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Research paper

A *Neospora caninum* vaccine using recombinant proteins fails to prevent foetal infection in pregnant cattle after experimental intravenous challenge

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ABSTRACT

The aim of the present study was to evaluate the immunogenicity and protective efficacy of rNcSAG1, rNcHSP20 and rNcGRA7 recombinant proteins formulated with immune stimulating complexes (ISCOMs) in pregnant heifers against vertical transmission of *Neospora caninum*. Twelve pregnant heifers were divided into 3 groups of 4 heifers each, receiving different formulations before mating. Immunogens were administered twice subcutaneously: group A animals were inoculated with three recombinant proteins (rNcSAG1, rNcHSP20, rNcGRA7) formulated with ISCOMs; group B animals received ISCOM-MATRIX (without antigen) and group C received sterile phosphate-buffered saline (PBS) only. The recombinant proteins were expressed in *Escherichia coli* and purified nickel resin. All groups were intravenously challenged with the NC-1 strain of *N. caninum* at Day 70 of gestation and dams slaughtered at week 17 of the experiment. Heifers from group A developed specific antibodies against rNcSAG1, rNcHSP20 and rNcGRA7 prior to the challenge. Following immunization, an statistically significant increase of antibodies against rNcSAG1 and rNcHSP20 in all animals of group A was detected compared to animals in groups B and C at weeks 5, 13 and 16 ($P < 0.001$). Levels of antibodies against rNcGRA7 were statistical higher in group A animals when compared with groups B and C at weeks 5 and 16 ($P > 0.001$). There were no differences in IFN-γ production among the experimental groups at any time point ($P > 0.05$). Transplacental transmission was determined in all foetuses of groups A, B and C by Western blot, immunohistochemistry and nested PCR. This work showed that rNcSAG1, rNcHSP20 and rNcGRA7 proteins while immunogenic in cattle failed to prevent the foetal infection in pregnant cattle challenged at Day 70 of gestation.

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1. Introduction

Neospora caninum is an intracellular apicomplexan parasite that causes abortion in cattle (Dubey and Schares, 2011). This leads to significant financial losses world-wide (Reichel et al., 2013). Transplacental transmission of the parasite from an infected dam to its foetus is the major natural route of infection (Dubey et al., 2007). Strategies for the control of this disease are based on general biosecurity procedures that aim interrupt the life cycle of the parasite and the culling of seropositive cattle, if economically viable (Reichel et al., 2014). Thus, the economic importance of neosporosis, especially in cattle, is evident and the development additional control measures, such as treatment or vaccination are urgently needed.

Protective immune responses against *N. caninum* in the host are associated with a T helper 1 immune response, mediated by cytotoxic T lymphocytes and the production of interferon-gamma (IFN-γ), interleukin-12 (IL-12), tumour necrosis factor (TNF) and immunoglobulin G_2 (IgG_2) (Staska et al., 2003; Innes, 2007). The main challenge will be to develop a vaccination strategy that will prevent *N. caninum* vertical transmission, and this vaccination strategy must result in a balanced immune response that is compatible with pregnancy. Although promising results were obtained by immunization with live parasites (Innes et al., 2001; Williams et al., 2007; Weber et al., 2013; Hecker et al., 2013), a vaccine based on a cocktail of defined antigens produced with recombinant gene technology has many advantages, such as safety, controllable composition, quality and simplified production (Reichel and Ellis, 2009).

A limited number of recombinant proteins have been investigated as vaccine candidates against bovine neosporosis. These include mostly immune-dominant proteins, such as the major surface antigens and proteins localized in secretory organelles. NcSAG1 is an immunodominant surface protein, involved in low-affinity contact between tachyzoite and host cell surface membrane (Hemphill et al., 2006). Several authors have shown that polyclonal and monoclonal antibodies directed against this surface antigen and SRS2 inhibited host cell adhesion and invasion (Hemphill et al., 1999; Nishikawa et al., 2000; Haldorson et al., 2005). It has been reported that the immunization of mice with recombinant SAG1 showed significant protection against cerebral infection with *N. caninum* (Cannas et al., 2003), but the immunogenicity of this protein has not yet been evaluated in cattle.

Small heat shock proteins (sHSPs) are recognized for their participation in protein trafficking, signal transduction and cytoskeleton dynamics being their principal function to assist in the folding of newly formed proteins during synthesis. De Miguel et al. (2005) identified at least five sHSPs in *Toxoplasma gondii.* One of them, HSP20, was reported to be localized on the outer leaflet of the inner membrane complex (IMC) and at the conoid of *T. gondii* (De Miguel et al., 2005, 2008). HSP20 was also observed in this subcellular location in *N. caninum.* Vonlaufen et al. (2008) and Cóceres et al. (2012) suggested that HSP20 could have a similar role in different apicomplexan parasites because this protein has a highly conserved structure. Montero et al. (2008) reported that antibodies against *Babesia divergens* HSP20 blocked that parasites growth. In addition, Cóceres et al. (2012) showed that rabbit anti-*T. gondii* HSP20 antibodies reduced gliding motility and invasion of not only *T. gondii* but also *N. caninum.* Furthermore, *B. bovis* HSP20 was found to be recognized by $CD4^+$ T lymphocytes from cattle that have recovered from infection and thus, HSP20 was proposed as a candidate vaccine antigen for inclusion in a vaccine for babesiosis (Norimine et al., 2004); we consider that the protective immunity role of NcHSP20 should be analyzed.

The dense granules are globular organelles containing molecules that are secreted shortly after the invasion of host cells (Hemphill et al., 1999). In addition, a number of dense granule proteins such as NcGRA1, NcGRA2 and NcGRA7 are secreted during *in vitro* stage conversion and are incorporated into the cyst wall (Vonlaufen et al., 2004). The dense granule protein NcGRA7 has shown be an immunodominant antigen which is highly immunogenic and associated with active replication of the parasite (Jenkins et al., 1997; Huang et al., 2007). It has shown promising results with regard to protection against *N. caninum* challenge in mice (Liddell et al., 2003; Jenkins et al., 2004) and cattle (Nishimura et al., 2013). Nishimura et al. (2013) demonstrated that M3-NcGRA7 could induce protective immune response in a model using male calves seronegative to *N. caninum*. As a pregnant cattle model would be more suitable for evaluating the vaccine efficacy against *N. caninum*, in the present study we have tried to evaluate the efficacy of rNcGRA7 against vertical transmission in pregnant cattle challenged with *N. caninum*.

