak labelling in MNCs (red arrows). (**C**) Nc-6 Argentina *N. caninum*-strain inoculated heifer. Intense labelling in BNCs (black arrows) and mild labelling in glycocalyx and MNCs (red arrows). (**D**) Nc Spain-7 *N. caninum* strain-inoculated heifer. Intense labelling in BNCs (black arrows) and mild labelling in glycocalyx and MNCs (red arrows).

3. Results

3.1. Histopathological analysis

There were differences in the severity of histopathological lesions among groups (Table 2). Placentae from group B heifers had multifocal, necrotizing placentitis, while placental lesions in heifers from groups C and D were more severe and characterized by a diffuse, necrotizing placentitis with mineralization. Although maternal uterine crypt architecture remained largely intact, fetal trophoblast cells and villi had a variety of changes, ranging from acute degeneration to coagulative necrosis. These lesions consisted of serum leakage between the maternal and fetal tissues and diffuse necrosis with abundant necrotic cellular debris. Foci of mineralization were occasionally associated with severe and diffuse inflammatory cell infiltration chiefly comprising macrophages with fewer lymphocytes and polymorphonuclear neutrophils (PMNs). At the base of the maternal septa there were focal inflammatory lesions characterized by lymphocyte and macrophage infiltration that extended into the deeper connective tissue, and focal infiltrations of lymphocytes and macrophages surrounding uterine glands. In group A (negative control group) lesions were not observed. *N. caninum* DNA and antigen was detected in the placentae from heifers of groups B, C and D. All group A samples were negative for *N. caninum* (Table 2).

3.2. Lectin histochemistry

There were differences in intensity of labelling between the control and infected groups ($P < 0.05$). The intensity of lectin labelling was higher in group A in the gland secretion by CON-A and DBA and in endothelium by LCA. It was lower in the cytoplasm of epithelial uterine glands by SBA, mononucleate trophoblast cells (MNCs) by PSA and PHA-e, and binucleate trophoblast cells (BNCs) by PHA-e. Statistically significant differences between groups ($P < 0.05$) were also identified in the supranuclear cytoplasm of epithelial uterine glands by SWGA, WGA, PNA, CON-A, LCA, PSA, RCA-I, SBA, DBA and BSA-I, in the apical cytoplasm by PNA, CON-A, LCA, PSA, SBA, DBA, PHA-e and RCA-I, and in uterine gland secretion by LCA and CON-A (Fig. 1). Similar differences were found in chorioallantoic mesenchyme by LCA and CON-A, endothelium by LCA, PSA and SBA, BNCs by SWGA, LCA, RCA-I and BSA-I, glycocalyx by PNA, LCA, PSA, PHA-e, RCA-I and DBA, and MNCs by WGA, PNA, CON-A, LCA, SBA, RCA-I, DBA and BSA-I (Figs. 2–4). The results are summarized in Tables 3–5.

4. Discussion

In previous studies, LHC has proven to be a valuable tool for investigating differences in the glycoconjugate pattern resulting from challenge with agents that affect the reproductive system [23,26,41,42]. Changes in the pattern of LHC in bovine female genital tissues have been described for campylobacteriosis [43], trichomoniasis [23] and *N. caninum* infection [42]. Furthermore, variations in the pathogenicity of different strains of *Tritrichomonas foetus* were observed in a murine model [32]. However, this is the first study in which LHC has been used to analyse the intraspecies variation among three strains of *N. caninum* in bovine genital tract tissues.

Table 3
Estimated mean ± SEM of the intensity of lectin labelling in placentae and uteri of *N. caninum*-infected and uninfected heifers for succinylated wheat germ agglutinin (SWGA), wheat germ agglutinin (WGA), peanut agglutinin (PNA) and concanavalin-A (CON-A)

