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Short communication

Safety and immunogenicity of a soluble native *Neospora caninum* tachyzoite-extract vaccine formulated with a soy lecithin/β-glucan adjuvant in pregnant cattle

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ABSTRACT

The global economic impact of *Neospora caninum* infection in cattle herds has promoted the development of vaccines that can be safely used during pregnancy. The aim of this study was to evaluate the safety and immunogenicity of a vaccine formulated with the soluble fraction of tachyzoite's lysate and a soy-based aqueous adjuvant (sNcAg/AVEC), which was protective in the mouse model and induced strong IFN-γ responses and high avidity antibodies in non-pregnant cattle. Ten pregnant heifers were vaccinated twice during the first trimester of gestation and 8 remained unvaccinated. Anti-*N. caninum* immune responses were efficiently primed by vaccination, evidenced by a quick induction of IgM serum titers (7 dpv) and a prompt switch to high avidity IgG shortly after infection (performed at 78 or 225 days of gestation; $n = 5$ each); while naïve cattle elicited lower IgG titers, with a delayed kinetics. High systemic IFN-γ levels were induced after infection which did not interfere with pregnancy. No local or systemic adverse effects were recorded along the study. Calves were born in term and in good health conditions, showing that the sNcAg/AVEC vaccine was safe when applied to healthy heifers during the first trimester of gestation.

1. Introduction

Neospora caninum is a protozoan parasite which has emerged as a major cause of reproductive failure in cattle worldwide (Dubey and Schares, 2011; Innes et al., 2005) affecting the economic performance of the dairy and beef industries (Reichel and Ellis, 2006). The major route of transmission is vertical, and infection during early pregnancy generally produces fetal death and abortion (Gibney et al., 2008).

There are indications that the risk of endogenous abortion is influenced by the parity of the dams (Dubey et al., 2007). Thurmond and Hietala (1997) observed a markedly increased abortion risk in congenitally infected heifers during their first gestation but not in later gestations, compared to the abortion risk in seronegative controls. Therefore, if the disease is diagnosed in endemic areas, vaccination of pregnant heifers early in gestation may reduce the incidence of abortions. However, although

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many vaccine candidates have been assessed, there is little information on the safety and immunogenicity of *N. caninum* vaccines in pregnant cattle. Two studies have reported interference with pregnancy when inactivated vaccines were applied during the first trimester; such as reabsorption (Andrianarivo et al., 2000) or embryonic death (Weston et al., 2012).

Here we evaluated the safety and immunogenicity of a vaccine composed of the soluble fraction of tachyzoites lysate (sNcAg) of a virulent *N. caninum* strain (Nc1) formulated with an aqueous soy-lecithin based adjuvant (*Providean*-AVEC®). In previous studies we demonstrated that this vaccine (called sNcAg/AVEC) conferred protective immunity to BALB/c mice (Mansilla et al., 2012). The same formulation given to non-pregnant cattle elicited a Th1-type response associated with CD4+/T-cell activation and systemic IFN-γ production (Mansilla et al., 2013), an immune profile similar to that described for controlling *N. caninum* infection (Maley et al., 2006; Rosbottom et al., 2007; Williams et al., 2000).

2. Materials and methods

2.1. Cells, parasite and antigen preparation

N. caninum tachyzoites (Nc1 strain) were cultured in VERO cells under conditions previously standardized (Moore et al., 2011). Partially purified tachyzoites were used either to formulate the live inoculum (1×10^8 tachyzoites/2 mL) or to obtain the native antigen extract. Soluble extracts of native antigen were prepared as described before (Mansilla et al., 2013; Mansilla et al., 2012; Moore et al., 2011).

2.2. Subunit vaccine

The vaccine was formulated following published procedures (Mansilla et al., 2013). Each dose of 2 mL contains 50 μg of sNcAg diluted in sterile PBS, mixed with an aqueous soy-lecithin/β-glucan adjuvant (30%; "*Providean*-AVEC®", Tecnovax S.A., Buenos Aires, Argentina). The vaccine was injected subcutaneously on the neck using a 21-gauge needle (BD Biosciences).