Numerous adjuvants have been evaluated in the formulation of inactivated vaccines against *N. caninum* (Andrianarivo et al., 1999; Moore et al., 2005; Williams et al., 2007). Immune stimulating complexes (ISCOMs) are 40 nm nanoparticles used as delivery system for vaccine antigens. The ISCOMs are made up of saponin, cholesterol, lipids and antigen (Morein et al., 2004). Immune stimulating complexes (ISCOMs) have been successfully used in the development of vaccines for ruminants (Morein et al., 2004). Promising results have been obtained in terms of immunogenicity and IFN-γ levels when using inactivated *Neospora* immunogens in calves (Moore et al., 2011). Moreover, partial protection against tissue cyst formation was provided by using crude rhoptries antigens from *T. gondii* formulated in ISCOMs in pigs (García et al., 2005).

Based on the mentioned previous studies, the aim of this study was to test whether a cocktail of NcSAG1, NcHSP20 and NcGRA7 recombinant proteins formulated with ISCOMs are able to stimulate an immune response that is protective against vertical transmission in pregnant cattle experimentally challenged.

2. Materials and methods

2.1. Recombinant protein production and vaccine preparation

N. caninum isolate used in the current study was strain NC-1 (Dubey et al., 1988). The cloning and purification of the recombinant protein NcSAG1 (rNcSAG1) was already described by Wilkowsky et al. (2011). Briefly, rNcSAG1

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was cloned and expressed only as a fragment of SAG-1. The truncated Nc-SAG1 gene does not include the hydrophobic signal peptide and the C-terminus as previously described (Chahan et al., 2003). The Nc-SAG1 gene was amplified by PCR using the oligonucleotide primers Nc-SAG1F (5′-GGCATCAGAAAAATCACC-3′) and Nc-SAG1R (5′-GACCAACATTTTCAGCCG-3′). Then, rNcSAG1 was cloned into the prokaryotic expression vector pRSETC (Invitrogen Corporation, Carlsbad, CA) and expressed as polyhistidine-tagged fusion proteins in *Escherichia coli*. rNcSAG1 was extracted from the inclusion bodies, resuspended in 2 ml of 8 M urea and incubated at 37 °C. After this, rNcSAG1 was purified from the soluble fraction with a nickel resin in denaturing conditions (Wilkowsky et al., 2011).

For the cloning of the open reading frames of NcHSP20 and NcGRA7 gene, the genomic DNA of purified *N. caninum* tachyzoites NC-1 strain (1×10^8) was extracted using a commercial kit as previously described (Cóceres et al., 2010). The NcHSP20 gene was amplified by PCR using the oligonucleotide primers NcHSP20F (5′-CACCATGAGTTGCTGTGGCGGCGCC-3′) and NcHSP20R (5′-TTACTTCTTTGACCCCTTCTC-3′). The NcGRA7 gene was amplified using the oligonucleotide primers NcGRA7F (5′-CACCATGCAAAGTTGTGGGTGCCA-3′) and NcGRA7R (5′-TTCGGTGTCTACTTCCTGCTC-3′). For production of *N. caninum* recombinant proteins NcHSP20 (rNcHSP20) and NcGRA7 (rNcGRA7), the corresponding gene fragments were cloned into expression vector pET 200 (Champion pET Directional TOPO Expression Kits, Invitrogen) and expressed in BL21 (DE3) bacteria. Protein expression was induced with 1 mM isopropylthio-β-galactoside (IPTG) at 37 °C for 4 h. Bacteria expressing rNcHSP20 and rNcGRA7 were lysed with lysis buffer pH 8 (300 mM NaCl, 50 mM $NaH_2PO_4 + H_2O$, 10 mM imidazole). Lysates were sonicated, centrifuged and the supernatants were collected and directly loaded onto a Ni–NTA column previously equilibrated with lysis buffer. The column was washed four times with washing buffer (300 mM NaCl, 50 mM $NaH_2PO_4 + H_2O$, 30 mM imidazole) and bound rNcHSP20 and rNcGRA7 were eluted with elution buffer (300 mM NaCl, 50 mM $NaH_2PO_4 + H_2O$, 250 mM imidazole). The purity of all recombinant proteins was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and staining with Coomassie brilliant blue (Fig. 1). Finally, protein concentrations were determined by the Bradford's method (Bradford, 1976).

For the immunizations of cattle in this trial, each vaccine dose was formulated as a mixture containing 30 μg of each protein (90 μg total recombinant proteins/dose) and 200 μl of ISCOMs (with approximately 750 μg/dose) according to the manufacturer's instructions (Abisco-300, ISCONOVA, Uppsala, Sweden). The ISCOMs were kindly provided by Dr. Morein. Formulations were maintained in an insulated box at room temperature (rt) for 1 h until vaccination.

2.2. Parasite culture

N. caninum NC1 tachyzoites were maintained *in vitro* by continuous passage in Vero cells using standard procedures described previously (Dubey et al., 1988) and were harvested when 80% of cells were infected. For challenge, NC-1 tachyzoites were harvested and viability was determined by counting in a haemocytometer. Organism numbers were adjusted to a concentration of 4.7×10^7 in a final volume of 3 ml of PBS, and used immediately to infect animals.

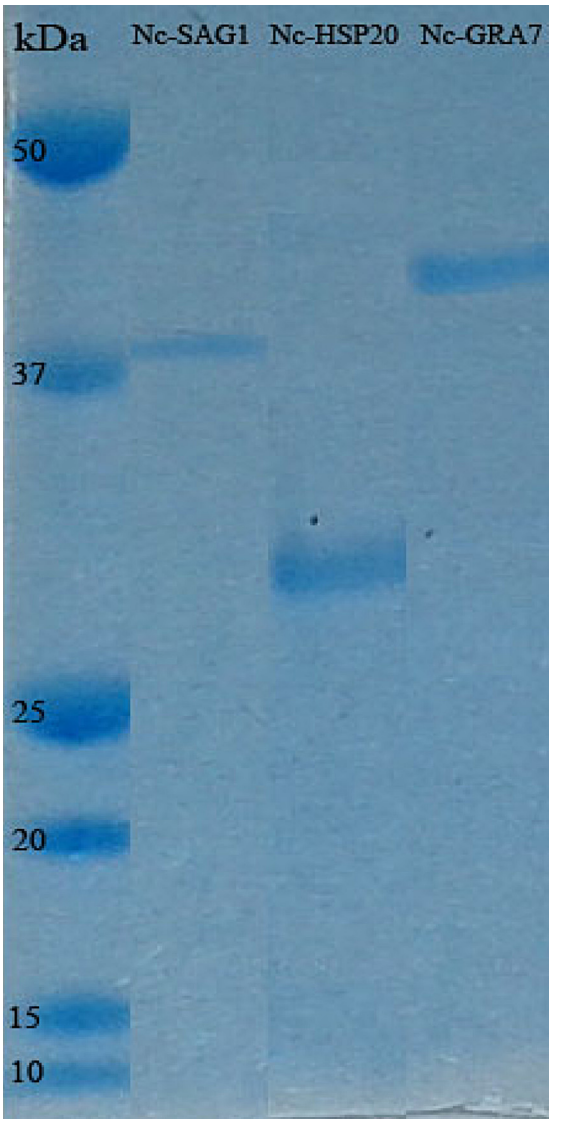

Fig. 1. Expression of *N. caninum* recombinant antigens, expressed fused to a 6-His tag at the N-terminus in *E. coli*, purified by Ni–NTA resin, run onto a SDS–PAGE and stained with Coomassie brilliant blue. On the left side, molecular weight markers are labelled in kDa.