Site	SWGA				WGA				PNA				CON-A			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Uterine glands																
Supranuclear cytoplasm	0.63[a] ± 0.09	0.61[a] ± 0.09	0.34[a,b] ± 0.09	0.13[b] ± 0.06	1.60[a] ± 0.11	1.16[b] ± 0.13	0.22[c] ± 0.08	0.66[d] ± 0.14	0.30[a] ± 0.09	0.74[b] ± 0.23	0.00[a] ± 0.00	0.00[a] ± 0.00	1.77[a] ± 0.08	1.45[b] ± 0.09	1.88[a] ± 0.06	0.21[c] ± 0.08
Apical cytoplasm	0.70[a] ± 0.09	0.65[a] ± 0.09	0.78[a] ± 0.08	0.91[a] ± 0.15	2.27[a] ± 0.08	2.54[a] ± 0.09	2.37[a] ± 0.09	2.34[a] ± 0.09	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[b] ± 0.00	1.00[b] ± 0.00	2.40[a] ± 0.09	3.00[b] ± 0.00	3.00[b] ± 0.00	2.21[a] ± 0.08
Secretion	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	2.73[a] ± 0.08	2.01[b] ± 0.05	2.00[b] ± 0.00	2.00[b] ± 0.00
Chorion																
Mesenchyme	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	2.73[a] ± 0.12	1.87[b] ± 0.18	2.72[a] ± 0.12	1.28[c] ± 0.12
Connective tissue	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00
Endothelium	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00
Binucleated cells	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.19[b] ± 0.14	0.10[a] ± 0.06	0.25[a] ± 0.08	0.34[a] ± 0.14	0.16[a] ± 0.07	0.00[a] ± 0.00	0.00[a] ± 0.00	0.18[a] ± 0.14	0.38[a] ± 0.18	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00
Glycocalyx	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	2.00[b] ± 0.18	3.00[c] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00
Mononuclear cells	0.00[a] ± 0.00	0.00[a] ± 0.00	0.38[a] ± 0.18	0.38[a] ± 0.18	0.60[a] ± 0.16	0.10[a,b] ± 0.05	0.00[b] ± 0.00	0.66[a] ± 0.02	2.00[a] ± 0.26	2.23[a] ± 0.24	0.38[b] ± 0.18	0.00[b] ± 0.00	2.10[a] ± 0.06	2.35[a] ± 0.09	2.06[a] ± 0.05	2.66[b] ± 0.09

A, negative control; B, inoculated with Nc-1 strain: C, inoculated with Nc-6 Argentina strain; D, inoculated with Nc Spain-7 strain.
Statistically significant differences between the groups within each tissue are shown by letters (same letter denotes no statistically significant difference between the groups).

Table 4
Estimated mean ± SEM of the intensity of lectin labelling in placentae and uteri of *N. caninum*-infected and uninfected heifers for *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* erythroagglutinin (PHA-e) and *Ulex europaeus*-I (UEA-I)

