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Effect of a killed whole *Neospora caninum* tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions

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Abstract

A standard field trial was carried out to assess the effect of a commercial *Neospora*-vaccine based on whole killed tachyzoites (Bovilis–Neoguard, Intervet®) on the abortion rate. Eight hundred and seventy-six cows, over 2.5 months pregnant, belonging to 25 Costa Rican dairy herds, were used in the analysis. For each cow vaccinated, a cow of the same herd, breed and age category, was selected as control. The period of administration of treatments extended from June to November of 2000. The treatments were administered in two, 5 ml doses 1 month apart, the first dose given between day 75 and 90 of gestation. The incidence of abortion among all treated cows was of 16.0% (140/876). The treatment specific incidence was 11.2% (49/438) and 20.8% (91/438) for the vaccinated and the placebo group, respectively. The prevented fraction by vaccination amounted to 0.46 (95% CI: 0.26, 0.61), and the cumulative incidence ratio for the vaccinated group was 0.54. The Cox hazard ratio was 0.51(95% CI: 0.37, 0.72), meaning that the force of abortion is reduced twice in the vaccinated group. The results of this study, the first one following this type of design, shows that the killed whole *Neospora caninum* tachyzoite preparation had a reasonable effect on the abortion rate in Costa Rican dairy cattle.

Keywords: *Neospora caninum*; Vaccine efficacy; Field trial; Dairy cattle; Costa Rica

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1. Introduction

Neospora caninum is a protozoan parasite (phylum Apicomplexa) discovered in 1984 by Bjerkas et al. and is recognised as one of the most important causes of foetal losses in both dairy and beef cattle around the world. It affects several species of mammals including cats, dogs, wild canids, camels, water buffaloes, rodents and cattle (Dubey, 1999).

Previously, neosporosis was misdiagnosed as toxoplasmosis (*Toxoplasma gondii*) due to close structural similarities and clinical signs (Dubey et al., 1988; Speer et al., 1999). Also some other aplicomplexan parasites should be taken into account as differential diagnosis of neosporosis, especially parasites of the *Hammondia* genera (Mugridge et al., 1999).

The life cycle of the parasite has been widely studied. The biology of the parasite is better known since the discovery that the dog is the definitive host of *N. caninum* (McAllister et al., 1998; Lindsay et al., 1999). The vertical way of transmission has been documented very well (Bjorkman et al., 1996; Paré et al., 1996; Thurmond et al., 1997; Schares et al., 1998; Davison et al., 1999a,b). Besides, several studies provide epidemiological evidence of the existence of horizontal transmission (McAllister et al., 2000; Dijkstra et al., 2001).

The economic impact of bovine abortion on dairy industry has encouraged the development of effective vaccines against bovine herpesvirus (BHV-1), bovine diarrhoea virus (BVDV), *Brucella abortus* and *Leptospira* sp. In the specific case of *N. caninum*, there is an increasing knowledge about some basic topics that are necessary to develop an effective vaccine and vaccination strategy against it such as: the molecular biology of the parasite, the role of the immune system of the host, the possible ways of acquisition of the disease and the critical periods during pregnancy in which the foetus may acquire congenital infection (Hemphill et al., 2000; Andrianarivo et al., 1999; Innes et al., 2001).

Experimental studies carried out in mice, described the effectiveness of a *N. caninum* crude lysate vaccine (Liddell et al., 1999) and of a recombinant vaccinia virus expressing the surface proteins NcSRS2 and NcSAG1. These vaccines rise the level of specific IgG antibodies, complement, interleukin-12 and IFN-γ which conferred protection against vertical transmission to the offspring (Nishikawa et al., 2000, 2001a,b). Experimental studies in cattle under laboratory and field conditions, have shown the effectiveness of several vaccinia preparations, based on killed tachyzoites with adjuvants, to elicit a response at both cellular and humoral level (Andrianarivo et al., 2000; Choromanski and Block, 2000). However, their efficacy to prevent *Neospora*-infection in cattle is not well demonstrated until now. Moreover, the effect of vaccination on the probability of bovine abortion and/or its effect on prevention of infection in susceptible animals have not yet been very well documented.