2.3. Animals and experimental design

Twenty-two seronegative 22 month old Angus heifers were selected in the experiment. The heifers belonged to a beef herd located at INTA Balcarce, Argentina. Heifers were bled monthly for a year prior to breeding and their seronegative status to *N. caninum* was confirmed using an indirect fluorescent antibody test (IFAT) (negative at

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1:25) (Venturini et al., 1999). In addition, heifers were seronegative to *T. gondii* in a micro-agglutination test (Desmonts and Remington, 1980), and by serum neutralization tests for Bovine Viral Diarrhoea virus (BVDV) and Bovine Herpesvirus-1 (BHV-1). The herd was also bovine brucellosis and tuberculosis free and a vaccination programme against Foot and Mouth disease routinely performed.

Heifers were in good body condition and maintained on pasture. Animals had adequate genital development and were cycling at the start of the trial. Routine clinical examination, pelvic area measurements and vaccination with inactivated vaccines against BVDV-1 and BHV-1 (Bioabortigen, Biogénesis Bagó, Argentina) were performed twice three months before breeding.

Cattle were randomly allocated into three groups (Fig. 2). Group A heifers ($n=9$) were immunized subcutaneously (sc) in the neck region with 2 doses of a vaccine designed with a mixture of rNcSAG1, rNcHSP20 and rNcGRA7 and ISCOMs 3 weeks apart, four weeks before breeding. Group B heifers ($n=5$) included heifers given sc ISCOM-MATRIX (without antigen) following the same immunization regimen as group A to evaluate immune response to adjuvant alone. Finally, group C ($n=8$) were mock inoculated sc with sterile PBS to ensure that PBS, used for vaccine formulation, did not produce any reaction. All animals were bred 4 weeks after the first immunization (Fig. 2). Local inflammatory reactions at the injection site were evaluated daily for one week after each inoculation.

Heifers were oestrus synchronized using cloprostenol according to the manufacturer's instructions (D cloprostenol, Tecnofarm®, Argentina), then allocated into pens with four healthy Angus bulls (campylobacteriosis and trichomoniasis-free) for natural breeding over 7 days. All the animals were fed on natural pasture and housed in dog- and fox-proof accommodations. Clean water was always available. Pregnancy was confirmed by transrectal ultrasonography at 35 days after mating. From the 22 cattle, 11 pregnant animals carrying single foetuses and one of the dams with twins were selected for the experiment.

All groups were challenged intravenously (iv) with 4.7×10^7 NC-1 tachyzoites at Day 70 of gestation. An aliquot of each parasite inoculum was seeded in VERO cells to confirm parasite viability. Parasite growth was observed *in vitro* after 5 days. Foetal viability was confirmed by using ultrasonography every week after NC-1 tachyzoite challenge until slaughter at the 17th week of the experiment (104 days of pregnancy). All animals used in this study were handled in strict accordance with good animal practice and the conditions defined by the Animal Ethics Committee at INTA Balcarce. All efforts were made to minimize suffering during slaughter.

2.4. Samples of dams

Blood samples (40 ml), with and without heparin, were collected from the jugular vein for serological and cellular immune response assays at weeks 0, 5, 13 and 16 of the trial.

Blood samples with heparin were centrifuged 40 min at $4000\times g$ to remove plasma; the layer of peripheral blood mononuclear cells (PBMC) was recovered. Cells were treated with ammonium chloride and centrifuged for 15 min at $2000\times g$ (Corpa et al., 2001). Finally, PBMC were cryopreserved with 20% bovine foetal serum and 5% dimethyl sulfoxide (DMSO) at held at −80 °C until use. Blood samples without heparin were centrifuged at $1600\times g$ for 10 min and serum was kept at −20 °C.

2.5. Western blot analysis

Anti *N. caninum*-specific antibodies were detected in sera of all animals as described (Álvarez García et al., 2002). Briefly, purified rNcSAG1, rNcHSP20 or rNcGRA7 were transferred from 12% SDS-PAGE gels to nitrocellulose membrane (Immobilon-NC, 0.45 μm, Millipore, USA) for 120 min at 30 V using a standard protocol. Then, membranes were washed in Tris–phosphate-buffered with 0.05% Tween-20 (TBS-T) and incubated overnight in blocking buffer (TBS-T containing 5% (w/v) skimmed milk). After being washed in TBS-T, the membranes were incubated with each bovine serum diluted to 1:100 in blocking buffer for 60 min at rt. Membrane was washed 3 times with TBS-T and then incubated with an anti-bovine IgG HRP-conjugated rabbit antibody (Sigma Chemical Co) at a dilution of 1:2000. Following incubation, membranes were washed and antigen–antibody reaction was visualized using 4-chloro-1-naphthol (Sigma Chemical Co).

2.6. Indirect ELISA for detecting specific IgG and its subisotypes

N. caninum-specific antibodies were determined by indirect ELISA (iELISA) as previously described (Cóceres et al., 2010) with brief modifications. Briefly, polystyrene 96-well microtitre plates (Nalge Nunc International) were coated with 2–5 μg/ml of the different recombinant proteins diluted in 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4 °C. One hundred microlitres of bovine serum diluted to 1:100 in blocking solution (5% milk in 100 mM Tris–HCl, pH 7.5, 0.9% NaCl, 0.1% TBS-T) was added to each well per duplicate. One hundred microlitres of a 1/5000 dilution of peroxidase-conjugated anti-bovine IgG polyclonal antibody (Sigma–Aldrich) diluted in blocking solution was added to each well, and incubated for 30 min at 37 °C. The peroxidase activity was detected with H_2O_2 and o-phenylenediamine dihydrochloride (OPD, Sigma–Aldrich, Argentina) for 30 min at room temperature. The reaction was stopped by the addition of 1 N H_2SO_4. Optical density (OD) at 415 nm was read in a microplate reader (Kayto model RT-2100C). The cut-off OD value for each protein was determined as the mean OD415 nm value plus two standard deviations for sera from week 0.