Site	LCA				PSA				PHA-e				UEA-I			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Uterine glands																
Supranuclear cytoplasm	1.87[a] ± 0.97	2.03[a] ± 0.95	2.09[a] ± 0.82	0.72[b] ± 0.63	1.00[a] ± 0.00	3.00[b] ± 0.00	0.00[c] ± 0.00	0.00[c] ± 0.00	0.16[a] ± 0.08	0.52[a] ± 0.16	0.25[a] ± 0.08	0.41[a] ± 0.09	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00
Apical cytoplasm	0.97[a] ± 0.81	2.03[b] ± 0.80	1.00[a] ± 0.80	2.09[b] ± 0.89	1.00[a] ± 0.00	1.52[b] ± 0.09	0.50[c] ± 0.09	0.00[d] ± 0.00	0.00[a] ± 0.00	0.58[b] ± 0.17	1.00[c] ± 0.00	0.75[b,c] ± 0.08	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00
Secretion	3.00[a] ± 0.00	3.00[a] ± 0.00	2.06[b] ± 0.44	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.07	2.35[a] ± 0.00	2.34[a] ± 0.14	3.00[a] ± 0.06	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00
Chorion																
Mesenchyme	3.00[a] ± 0.00	2.00[b] ± 0.00	1.00[c] ± 0.00	2.00[b] ± 0.00	2.87[a] ± 0.10	2.74[a] ± 0.07	2.75[a] ± 0.05	2.88[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00
Connective tissue	1.00[a] ± 0.00	2.00[b] ± 0.09	1.00[a] ± 0.12	2.00[b] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00a ± 0.00	0.00a ± 0.00	0.00a ± 0.00
Endothelium	3.00[a] ± 0.00	1.48[b] ± 0.51	1.22[b] ± 0.68	1.50[b] ± 0.84	2.00[a] ± 0.15	1.77[a] ± 0.12	0.47[b] ± 0.09	0.28[b] ± 0.08	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00
Binucleated trophoblast cells	2.00[a] ± 0.00	0.00[b] ± 0.00	1.50[a] ± 1.43	1.96[a] ± 1.03	0.23[a] ± 0.10	0.26[a] ± 0.09	0.09[a] ± 0.05	0.06[a] ± 0.06	2.23[a] ± 0.14	3.00[b] ± 0.00	3.00[b] ± 0.00	3.00[b] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00
Glycocalyx	1.00[a] ± 0.00	3.00[b] ± 0.00	2.53[c] ± 0.50	3.00[b] ± 0.00	0.30[a] ± 0.10	0.06[ab] ± 0.07	0.09[ab] ± 0.05	0.00[b] ± 0.00	3.00[a] ± 0.00	2.35[b] ± 0.20	2.34[b] ± 0.22	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00
Mononuclear trophoblast cells	1.00[a] ± 0.00	0.00[b] ± 0.00	1.31[a] ± 0.28	1.50[a] ± 0.28	2.47[a] ± 0.15	2.84[b] ± 0.09	2.91[b] ± 0.05	2.91[b] ± 0.05	1.17[a] ± 0.14	3.00[b] ± 0.00	3.00[b] ± 0.00	3.00[b] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00

A, negative control; B, inoculated with Nc-1 strain: C, inoculated with Nc-6 Argentina strain; D, inoculated with Nc Spain-7 strain.
Statistically significant differences between the groups within each tissue are shown by letters (same letter denotes no statistically significant difference between the groups).

Table 5

Estimated mean ± SEM of the intensity of lectin labelling in placentae and uteri of *N. caninum*-infected and uninfected heifers for soybean agglutinin (SBA), *Ricinus communis* agglutinin (RCA-1), *Dolichos biflorus* agglutinin (DBA) and *Griffonia* (*Bandeiraea*) *simplifolia* agglutinin-I (BSA-I).

Site	SBA				RCA-I				DBA				BSA-I			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Uterine glands																
Supranuclear cytoplasm	2.20[a] ± 0.40	3.00[b] ± 0.00	3.00[b] ± 0.00	3.00[b] ± 0.00	1.00[a] ± 0.07	1.00[a] ± 0.00	1.00[a] ± 0.00	1.40[b] ± 0.51	2.00[a] ± 1.00	2.00[a] ± 0.00	2.63[b] ± 0.79	2.57[b] ± 0.54	2.00[a] ± 0.00	2.00[a] ± 0.00	2.00[a] ± 0.00	2.31[b] ± 0.93
Apical cytoplasm	2.50[b] ± 0.51	3.00[b] ± 0.00	3.00[b] ± 0.00	3.00[b] ± 0.00	1.17[a] ± 0.34	2.38[b] ± 0.49	2.03[c] ± 0.18	2.75[d] ± 0.44	1.03[a] ± 0.31	1.52[b] ± 0.57	2.56[c] ± 0.56	2.00[d] ± 0.51	2.20[a] ± 0.41	2.12[a] ± 0.34	2.21[a] ± 0.42	2.00[a] ± 0.67
Secretion	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	2.20[b] ± 1.21	1.00[b] ± 0.00	0.16[b] ± 0.37	0.47[b] ± 0.51	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00
Chorion																
Mesenchyme	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	2.07[a] ± 0.19	2.06[a] ± 0.18	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	2.07[a] ± 0.00	2.06[a] ± 0.00	2.13[a] ± 0.25	2.13[a] ± 0.00
Connective tissue	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.70[a] ± 0.47	0.71[a] ± 0.46	0.78[a] ± 0.42	0.69[a] ± 0.47
Endothelium	3.00[a] ± 0.00	2.65[b] ± 0.49	2.68[b] ± 0.47	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.73[a] ± 0.45	0.74[a] ± 0.44	0.69[a] ± 0.47	0.69[a] ± 0.47
Binucleate cells	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.53[a] ± 0.51	0.85[a] ± 0.44	2.78[b] ± 0.42	2.84[b] ± 0.40	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	0.46[a] ± 0.51	0.84[b] ± 0.37	0.00[c] ± 0.00	0.00[c] ± 0.00
Glycocalyx	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	3.00[a] ± 0.00	2.00[a] ± 0.01	1.50[a] ± 0.02	2.81[b] ± 0.40	3.00[c] ± 0.00	0.00[a] ± 0.00	0.87[b] ± 0.34	1.43[c] ± 0.56	2.37[d] ± 0.98	2.07[a] ± 1.50	2.06[b] ± 0.48	2.12[b] ± 0.50	2.13[b] ± 0.42
Mononuclear cells	0.13[a] ± 0.34	0.97[a] ± 0.34	1.13[b] ± 0.75	1.48[b]	1.00[a] ± 0.00	1.25[ab] ± 0.36	1.68[b] ± 0.96	2.53[c] ± 0.50	0.00[a] ± 0.00	0.00[a] ± 0.00	0.00[a] ± 0.00	0.34[b] ± 0.09	0.33[a] ± 0.76	0.06[a] ± 0.25	0.09[a] ± 0.30	0.19[a] ± 0.74