2.3. Animal procedures and experimental design

Animal procedures were performed according to standard protocols and guidelines from the Animal Ethics Committee at INTA, Argentina. Forty 2–3-year-old healthy Aberdeen Angus heifers (*Bos taurus*) were initially involved in this study. They were seronegative for *N. caninum* by ELISA ID Vet, Montpellier, France and IFAT (<1:25 serum dilution) and certified free from brucellosis, tuberculosis, leptospirosis, campylobacteriosis and trichomonosis (sampled 10 days before the beginning of the experiment).

Each animal received 2 mg (2 mL) of estradiol benzoate (Syntex®, Syntex S.A., Argentina) to synchronize estrus, and an intravaginal progesterone-releasing device was used (Syntex DIB 0.5®, Syntex S.A.). After 7 days, devices were removed and animals were inoculated with a 2 mL dose of Ciclase DL® (Syntex) and 0.5 mg (1 mL) of estradiol cypionate (Von Franken S.A.I.C., Buenos Aires, Argentina). Artificial insemination was carried out 52–56 h after devices were removed. At the same time, one 2.5 mL dose (0.0105 mg) of gonadotropin releasing hormone (Río de Janeiro®, Allignani Hnos S.R.L., Argentina) was applied. Animals stayed in the pasture for 35 days, until pregnancy was diagnosed by ultrasound.

Time-line of interventions and sampling points are depicted in Fig. 1. Briefly, on day 65 and 75 of gestation, ten animals were immunized subcutaneously with 50 μg of the subunit vaccine and eight remained unvaccinated. Thirteen days post-vaccination (dpv) five randomly selected animals from each group (vaccinated or non-vaccinated) were inoculated with 1×10^8 tachyzoites (Nc1 strain) by the intravenous route (iv) following published procedures (Hecker et al., 2013). A second inoculation round was performed at 147 dpv (225 days of pregnancy) on 5 vaccinated and 4 non-vaccinated dams. The control group ended up with 3 animals, because one of them has to be removed from the herd a few days after the experimental infection due to a bacterial infection in the legs.

Heifers were observed by qualified veterinarians daily throughout the experimental period. Rectal temperatures were recorded during 4 days after vaccination and parasite inoculation. Animals with temperatures above 39.5 °C were considered to be febrile. Animals were inspected daily for local inflammatory reactions at injection sites during the first 2 weeks after vaccination. Ultrasounds and digital rectal exams were performed weekly during the first 2 months following challenge and monthly thereafter up to birth. At parturition, calves underwent a complete standard clinical examination. Briefly, pulse (beats per minute) and respiration (breath per minute) rates were recorded. Ear tickle, neck and back inspection were performed to evaluate the correct alignment of the vertebral column. Cranial nerve function was assessed by nasal stimulation, thoracolumbar stimulus and the observation of facial expression. The rest of the clinical assessment consisted on the inspection of different anatomical structures for congenital malformations.

2.4. Humoral responses

Specific serum-antibodies were tested as described before (Mansilla et al., 2013). ID Screen® *N. caninum* (ID Vet) commercial ELISA kit was used to detect total antibodies following the manufacturer's instructions. The same kit platform was applied to titrate IgG1 and IgG2; performing serial two-fold dilutions of the sera (from 1:25) and a sheep anti-bovine IgG1 and IgG2 peroxidase conjugates (1:750 in the kit's dilution buffer). IgM was measured using a single dilution of the sera (1:25) and a sheep anti-IgM peroxidase conjugate (1:5000). Conjugates were purchased from AbD Serotec (Oxford, UK). Avidity was determined adding a washing step with a 6 M urea solution after incubating the serum samples diluted 1:50, as described before (Mansilla et al., 2013, 2012). Percentage of residual reactivity after the urea-wash treatment was calculated and expressed as avidity index "AI%" (Lavoria et al., 2012).