In addition, anti-bovine IgG_1 or IgG_2 mAbs (1:100; Serotec™, Oxford, UK) were used as described (Aguado-Martínez et al., 2009; Moore et al., 2011). One microgram of solubilized *N. caninum* tachyzoite antigens diluted in 0.06 M carbonate/bicarbonate buffer (pH 9.6) was distributed and adsorbed to each flat bottom well of 96-well plates (Polysorp, Nunc). For IgG_1 and IgG_2, a kinetic reading was determined at an OD 405 when the *N. caninum* high-positive control with anti-IgG_1 reached

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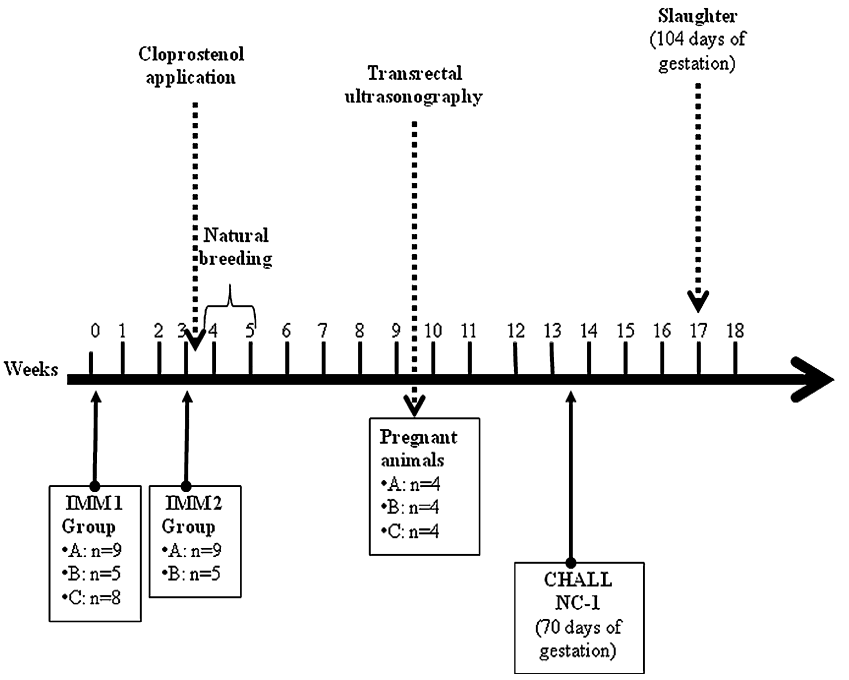

Fig. 2. Schematic of the experimental design over time. This figure shows immunization (IMM1) mix of rNcSAG1, rNcHSP20 and rNcGRA7 formulated in ISCOMs (group A) (IMM1, IMM2) and challenge (CHALL) with *N. caninum* NC-1 tachyzoites at Day 70 of gestation.

1.0 ± 25%. Data were expressed as a ratio of OD values for IgG_1OD/IgG_2OD.

2.7. IFN-γ assay

Immune stimulation was performed as previously described (Serrano-Martínez et al., 2007) with brief modifications. Briefly, duplicate aliquots of 0.9 ml of heparinized blood, collected from each heifer at weeks 0, 5, 13 and 16 were cultured in duplicate wells in 24-well tissue culture plates (Cellstar Greiner, Monroe, USA). Cells were also cultured with 0.1 ml of PBS (unstimulated control) or concanavalin A (Con-A, Sigma) at 10 μg/ml (positive control) or with rNcSAG1, rNcHSP20 and rNcGRA7 (10 μg/ml). Plates were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere as previously mentioned (Nishimura et al., 2013). Culture supernatants were collected and assessed for IFN-γ content using a commercial sandwich iELISA (BOVIGAM, CSL, Victoria, Australia) following manufacturer's instructions. IFN-γ concentrations in supernatants were analyzed using a spectrophotometer (Labsystems Multiskan Plus). A standard curve, derived from a series of dilutions of a recombinant bovine IFN-γ standard (Serotec™, Oxford, UK), was used to estimate IFN-γ levels in the test samples.

2.8. Phenotypic analysis of PBMC using flow cytometry

Percentages of T-cells ($CD4^+$, $CD8^+$ and $\gamma\delta^+$) and monocytes in PBMC were analyzed using flow cytometry after immune-labelling with mAbs specific for bovine leucocyte subpopulations: CC8 (IgG_{2a}, anti-CD4), CC63 (IgG_{2a}, anti-CD8), CC-G33 (IgG_1, anti-CD14), CC15 (IgG_{2a}, anti-$\gamma\delta^+$) (AbD Serotec) and secondary antibodies rat anti-bovine IgG isotypes conjugated with FITC or PE (AbD Serotec).

Cell suspensions (5×10^5 cells/well) were incubated with mAbs diluted in PBS at 4 °C for 20 min. Cells were then washed with PBS, re-suspended in 100 μl of fluorescence-activated cell sorting (FACS) buffer flow (Becton, Dickinson and Company, Franklin Lakes, USA) and fixed with 0.05% formaldehyde. Isotype controls were used as negative controls. Analysis was performed using a FAC Scan cytometer and Cell Quest software (Becton, Dickinson and Company). Ten thousand cells were analyzed and the results expressed as the percentage of positively immunolabelled cells.

2.9. Necropsy, sampling, histopathology and immunohistochemistry

Following slaughter, foetal and placental tissues were immediately removed and examined following standard gross pathology procedures (Campero et al., 2003). Foetal length was estimated using crown-rump measurement (Kirkbride, 1986).

Foetal tissue samples including lung, spleen and abomasal content were extracted to determine presence of aerobic and microaerophilic organisms, BVDV and BHV, *Campylobacter fetus* and *Tritrichomonas foetus*, according to methods described by Campero et al. (2003). Foetal cavity fluids were collected and stored at −20 °C until assessment by immunoblotting. Placental and foetal tissue samples were collected for DNA extraction and histopathological analysis. Foetal tissues, including whole central nervous system (CNS), liver, heart, lung, striated muscle, periorbital muscle, adrenal gland, kidney, small and large intestine, and spleen, were collected and fixed in 10% buffered

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formalin, processed by standard methods and embedded in paraffin-wax blocks. Five μm-thick sections of tissue were cut, mounted on glass microscope slides and stained with haematoxylin and eosin (H&E). Similarly, placental samples were taken from different placentomes and the inter-cotyledonary chorion (5 samples of every region). Maternal CNS samples were also taken and analyzed.