A, negative control; B, inoculated with Nc-1 strain; C, inoculated with Nc-6 Argentina strain; D, inoculated with Nc Spain-7 strain.

Statistically significant differences between the groups within each tissue are shown by letters (same letter denotes no statistically significant difference between the groups).

Variations in the lectin binding pattern for the same mammalian species have been reported by various investigators. These may be related to different stages of gestation, making it essential to define a standard pattern to determine pathological changes in tissues of the reproductive tract [23,44]. This study was designed to ensure that any differences in the lectin binding patterns between groups were related to the strains of *N. caninum* used Therefore, all heifers, including the control animals, were of similar age, came from the same herd and were raised under similar sanitary conditions. Furthermore, all heifers were mated within a 24-h period, thereby facilitating precise determination of the stage of pregnancy and elimination of the possibility of variation in the pattern of lectin binding due to the stage of pregnancy. Additionally, randomly assigning heifers to each group reduced the scope for potential idiosyncratic variation between animals.

Experimental infection with *N. caninum* in early gestation was associated with cellular damage and inflammatory infiltration in both the maternal and fetal components of the placentome, with a predilection for blood vessels of the placental septum and plates and the fetal villus [8,10,45,46]. Typically, the lesions associated with this agent comprise multifocal to diffuse severe non-suppurative and necrotizing placentitis [10,40,45,47,48]. In this study, the groups infected with the Nc-6 Argentina and Nc Spain-7 strains had more severe placental lesions than the Nc-1 inoculated groups and the control group. The more severe placentitis in these groups was associated with lower lectin binding to α-Man (bound by LCA and PSA) on vascular endothelium and to α1-D-Gal (bound by BSA-I). A lower level of α-Man (bound by LCA, CON-A and PSA) has been related to intrauterine infection and preterm birth in humans [49,50], which suggests that there may also be an association between these glycoside changes in blood vessels and *N. caninum* infection (Fig. 2).

Some adhesin proteins with Gal/GalNAc residues (bound by SBA, DBA and PNA) play an essential role in adhesion, invasion and intracellular stages in sporozoites of other protozoa such as *Cryptosporidium parvum* and *Entamoeba histolytica* [51,52]. Those galectins bind β-galactose (β-Gal) and are associated with interaction with host membrane glycans to form a cell−surface network for optimal endocytosis [53,54]. Differences in lectin labelling of endothelium (by SBA), apical cytoplasm of epithelial cells of endometrial glands (by DBA) and glycocalyx and mononuclear cells (by PNA) were observed among groups and may be related to the passage of the parasite through the different tissues from maternal blood to trophoblast cells (Fig. 3). This could also be associated with the more severe necrosis observed in MNCs than in BNCs, which may indicate the preference of *N. caninum* for those cells during placental infection. Furthermore, the difference in the α-D-GlcNAc binding (by WGA) in the MNCs suggests interaction between those cells and the parasite. α-D-GlcNAc has a role in complement activation and as an opsonin and phagocyte receptor in humans and mice [55−57]. The differences in MNCs observed among groups may indicate an intracellular function of the parasite for modulating immune activation and opsonization by reducing the superficial expression of this molecule to avoid the immune response.