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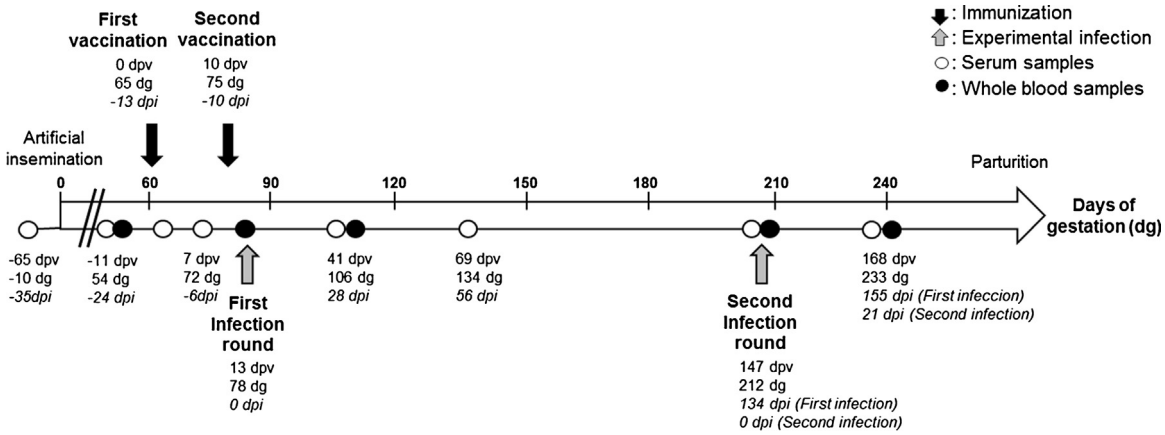

Fig. 1. Cartoon showing the time-line of interventions and sampling points from artificial insemination to parturition. The main time line corresponds to days of gestation (dg). Days post vaccination (dpv) and post infection (dpi) are indicated at each intervention step. References are shown in the figure.

2.5. Cell-mediated immune responses

IFN-γ was measured in stimulated plasma using a commercial ELISA (ID Screen® Ruminant IFN-γ; ID Vet) as described before (Mansilla et al., 2013). Briefly, whole blood samples were taken before vaccination (−11 dpv) and at 28, 132 and 154 dpv (Fig. 1). Each sample was cultured by triplicate in 24-well sterile plates and incubated overnight (37 °C, 5% CO_2) with sNcAg (1 μg/mL); Pokeweed mitogen (PWM, 10 μg/mL, Sigma) or phosphate buffer saline (PBS) as positive or negative control, respectively. After this incubation period, plasma samples were collected and run by ELISA.

2.6. Statistical analysis

Results between different experimental groups were compared by ANOVA 2-factor repeated measures followed by Bonferroni multiple comparisons test. When two groups were compared, Mann–Whitney test was used. The confidence interval was 99%. Statistical analyses were carried out using GraphPad Prism v5.0 (GraphPad Software).

Table 1
IgG1 and IgG2 serum titers measured by ELISA in vaccinated and non-vaccinated animals 28 and 21 days after the first and second infection rounds, respectively. Gray scale (brighter to dark) represents increasing titers.

Infection round	Group	Animal ID	IgG1	IgG2
1st (78 dg)	Vaccinated	1	200	>200
		11	100	150
		15	200	>200
		17	200	100
		19	200	>200
	Non-vaccinated	2	125	100
		4	50	50
		6	100	30
		10	100	>200
		12	50	25
2nd (212 dg)	Vaccinated	3	100	100
		5	150	25
		7	>200	50
		9	200	50
		13	>200	100
	Non-vaccinated	14	50	25
		16	100	25
		18	100	50

3. Results and discussion

Subcutaneous administration of the sNcAg/AVEC vaccine to healthy, pregnant heifers did not produce unwanted injection site reactions neither systemic adverse effects. All animals showed good health conditions throughout the whole experiment. Experimental infection did not cause evident clinical signs, neither miscarriages, although all animals were febrile (~39.5–41.0 °C) at least one day during the first 4 days post-infection (dpi). All calves were born in term, healthy and with normal weight, except one calf born to a naïve dam infected at 78 days of gestation (dg) which had low weight and survived due to the post-natal care provided. As no abortion was recorded in the naïve/infected groups, it was not possible to evaluate protection. However, macroscopic lesions in the placenta, such as severe autolysis or edema were observed only in some of the not-vaccinated animals (data not shown), suggesting that the vaccine may induce protection and encouraging us to repeat the trial, adjusting some of the variables.