Based on the severity of lesions, four scores for histological lesions were established: absent (0); mild (1); moderate (2) and severe (3) according to Caspe et al. (2012). Similarly, a pathology score was calculated by adding individual lesion scores assessed in the CNS, heart, lung, liver and placenta (minimum score = 0; maximum score = 15). Finally, a mean foetal pathology score from each group was calculated and the foetal pathology score was correlated with the ratio $CD4^+/CD8^+$ of dams before the challenge.

Tissues where *N. caninum*-compatible lesions were observed were selected and analyzed by immunohistochemistry (IHC) using a polyclonal antibody rabbit against NC-1 tachyzoites (kindly provided by Dr. Mark Anderson, UCDavis, Davis, USA) and an avidin–biotin complex procedure (ABC) (Vectastain Elite ABC kit; Vector Laboratories, USA) as previously described (Campero et al., 2003).

2.10. Immunoblotting analysis of foetal fluids

N. caninum-specific antibodies were detected in foetal fluids as described (Álvarez García et al., 2002). Briefly, nitrocellulose membranes (Immobilon-NC, 0.45 μm, Millipore, USA) with *N. caninum*-native antigen were incubated with foetal fluids diluted to 1:50 and then incubated with an anti-bovine IgG HRP-conjugated rabbit antibody (Sigma Chemical Co) at a dilution of 1:2000. Following incubation, membranes were washed and antigen-antibody reaction was visualized using 4-chloro-1-naphthol (Sigma Chemical Co).

2.11. Nested PCR (nPCR)

DNA was isolated from tissue samples using a commercially available kit according to the manufacturer's recommendations (DNeasy Tissue Kit, QIAGEN Group, Germany). DNA concentration was measured using an Epoch micro-volume spectrophotometer system (Epoc, Bioteck® instruments, Inc., Vermont, USA) and samples were diluted to a final concentration of 60 ng/μl for DNA detection by nPCR. For detection of parasite DNA, an nPCR on the internal transcribed spacer (ITS1) region of *N. caninum* was carried out with four oligonucleotides as described by Buxton et al. (1998). Secondary amplification products were visualized by electrophoresis in 2% agarose gel and ethidium bromide staining. DNA equivalent to 10^2 tachyzoites was used as the positive PCR control. To avoid false positive reactions, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips and disposable gloves. Moreover, negative control samples were included in each set of DNA extractions and PCRs.

2.12. Statistical analysis

OD values from serum antibody responses and IFN-γ production 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).

Parasite detection frequency was compared by Fisher's exact test. Lesion score values were analyzed using the Kruskal–Wallis test followed by a non-parametric multiple-comparison test. A correlation was calculated between the ratio of $CD4^+/CD8^+$ cells from each dam and the pathology score of its foetus. All statistical analyses were performed using Graph-Pad Prism 5 v.5.01 (GraphPad Software, San Diego, CA, USA).

3. Results

No apparent clinical signs were observed in any heifer throughout the study. No significant local reactions were observed at the site of injection in heifers receiving the experimental vaccine.

3.1. Serum antibody responses

3.1.1. Western blot analysis

Detection of rNcSAG1, rNcHSP20 and rNcGRA7 antigens is summarized in Fig. 3. Antibodies against the three recombinant proteins were detected in group A animals at week 5 and persisted until week 16 of the assay (Fig. 3b and c) and were absent at week 0 (Fig. 3a). Animals inoculated either with ISCOM-MATRIX (group B) (Fig. 3d) or sterile PBS alone (group C) (Fig. 3e) never produced antibodies against any of the proteins.

3.1.2. iELISA

The antibody responses in heifers analyzed by iELISA are shown in Fig. 4. Following immunization, a highly statistically significant increase in antibody levels against rNcSAG-1 and NcHSP20 were noted in all animals of group A compared to animals from group B and C at weeks 5, 13 and 16 ($P < 0.001$) (Fig. 4a and b). Similar results were obtained for IgG responses against NcGRA7 although the statistically significant differences compared to groups B and C were observed at week 5 and week 16 ($P < 0.001$) (Fig. 4c), only.

3.1.3. IgG subisotypes

The IgG1, IgG2 Neospora-specific antibodies values and ratio of IgG1/IgG2 subclass in response to immunization of animals in group A are shown in Table 1. Heifers from group A showed increased ratios close to 2 following the second immunization (week 5) and after challenge the ratio increased to above 4 (week 16). By week 16 (3 weeks post challenge) heifers from group B and C had ratios close to 1.

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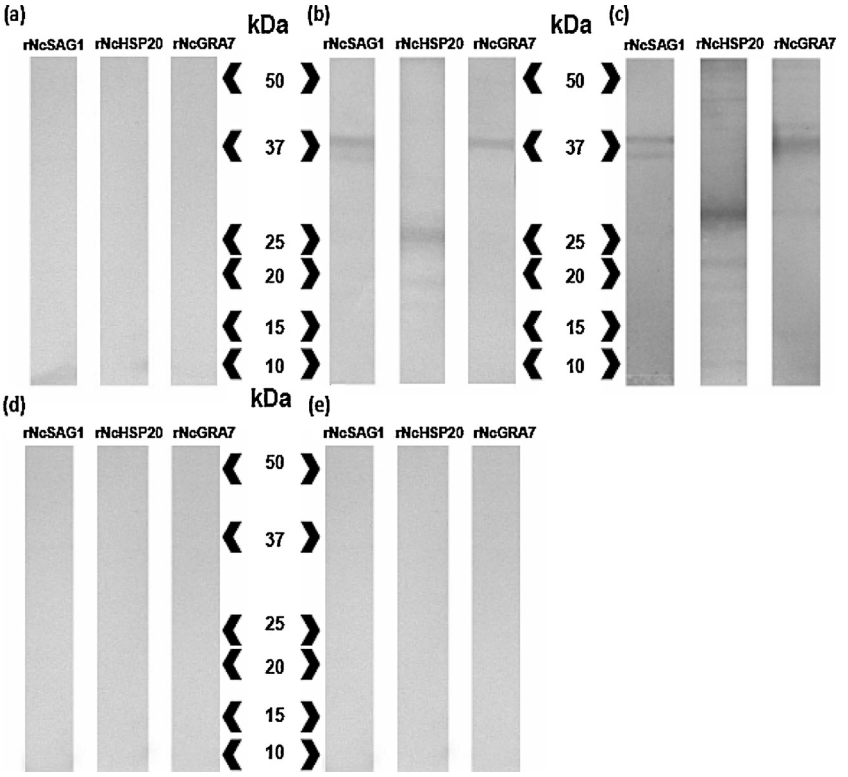

Fig. 3. Examples of immunoblot analysis of rNcSAG1, rNcHSP20 and rNcGRA7. (a) Sera from animals of group A at week 0, (b) sera from animals of group A at week 5, (c) sera from animals of group A at week 16, (d) sera from animals of group B at week 13, (e) sera from animals of group C at week 13.