BNCs expressed N-acetyl-galactosamine residues recognized by SBA and DBA and α-D-man bound by CON-A, as reported [20,44,58], with no differences between groups. Although differences in CON-A binding have been reported in heifers infected with *N. caninum* at mid-gestation (150 ± 7 days of gestation) [42], no differences were found between any of the strains evaluated when they were inoculated at early gestation (70 days of gestation). On the contrary, differences in the expression of bisected complex N-Linked sequences identified by PHA-e and RCA-I in BNCs were found between control and infected groups (Fig. 4).

The significant changes in the lectin labelling patterns in the placentae of the inoculated heifers in this study suggest that infection of heifers with *N. caninum* during early pregnancy produces carbohydrate expression modifications, demonstrable by LHC. These changes may induce alterations in placental function, which could be incompatible with gestation. Furthermore, the differences observed in this study between infected groups may also indicate that infection with different strains of *N. caninum* can produce lesions of different severity, which are also associated with a change in the expression of carbohydrates in the tissues. The histopathological lesions produced in the trophoblast cells in the group inoculated with strains Nc Spain-7 and Nc-6 Argentina were more severe than those in the group inoculated with the reference strain Nc-1. In addition to these differences, a lectin binding pattern was also found in the trophoblast cells, which may indicate strain differences in pathogenesis. These differences in lectin binding patterns could be due to intraspecific differences among isolates and may be associated with different pregnancy outcomes and the severity of the fetopathy between strains of *N. caninum*, as reported [9,10,15,59].

5. Conclusion

The differences in pathogenesis and lectin binding pattern in the placentae suggest that abortion resulting from *N. caninum* infection in early gestation may be associated with changes in the cell structure of the placenta, resulting in variable severity of lesions and, consequently, different pregnancy outcomes in *N. caninum*-infected heifers.

Ethical statement

This study was carried out in strict accordance with the Animals (Scientific Procedures) Res No. 16/07 Disp. 174/09 of the Argentinian National Institute of Agricultural Technology (CICUAE−INTA). The experimental protocol was approved by the Animal Ethics Committee of CICUAE-INTA. The number of animals was determined according to the 3R principles of animal research (replacement, reduction and refinement). All animals used in this study were handled in strict accordance with good animal practices and conditions, fed on natural pasture grass in one paddock and maintained using standard animal husbandry procedures. Clean water was available ad libitum. Veterinary monitoring was performed daily for any clinical signs, and treatment for any secondary disease was administered following standard veterinary practices.

Declaration of competing interests

The authors declared no conflicts of interest in relation to the research, authorship or publication of this article. They also declared no financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

References

[1] Donahoe SL, Lindsay SA, Krockenberger M, Phalen D, Šlapeta J. A review of neosporosis and pathologic findings of *Neospora caninum* infection in wildlife. Int J Parasitol Parasites Wildl 2015;4(2):216−38.

[2] Dubey JP, Schares G, Ortega-Mora LM. Epidemiology and control of neosporosis and *Neospora caninum*. Clin Microbiol Rev 2007;20(2):323−67.

[3] Buxton D, McAllister MM, Dubey JP. The comparative pathogenesis of neosporosis. Trends Parasitol 2002;18(12):546−52.

[4] Dubey J. Neosporosis in cattle. J Parasitol 2003;89:S42−56.

[5] Atkinson R, Harper PA, Reichel MP, Ellis JT. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. Parasitol Today 2000;16(3):110−4.

[6] Schock A, Innes EA, Yamane I, Latham SM, Waastling JM. Genetic and biological diversity among isolates of *Neospora caninum*. Parasitology 2001;123(1): 13−23.