Costa Rica is a country with an important dairy industry, with a sufficient production to supply the internal necessities and to export it to some countries in Latin America (FAOSTAT, 2000). Recently, it has been described that *N. caninum* is present in cattle of the most important Costa Rican dairy regions (Pérez et al., 1998; Romero et al., 2000). A serological study reported 100% of seropositive herds and within herd seroprevalences ranged between 25% and 70% in the Poás area (Romero et al., 2002). Besides, the horizontal transmission rate was estimated to be higher than reported elsewhere (Romero and Frankena, 2003). Furthermore, in a previous case-control study it was assessed that

seropositive cows had a 12 times increased probability to abort compared to seronegative cows (Pérez et al., 1998).

The objective of this study was to assess the effectiveness of a commercially available killed whole *N. caninum* tachyzoite preparation on the crude abortion rate under field conditions, in dairy cows from the most important dairy regions in Costa Rica.

2. Materials and methods

2.1. Study population

Nine hundred and thirty-one pregnant dairy cows from 25 Costa Rican dairy herds were included. The herds were located in five ecological zones (Holdridge, 1967): moist-low montane forest, moist-pre montane forest, very moist-low montane forest, very moist-pre montane forest and rainy-low montane forest, of three dairy areas: Poás, Cartago and San Carlos, were used in this study. The farms involved were selected intentionally because all of them use VAMPP 5.1 (Veterinary Automated Management and Production Control Programme, Noordhuizen and Buurman, 1984) and were disposed to collaborate in this study. Besides, these farms have a veterinary practitioner who is responsible for the herd health program. Abortive diseases such as bovine herpesvirus (BHV-1), bovine diarrhoea virus and leptospirosis were previously diagnosed in the farms by serologic testing at least once but not as part of a serological survey (or serological sampling), but not as part of this study. Prevalence or incidence of these diseases for each farm was unknown. The within-herd seroprevalence for brucellosis did not reach 2% in those herds in which it was present, but most of the herds were free of brucellosis (source: Ministry of Agriculture and Livestock). The incidence of abortions ranged from 10% to 33% in the 2 years previous to this study, with a global rate of 19.0% (source: Centro Regional de Investigación en Producción Animal Sostenible project [CRIPAS]) of the Escuela de Medicina Veterinaria of the Universidad Nacional, Costa Rica (Baaijen and Pérez, 1996).

2.2. Study design

This study was designed as a standard field trial based on a cohort study. The number of cows selected from a herd was proportional to the farm size. All females over 2 months in pregnancy (confirmed by rectal palpation by a veterinary practitioner) were eligible for inclusion. For each cow treated with the active component, a control animal of the same herd, breed and age category was selected. Four age categories were considered: parity 0 (heifers), parity 1, parity 2 and 3, parity 4 or higher. The sample size was calculated using the formula to estimate a difference between percentages in WinEpiscope 2.0 (Thrusfield et al., 2001) with an expected percentage of abortion of 8% in vaccinated and 15% in controls, which is the global percentage of abortion as described above. With a confidence level of 95% and a power of 90%, the sample size calculation resulted in 433 animals per group.

Cows with a previous positive diagnosis of any abortive disease such as BHV-1, BVDV, brucellosis, leptospirosis and neosporosis were not included. The serological status towards abortive diseases for the rest of the selected cows was unknown but due to the

large number of animals it is expected that cows with an unknown history of potentially abortive infections are evenly distributed over the treatment groups. Selected cows that were culled during the trial before parturition and without abortion were dropped from the database.