Vaccination efficiently primed an anti-*N. caninum* immune response, which was evidenced by a quick increase in IgM serum titers (7 dpv, Fig. 2B) and a prompt switch to IgG after challenge; while naïve cattle elicited lower levels of IgG with a delayed kinetics (Fig. 2A and Table 1). The experimental infection performed at 78 dg boosted specific total antibody levels, which was higher in vaccinated than in non-vaccinated animals from 28 to 57 dpv ($p < 0.01$). Vaccinated dams infected at 225 dg also induced higher anti *N. caninum* antibody levels that naïve animals at 154 dpv ($p < 0.01$) (Fig. 2A).

The increased antibody levels observed in vaccinated dams after infection corresponded to an evident secondary response. Avidity of antibodies induced in vaccinated dams infected at 78 dg, measured at 28 dpi, was significantly higher compared to naïve-infected animals (Fig. 2C). A

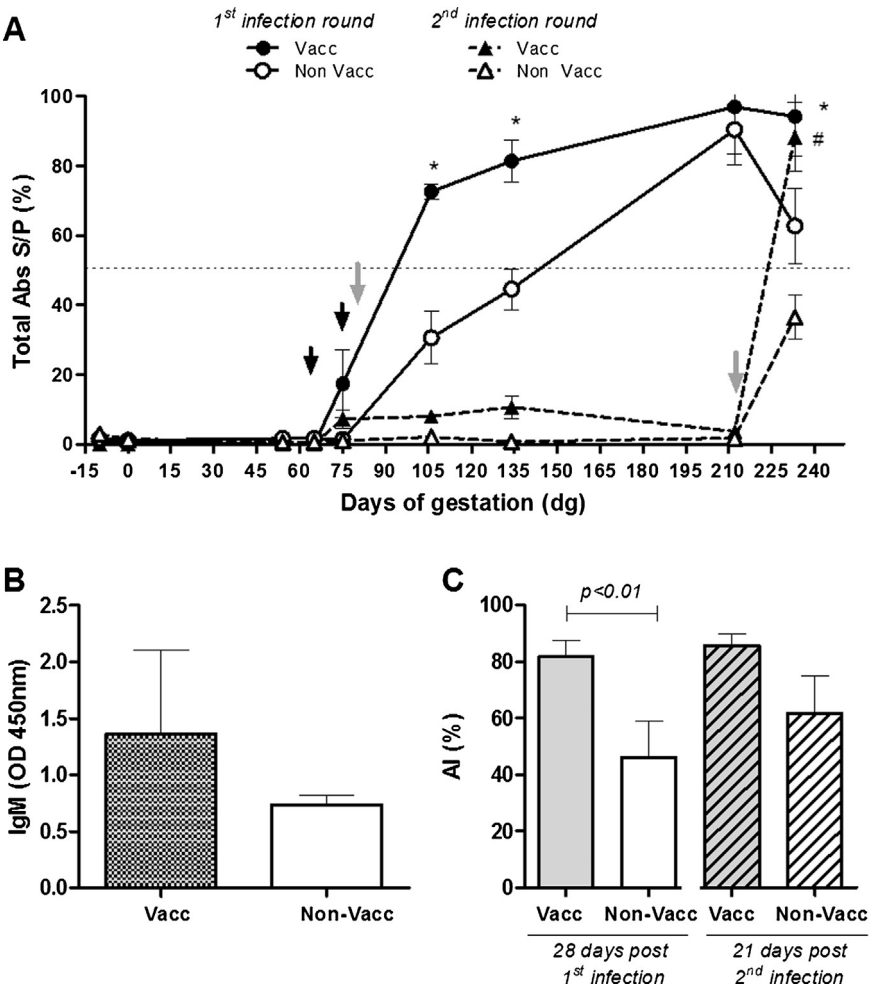

Fig. 2. (A) Time course of serum antibody response of pregnant cattle immunized with two doses of sNcAg (50 μg) formulated with *Providean*-AVEC® administered at 65 dg (0 dpv) and 10 days later, indicated by black arrows. Challenge was performed by inoculating 1×10^8 live tachyzoites by the iv route at 78 or 225 dg (13 and 147 dpv, indicated with gray arrows). Individual serum samples were assessed using a commercial ELISA test. Mean percent reactivity (±SD) relative to the kit positive control is depicted. Horizontal dotted line indicates the assay's cut-off value. (*) Significant differences between vaccinated and non-vaccinated animals infected at 13 dpv ($p < 0.01$ at 28, 57 and 154 dpv). (#) Significant differences between vaccinated and non-vaccinated animals infected at 147 dpv ($p < 0.01$ at 154 dpv). (B) IgM levels (OD values) in vaccinated and non-vaccinated animals measured at 7 dpv by ELISA. (C) Avidity of specific antibodies anti *N. caninum* in serum samples from vaccinated and non-vaccinated animals, 21 days after the first infection (plain bars) and 28 days after the second infection (striped bars). Significant differences between groups are indicated.

similar trend was observed after the second parasite inoculation (225 dg), although differences were not statistically significant (Fig. 2D). CD4+ T-cell responses were also primed by vaccination (data not shown).