[7] Quinn HE, Miller CMD, Ryce C, Windsor PA, Ellis JT. Characterization of an outbred pregnant mouse model of *Neospora caninum* infection. J Parasitol 2002;88(4):691−6.

[8] Wouda W, Moen AR, Schukken YH. Abortion risk in progeny of cows after a *Neospora caninum* epidemic. Theriogenology 1998;49(7):1311−6.

[9] Regidor-Cerrillo J, Gómez-Bautista M, Del Pozo I, Jiménez-Ruiz E, Aduriz G, Ortega-Mora LM. Influence of *Neospora caninum* intra-specific variability in the outcome of infection in a pregnant BALB/c mouse model. Vet Res 2010;41(4):52.

[10] Caspe SG, Moore DP, Leunda MR, Cano DB, Lischinsky L, Regidor-Cerrillo J, et al. The *Neospora caninum*-Spain 7 isolate induces placental damage, fetal death and abortion in cattle when inoculated in early gestation. Vet Parasitol 2012;189(2−4):171−81.

[11] Rico-San Román L, Horcajo P, Regidor-Cerrillo J, Fernández-Escobar M, Collantes-Fernández E, Gutiérrez-Blázquez D, et al. Comparative tachyzoite proteome analyses among six *Neospora caninum* isolates with different virulence. Int J Parasitol 2020;50(5):377−88.

[12] Regidor-Cerrillo J, Pedraza-Díaz S, Gómez-Bautista M, Ortega-Mora LM. Multilocus microsatellite analysis reveals extensive genetic diversity in *Neospora caninum*. J Parasitol 2006;92(3):517−24.

[13] Regidor-Cerrillo J, García-Lunar P, Pastor-Fernández I, Álvarez-Garcia G, Collantes-Fernández E, Gómez-Bautista M. *Neospora caninum* tachyzoite immunome study reveals differences among three biologically different isolates. Vet Parasitol 2015;212(3-4):92−9.

[14] Pedraza-Díaz S, Marugán-Hernández V, Collantes-Fernández E, Regidor-Cerrillo J, Rojo-Montejo S, Gómez-Bautista M, et al. Microsatellite markers for the molecular characterization of *Neospora caninum*: application to clinical samples. Vet Parasitol 2009;166(1-2):38−46.

[15] Rojo-Montejo S, Collantes-Fernández E, Regidor-Cerrillo J, Alvarez-García G, Marugan-Hernández V, Pedraza-Díaz S, et al. Isolation and characterization of a bovine isolate of *Neospora caninum* with low virulence. Vet Parasitol 2009;159(1):7−16.

[16] García-Sánchez M, Jiménez-Pelayo L, Vázquez P, Horcajo P, Regidor-Cerrillo J, Jiménez-Meléndez A. Maternal and foetal cellular immune responses in dams infected with high- and low-virulence isolates of *Neospora caninum* at mid-gestation. Front Cell Infect Microbiol 2021;11:684670.

[17] Dong J, Zhang N, Zhao P, Li J, Cao L, Wang X, et al. Disruption of dense granular protein 2 (GRA2) decreases the virulence of *Neospora caninum*. Front Vet Sci 2021;8:634612.

[18] García-Sánchez M, Jiménez-Pelayo L, Horcajo P, Collantes-Fernández E, Ortga-Mora LM, Regidor-Cerrillo J. *Neospora caninum* infection induces an isolate virulence-dependent pro-inflammatory gene expression profile in bovine monocyte-derived macrophages. Parasites Vectors 2020;13(1):374.

[19] Fuchs N, Sonda K, Bütikofer P, Hemphill A. Detection of surface-associated and intracellular glycoconjugates and glycoproteins in *Neospora caninum* tachyzoites. Int J Parasitol 1999;29(10):1597−611.

[20] Munson L, Kao JJ, Schlafer DH. Characterization of glycoconjugates in the bovine endometrium and chorion by lectin histochemistry. J Reprod Fertil 1989;87(2):509−17.

[21] Sharon N, Lis H. Lectins. In: Lennarz WJ, Lane MD, editors. Encyclopedia of biological chemistry. 2nd ed. Waltham: Academic Press; 2013. p. 701−5.