Table 1
Neospora-specific antibodies values (IgG1, IgG2) and IgG1/IgG2 ratio in response to immunization of animals in group A ($n=4$). Immunizations were performed at weeks 0 and 3, the challenge was performed at week 13.

Animal		Weeks			
		0	5	13	16
1	IgG1	0.15	0.28	0.18	0.77
	IgG2	0.13	0.11	0.10	0.18
	IgG1/IgG2	1.20	2.50	1.70	4.17
2	IgG1	0.12	0.15	0.19	0.74
	IgG2	0.10	0.09	0.10	0.16
	IgG1/IgG2	1.28	1.66	1.84	4.67
3	IgG1	0.14	0.19	0.17	0.68
	IgG2	0.11	0.13	0.11	0.14
	IgG1/IgG2	1.32	1.49	1.51	4.98
4	IgG1	0.14	0.18	0.17	0.78
	IgG2	0.01	0.11	0.11	0.16
	IgG1/IgG2	1.45	1.70	1.57	4.92

3.2. Cellular immune response

3.2.1. IFN-γ production

There was neither any specific IFN-γ production nor differences in the responses among the experimental groups A, B and C at week 0, 5, 13 and 16 ($P>0.05$). IFN-γ production in PBMC stimulated with PBS was similar to that observed in whole blood stimulated with rNcSAG1, rNcHSP20 and rNcGRA7 at all time points throughout the experiment ($P>0.05$). Production of IFN-γ in PBMC incubated with Con A was high in all groups at week 0, 5, 13 and 16. Neither group nor time differences in IFN-γ levels produced by Con A stimulated blood samples were observed ($P>0.05$).

3.2.2. Phenotypic analysis of PBMC using flow cytometry

A decrease in the percentages of $CD4^+$ cells in heifers from groups A, B and C was observed over time ($P<0.001$) (Fig. 5). This change was more evident after challenge in heifers from group A (week 16). However, this decrease was never significantly different from groups B and C ($P>0.05$). Percentages of $CD8^+$ cells were similar throughout the experiment in heifers from groups A, B and C ($P>0.05$) (Fig. 5).

$CD4^+/CD8^+$ percentage ratios were analyzed throughout the experiment. At all times, there were significant differences in the $CD4^+/CD8^+$ percentage ratios among groups. The $CD4^+/CD8^+$ ratio observed in groups A and B before the NC-1 challenge (week 13) were lower compared with those observed in heifer of group C. After challenge (week 16) the $CD4^+/CD8^+$ ratios increased in all groups but this change was not significant. In groups A and B the ratios increased slightly post challenge and later decreased again.

The percentages of $\gamma\delta^+$ T cells and monocytes did not change between treatments or over time ($P>0.05$).

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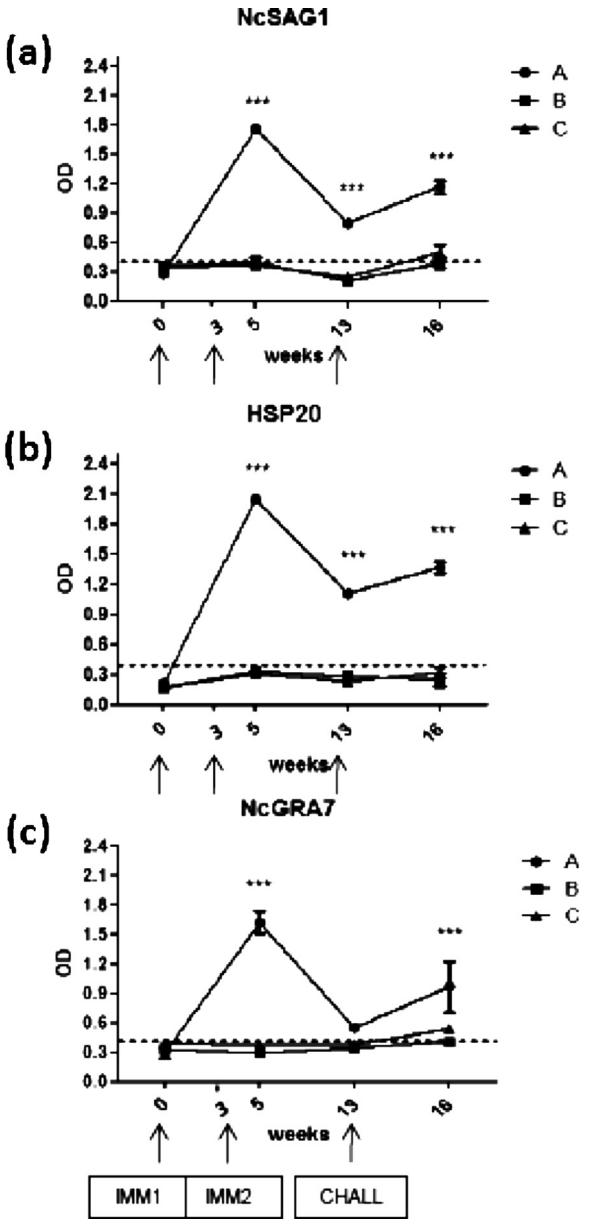

Fig. 4. Antibody responses in cattle after two immunizations (IMM1, IMM2) with the experimental vaccine (group A), ISCOM-Matrix (group B) or PBS control (group C) and subsequently challenged (CHALL) with *N. caninum* NC-1 tachyzoites on Day 70 of gestation. (a) Level of serum antibody to NcSAG1. (b) Level of serum antibody to NcHSP20. (c) Level of serum antibody to NcGRA7. The horizontal dotted lines represent the cut-off OD value determined as the mean OD415 nm (plus two standard deviations) value for sera at the week 0 sampling for each protein. $^{*}P < 0.05$, $^{***}P < 0.001$.

3.3. Foetal study

All foetuses were viable and no gross lesions were observed following slaughter and *post mortem* examination. One of the dams from group A delivered twins (#6a and #6b foetuses).

When foetal and placental histopathology was performed, *N. caninum* characteristic lesions were scored according to severity (Table 2). Moderate lesions (grade 2) in lung, heart and placenta were observed in all foetuses recovered from group A. However, only mild lesions (grade 1) were detected in the CNS of all foetuses in that group. On the other hand, foetuses from groups B and C showed lesions ranging from absent to severe. Histopathological lesions were not observed in maternal CNS samples. The lowest mean pathology score was observed in group A (mean = 5.80; standard error = ±0.83) when compared with groups B (6.50; ±1.29) and C (7.00; ±0.81), however that difference was not statistically significant ($P > 0.05$).