Anti *N. caninum* IgG1 and IgG2 titers were higher in vaccinated compared to naïve dams after infection, however the IgG1/IgG2 titer ratio was similar depending on the time of infection. Animals infected in the first trimester of pregnancy elicited similar IgG1 and IgG2 titers. However, those infected during the third trimester had higher IgG1 than IgG2 titers. These differences can be due to the immune-modulation described along pregnancy, characterized by different (pro or anti-) inflammatory profiles depending on the stage of gestation (Innes et al., 2002).

Vaccination did not induce IFN-γ anamnestic responses. After both experimental infections, vaccinated and non-vaccinated animals had similar high levels of IFN-γ measured at 28 and 21 days after the first and second infection, respectively (Fig. 3). An interesting observation of this experiment was that the burst in IFN-γ systemic levels at 106 and 233 dg did not interfere with pregnancy. Published data is still conflicting about the role of a sharp increase in the systemic levels of IFN-γ in abortion. Serrano-Perez et al. (2014) proposed recently that IFN-γ production could be linked to the transplacental migration of tachyzoites, while other authors demonstrated that IFN-γ production during pregnancy may be effective in preventing abortion in naturally infected cattle (Almeria et al., 2010; Lopez-Gatius et al., 2007).

Efforts toward a vaccine based on sNcAg are worthy to pursue; supported by the fact that there are different successful marketed vaccines containing soluble antigens from other protozoan parasites i.e.: CoxAbic™ against *Eimeria maxima* and NobivacPiro™ against Babesiosis

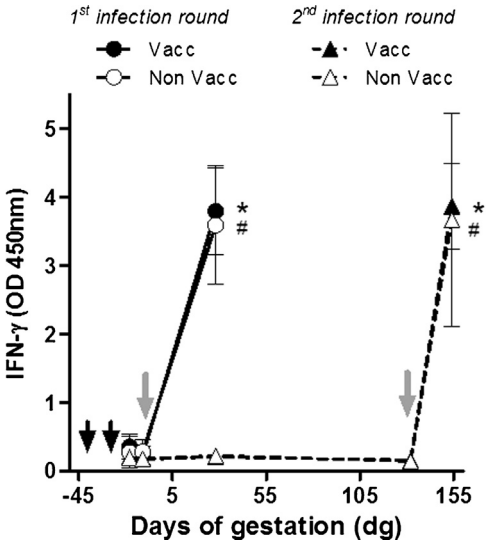

Fig. 3. Kinetics of systemic IFN-γ production in sNcAg-stimulated plasma measured by ELISA before vaccination (−11 dpv; 54 dg) and at different time points along gestation. The black arrows indicate the immunizations and the gray arrows, the time of each infection round. Levels were significantly higher from pre-immune determinations (−11 dpv; 54 dg) in vaccinated/infected (*) or non-vaccinated/infected (#) animals ($p < 0.01$).

(Schetters et al., 2009; Wallach et al., 2008); and that formulations containing sNcAg are immunogenic in cattle (Mansilla et al., 2013; Moore et al., 2011, 2005). Moreover, here we demonstrated that the vaccine based on sNcAg and *Providean*-AVEC®, a soy-lecithin based adjuvant that contains TLR agonists is safe and immunogenic when applied to pregnant cattle in the first trimester of gestation.

Conflict of interest

None declared.

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