[22] Jiménez-Pelayo L, García-Sánchez M, Regidor-Cerrillo J, Horcajo P, Collantes-Fernández E, Gómez-Bautista M, et al. Differential susceptibility of bovine caruncular and trophoblast cell lines to infection with high and low virulence isolates of *Neospora caninum*. Parasites Vectors 2017;10(1):463.

[23] Cobo ER, Campero CM, Gimeno EJ, Barbeito CG. Lectin binding patterns and immunohistochemical antigen detection in the genitalia of *Tritrichomonas foetus*-infected heifers. J Comp Pathol 2004;131(2−3):127−34.

[24] Doumecq ML, Soto P, Casalina MB, Gimeno EJ, Barbeito CG, Monteaevaro CE. Variation in the saccharide lectin binding pattern from different isolates of *Tritrichomonas foetus*. Exp Parasitol 2014;147:48−53.

[25] Woodwyk MA, Gimeno EJ, Soto P, Barbeito CG, Monteavaro CE. Lectin binding pattern in the uterus of pregnant mice infected with *Tritrichomonas foetus*. J Comp Pathol 2013;149(2−3):341−5.

[26] Monteavaro CE, Soto P, Gimeno EJ, Echevarría HM, Catena M, Portiansky EL, et al. Histological and lectin binding changes in the genital tract of mice infected with *Tritrichomonas foetus*. J Comp Pathol 2008;138(1):40—5.

[27] Monteavaro CE, Aguirre JI, Soto P, Echevarría HM, Catena MC, Portiansky EL, et al. Interaction of *Tritrichomonas foetus* with the reproductive tract of experimentally infected female BALB/c mice: ultrastructural evaluation. Vet J 2007;173(1):204—8.

[28] Fiorentino MA, Paolicchi FA, Campero CM, Barbeito CG. Lectin binding patterns and immunohistochemical antigen detection in placenta and lungs of *Brucella abortus*-bovine infected fetuses. Open Vet J 2018;8(1):57—63.

[29] Sun X, Jiamin J, Zhang Y-L, Ma Y, Zhang S, Tong X. Decreased histidine-rich glycoprotein and increased complement C4-B protein levels in follicular fluid predict the IVF outcomes of recurrent spontaneous abortion. Clin Proteomics 2022;19(1):47.

[30] Campero CM, Moore DP, Odeón AC, Cipolla AL, Odriozola E. Aetiology of bovine abortion in Argentina. Vet Res Commun 2003;27(5):359—69.

[31] Lindsay DS, Dubey JP. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am J Vet Res 1989;50(11):1981—3.

[32] Falcon JE, Rivero MB, Doumecq ML, Soto P, Monteavaro CE, Woudwyk MA, et al. Variations in the carbohydrate expression pattern and in lesions of the uterine horns of BALB/c mice infected with different *Tritrichomonas foetus* isolates. Res Vet Sci 2022;152:633—9.

[33] R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2021.

[34] Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Software 2015;67(1):1—48.

[35] Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. R J 2017;9(2):378—400.

[36] Lenth RV. Estimated marginal means, aka least-squares means. R package version 1.7.3. Available from: https://CRAN.R-project.org/package=emmeans; 2022.

[37] Fox J, Weisberg S. An R companion to applied regression. 3rd ed. Thousand Oaks: Sage Publications; 2019.

[38] Hartig F. DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models. R package version 0.4.5 Available from: https://CRAN.R-project.org/package=DHARMa; 2022.

[39] Lüdecke D, Ben-Shachar MS, Patil I, Waggoner P, Makowski D. performance: an R package for assessment, comparison and testing of statistical models. J Open Source Softw 2021;6(60):3139.

[40] Buxton D, Maley SW, Wright S, Thomson KM, Rae AG, Innes EA. The pathogenesis of experimental neosporosis in pregnant sheep. J Comp Pathol 1998;118(4):267—79.

[41] Morrell EL, Moore DP, Odeón AC, Poso MA, Odriozola E, Cantón G, et al. Retrospective study of bovine neonatal mortality: cases reported from INTA Balcarce, Argentina. Rev Argent Microbiol 2008;40(3):151—7.