IHC analysis showed positive labelling in 3/5 foetuses from group A in the CNS sample (#4, #6b), placenta (#6a, #6b) and in another foetus from group B in lung (#1) (Fig. 6a) and placenta (#19). In addition, positive *N. caninum* labelling was detected in the placenta of two dams from group C (Fig. 6b). A 17–18 kDa antigen was detected by western blot in 4/5 foetuses from group A and 2/4 foetuses from group B and 2/4 foetuses from group C (Table 2).

N. caninum DNA was detected more frequently using Nested PCR (nPCR) in foetal CNS and lung, as well as placental caruncles where *N. caninum* DNA was amplified from all the foetuses from groups A, B and C (Table 2), respectively. There were not significant differences in the frequency of detection of *Neospora* DNA from caruncles: groups A (7/16), B (8/16) and C (12/16) ($P > 0.05$).

From each group, 16 foetal CNS samples were collected and *N. caninum* DNA was amplified in 7, 6 and 3 samples from groups A, B and C, respectively, however these differences were not statistically significant ($P > 0.05$) (Fig. 7). Similarly, when the foetal lung samples were analyzed, parasitic DNA was amplified in 16 out of 16 samples from group A and 12 and 9 out of 16 in groups B and C, respectively. These results were not significantly different ($P > 0.05$).

4. Discussion

In the present study, we evaluated the immunogenicity and protective efficacy against vertical transmission of three *N. caninum* recombinant antigens (NcSAG1, NcHSP20 and NcGRA7) formulated in ISCOMs in a pregnant bovine model. However, the results showed that although rNcSAG1, rNcHSP20 and rNcGRA7 were immunogenic, they did not prevent vertical transmission in cattle.

Previous works has reported that antibodies against NcSAG1 inhibited parasite invasion into host cells (Cho et al., 2005) and the immunization with recombinant NcSAG1 showed significant protection against cerebral *N. caninum* infection in mice (Cannas et al., 2003). It has also been mentioned that the immunization with NcGRA7 DNA and a booster with its recombinant form showed partial protection in gerbils (Cho et al., 2005) and mice (Liddell et al., 2003; Jenkins et al., 2004; Vemulapalli et al., 2007). In addition, Nishikawa et al. (2009) demonstrated that NcGRA7 entrapped in liposomes coated with mannotriose (M3-NcGRA7) could induce parasite-specific Th1 immune responses and humoral antibodies that resulted in increased offspring survival and decreased infection rates in the brains of mouse dams immunized before pregnancy. Moreover, Nishimura et al. (2013) demonstrated

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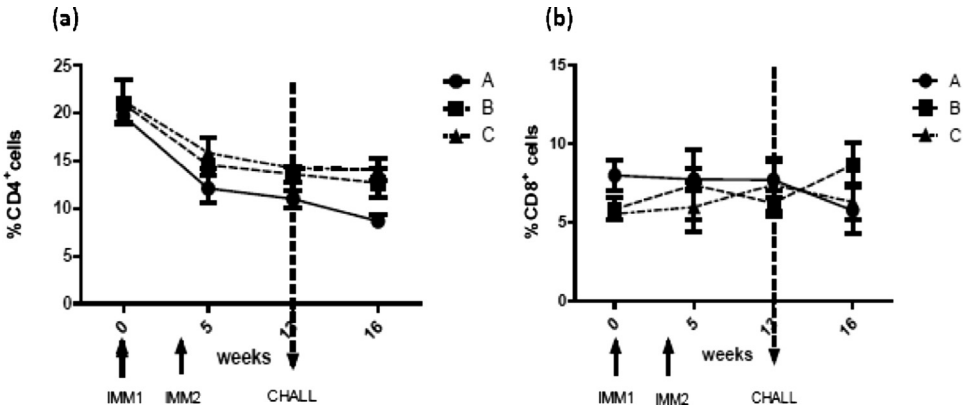

Fig. 5. Percentages of $CD4^+$ and $CD8^+$ cells in cattle after two immunizations (IMM1, IMM2) with recombinant vaccines (group A), ISCOMs (group B) or PBS (group C) and subsequent challenge (CHALL) with *N. caninum* NC-1 tachyzoites on Day 70 of gestation.

Table 2
Results obtained from foetuses in groups immunized twice with either recombinant antigens (group A), ISCOMS (group B) or PBS (group C) by Western blot, PCR, immunohistochemistry and histopathology of different tissues.

Groups	Treatment	Foetus #	Antibodies in foetal fluids by WB	nPCR	IHC	Pathology scores				Mean pathology score
						CNS	Heart	Lung	Placenta	
A	rNcSAG1, rNcHSP20, rNcGRA7 and ISCOMs	4	+	+	+	1	1	2	2	5.8
		6a	–	+	+	1	2	2	2	
		6b	+	+	+	0	2	1	2	
		13	+	+	–	1	1	2	1	
		14	+	+	–	1	2	2	1	
B	ISCOMs	1	+	+	+	0	2	2	2	6.5
		7	+	+	–	0	2	3	2	
		18	–	+	–	1	2	1	1	
		19	–	+	+	2	2	2	2	
C	PBS	2	+	+	–	0	2	3	2	7.0
		3	–	+	–	1	2	2	2	
		11	+	+	+	1	1	2	2	
		16	–	+	+	1	2	2	3	

that oligomannose-coated liposome-entrapped NcGRA7 induces protective immune response to *N. caninum* including antigen specific antibodies, IFN-γ production and low parasite load in brain of calves challenged with live parasites. In the present study, we evaluated the immunogenicity of NcGRA7 with a model of vaccination-challenge in pregnant heifers and although the animals developed antigen specific antibodies against NcGRA7, there was not

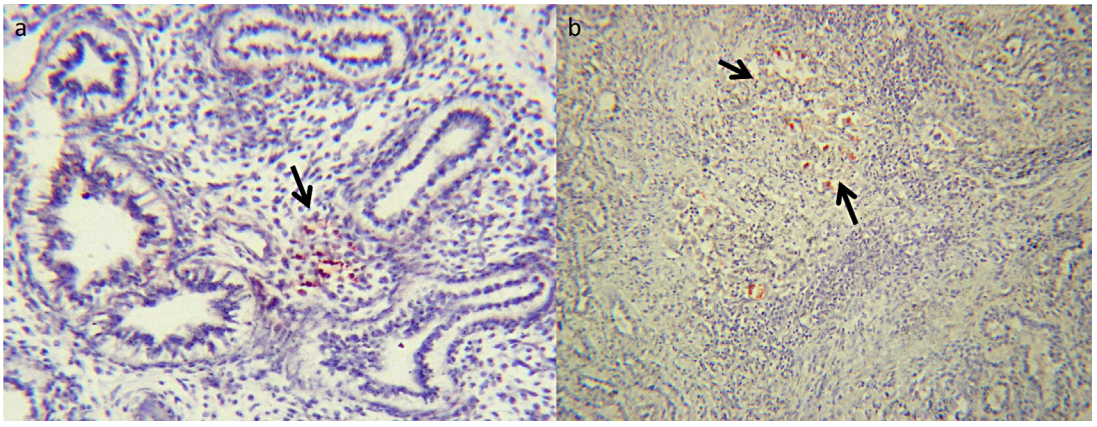

Fig. 6. Immunohistochemistry performed using a polyclonal antibody rabbit against NC-1 tachyzoites and Avidin Biotin Complex technique. Immunostaining was visualized with amino-ethylcarbazol substrate (Dako Inc.), and sections were counterstained with Mayer's haematoxylin (Sigma Diagnostics, St. Louis, MO). Arrows indicating clusters of *N. caninum* tachyzoites detected by in the lung (a) of a foetus from group B and the placenta (b) of a dam from group C (100×).

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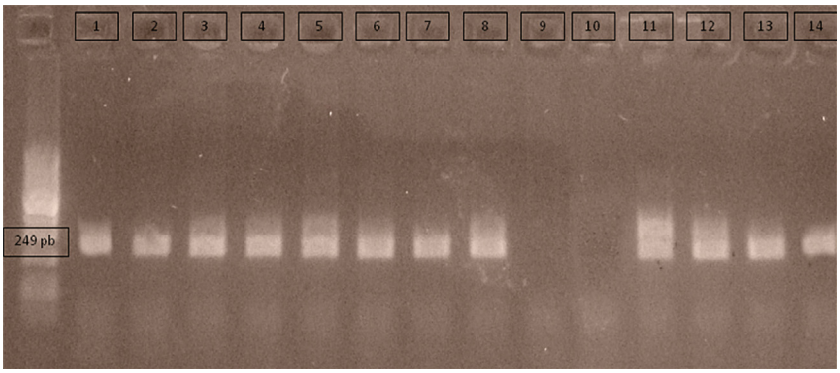

Fig. 7. Agarose gel showing positive PCR amplifications of *N. caninum*. Lanes 1, 2, 3, 4, 5, 6, 7, 8 11, 12 and 13 are showing positive samples from the CNS of foetuses from group A (#4, #6a, #6b, #13 and #14), group B (#1, #7, #18 and #19) and group C (#11 and #16), respectively. Lanes 9 and 10 are negative controls of the first and second round of the nPCR. Lane 14 is showing the positive control.

IFN-γ production and protection against foetal infection. In agreement with our findings, however Aguado-Martínez et al. (2009) reported no significant protection against cerebral infection or vertical transmission in a mouse model when the efficacy of the recombinant proteins rNcSAG4 and rNcGRA7 was assessed. Finally, rNcHSP20 was used for the first time in the present study and although this protein was immunogenic, it failed to confer significant protection against vertical transmission when used in combination with rNcSAG1, rNcGRA7 and ISCOMs.

Different IgG isotypes and IFN-γ levels have been detected in mouse and bovine models receiving inactivated and recombinant vaccines (Andrianarivo et al., 1999, 2000; Moore et al., 2005, 2011; Pinitkiatisakul et al., 2007; Baszler et al., 2008; Aguado-Martínez et al., 2009; Hecker et al., 2013). In the present study, the ratio of IgG_1/IgG_2 was higher than 2 at all times, indicating predominant levels of IgG_1. There was neither specific any IFN-γ production nor differences in the responses between the experimental groups at week 0, 5, 13 and 16. The predominant levels of IgG_1 and the absent of IFN-γ production may be associated with vertical transmission.

Recently, we observed that pregnant heifers that receive an extract soluble antigen formulated in ISCOMs, showed a decrease in $CD4^+$ and $CD8^+$ levels two weeks after the second dose (Hecker et al., 2013). In the current study, a decrease in percentages of $CD4^+$ cells in heifers from groups A, B and C was observed over time, however percentages of $CD8^+$ cells were similar throughout the experiment in heifers from all groups. Possibly, the reason of decrease in $CD4^+$ T cells in groups B and C could be due to pregnancy effect; unfortunately, unchallenged pregnant females were not evaluated in this study. The $CD4^+/CD8^+$ ratio observed in groups A and B before the NC-1 challenge (week 13) were lower compared with those observed in heifers of group C. This increment of T $CD8^+$ lymphocytes or decrease in T $CD4^+$ lymphocytes in specimens from groups A and B would be related to the ISCOM formulation rather than the recombinant proteins.

The similar leucocyte populations observed in the experimental groups after immunization were associated with similar pathology score in foetuses. The low ratio $CD4^+/CD8^+$ observed in all groups may partially explain the high pathological score in foetal tissues. $CD4^+$ cytotoxic T-cells and IFN-γ are associated to control *N. caninum* infection (Staska et al., 2003; Williams et al., 2007) but those parameters were not observed in animals receiving the experimental formulation.

In this study all foetuses were viable at the time of slaughter and had similar pathology scores. Infection was determined in all foetuses of all groups suggesting that there was not protection against vertical transmission in any dam. The lack of abortion in this study might be explained by the moderate pathogenicity of the NC-1 strain (Dubey et al., 1988); however, a similar dose sufficient to evaluate vertical transmission was used in previous work (Hecker et al., 2013). Although vertical transmission has been more effectively evaluated when challenge is performed in the last trimester of pregnancy (Williams et al., 2000), we tried to evaluate the pathology score in foetal tissues by challenging in early gestation. Moreover, Caspe et al. (2012) reported foetal serological immune responses in bovine foetuses from dams experimentally challenged on Day 65 of gestation.

To conclude, this work showed clearly that rNcSAG1, rNcHSP20 and rNcGRA7 proteins are immunogenic in cattle but the formulation of these proteins with ISCOMs failed to prevent the foetal infection in pregnant cattle challenged to 70th day of gestation. On the other hand, if this vaccine prevents the abortion induced by *N. caninum* should be determined because integrated herd management including the use of inactivated vaccines to prevent abortions due to this parasite has been proposed (Romero et al., 2004; Weston et al., 2012).

Conflict of interest

There are no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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