[42] Dorsch MA, de Yaniz MG, Fiorani F, Hecker YP, Odeón AC, Morrell EL, et al. A descriptive study of lectin histochemistry of the placenta in cattle following inoculation of *Neospora caninum*. J Comp Pathol 2019;166:45—53.

[43] Cipolla AL, Paolicchi FA, Poso MA, Morsella CG, Casaro AP, Massone AR, et al. Lectin-binding sites in uterus and oviduct of normal and *Campylobacter fetus* subspecies *venerealis*-infected heifers. Eur J Histochem 1998;42(1):63—70.

[44] Caspe SG, Konrad JL, Moore DP, Sala JM, Lischinsky L, Campero AM, et al. A comparative pattern of lectin-binding in the endometrial glands of the uterus and placenta of healthy buffaloes and bovines at early gestation. Biocell 2021;45(2):331—7.

[45] Bacigalupe D, Basso W, Caspe SG, Moré G, Lischinsky L, Gos ML, et al. *Neospora caninum* NC-6 Argentina induces fetopathy in both serologically positive and negative experimentally inoculated pregnant dams. Parasitol Res 2013;112(7):2585—92.

[46] Barr BC, Rowe JD, Sverlow KW, BonDurant RH, Ardans AA, Oliver MN, et al. Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. J Vet Diagn Invest 1994;6(2):207—15.

[47] Maley SW, Buxton D, Rae AG, Wright SE, Schock A, Bartley PM, et al. The pathogenesis of neosporosis in pregnant cattle: inoculation at mid-gestation. J Comp Pathol 2003;129(2—3):186—95.

[48] Buxton D, Barlow RM, Finlayson J, Anderson IE, Mackellar A. Observations on the pathogenesis of *Chlamydia psittaci* infection of pregnant sheep. J Comp Pathol 1990;102(2):221—37.

[49] Wang LK, Huang M-C, Liu C-C, Chen C-P. Second-trimester plasma mannose-binding lectin levels and risk of preterm birth. J Matern Fetal Neonatal Med 2017;30(6):678—83.

[50] Çalkavur Ş, Erdemir G, Onay H, Köroğlu ÖA, Yalaz M, Zekioğlu O, et al. Mannose-binding lectin may affect pregnancy outcome. Turk J Pediatr 2015;57(1):26—33.

[51] Bhat N, Joe A, PereiraPerrin M, Ward HD. Cryptosporidium p30, a galactose/N-acetylgalactosamine-specific lectin, mediates infection in vitro. J Biol Chem 2007;282(48):34877—87.

[52] Cornick S, Chadee K. *Entamoeba histolytica*: host parasite interactions at the colonic epithelium. Tissue Barriers 2017;5(1):e1283386.

[53] Liu FT, Rabinovich GA. Galectins as modulators of tumour progression. Nat Rev Cancer 2005;5(1):29—41.

[54] Nieminen J, Kuno A, Hirabayashi J, Sato S. Visualization of galectin-3 oligo-merization on the surface of neutrophils and endothelial cells using fluores-cence resonance energy transfer. J Biol Chem 2007;282(2):1374—83.

[55] Matsushita M. Ficolins: complement-activating lectins involved in innate immunity. J Innate Immun 2010;2(1):24—32.

[56] Liu FT. Regulatory roles of galectins in the immune response. Int Arch Allergy Immunol 2005;136(4):385—400.

[57] Lowe JB. Glycan-dependent leukocyte adhesion and recruitment in inflam-mation. Curr Opin Cell Biol 2003;15(5):531—8.

[58] Jones CJ, Wilsher SA, FBP Wooding, Benirschke K, Allen WR. The binucleate cell of okapi and giraffe placenta shows distinctive glycosylation compared with other ruminants: a lectin histochemical study. Mol Phylogenet Evol 2015;83:184—90.

[59] Pereira García-Melo D, Regidor-Cerrillo J, Collantes-Fernández E, Aquado-Martínez A, Del Pozo I, Minquijón E, et al. Pathogenic characterization in mice of *Neospora caninum* isolates obtained from asymptomatic calves. Parasitology 2010;137(7):1057—68.