Treatments were applied during the herd health routine visits of the veterinarians. These visits had an interval of 2–4 weeks between each other. Because of the large number of cows involved in this trial, the period of application of treatments was almost 5 months, beginning at June 2000. A single blind design was used to reduce bias; in this way, farmers and veterinarians were blinded regarding to application of vaccine and placebo. To reach this objective, vaccine and placebo were packed up in identical bottles.

Veterinarians followed all treated cows from the first application of the treatment until birth or foetal loss. There were two ways for detecting foetal loss (1) direct observation of the aborted foetus, (2) cow diagnosed as empty by rectal palpation after it had been found pregnant previously during the biweekly or monthly routine herd health visit of the veterinarian. The cows were routinely checked at the 4th and 7th month of gestation, and/or after the notice of vaginal discharge emerging from the vagina or by repetition of heat. If the veterinarian reports the cow as empty or the cow returns to heat, VAMPP assigns a date of abortion 21 days before the reported day of the vet check or heat. In the case of cows with vaginal discharge, VAMPP interrupts the gestation at the day the farmer made the report when the veterinarian confirms the foetal loss.

2.3. Treatments

The vaccine administered was a commercial preparation (NeoGuard, Intervet®), based on inactivated *N. caninum* tachyzoites (3×10^6 ml^{-1} at harvest), 10% of adjuvant, 5% of stabilisers and 5% of phosphate buffered saline. The placebo consisted of isotonic saline infusion specially prepared for this trial. Thus, the placebo had an appearance like the vaccine and both were packed in identical bottles.

The first treatment (vaccine or placebo) was administered between day 75 and 90 of gestation. A booster was administered 4 weeks later. Vaccines and placebos were stored at 4 °C in our laboratory. Veterinarians transported the bottles from our laboratories to the farms in an icebox, keeping the recommended storing temperature. A dose of 5 ml of vaccine was injected subcutaneously (in the neck with a disposable needle 18 in. × 1.5 in.), following instructions of the manufacturer.

2.4. Data collection

Characteristics of the cows such as age, parity, breed, gestational age at the end of the pregnancy, previous diagnosis for other diseases, etc., were taken from the VAMPP central database. The farmer captures the data about the events at the farm using a notebook. Then, this information is transferred to VAMPP by him/her self, or by technicians that give technical assistance to the farms. The staff of the CRIPAS project periodically collects the VAMPP information.

The outcome variable had a dichotomous nature indicating the type of end of the gestation: (0) parturition of a healthy calf after day 270 of gestation or (1) any event apart

from a normal parturition such as: mummification, abortion, neonatal death or stillbirth with neurological disorders. The abortion was detected as described above. Mummification was diagnosed by rectal palpation.

2.5. Data analysis

A description of proportions of treatments administered by farm, breed, ecological zone and parity was performed. The prevented fraction (Rothman and Greenland, 1998) was used to assess the efficacy of the vaccine. This measure was calculated with the formula:

$$\mathrm{PF}_{\mathrm{vaccine}} = 1 - \frac{\mathrm{incidence}_{\mathrm{vaccine}}}{\mathrm{incidence}_{\mathrm{placebo}}}$$

Also, the cumulative incidence ratio (CIR) with its 95% confidence interval was calculated using WinEpiscope 2.0.

A survival analysis was performed to compare the failure curves of both treatment groups and to assess the relation between treatment and survival time. This analysis was performed using a Kaplan–Meier survival graph and a Wilcoxon test using SAS/STAT release 8.0 (SAS Institute Inc., 1990a,b). The survival functions for vaccinated and placebos were also compared using Cox proportional hazard regression while taking the herd effect and the pair effect into account (frailty model) using S-PLUS 2000 release 3 (S-PLUS 2000, MathSoft Inc.).

3. Results

The total number of treatments administered was 931 (464 in the vaccine group and 467 in the placebo group). Major breeds were Jersey (616), Holstein (262) and Holstein × Jersey cross breed (53). Regarding to parity, 200 were heifers, 286 were first calving, 233 were of parity 2 or 3 and 212 cows had 4 or more parities. The distributions of cows toward ecological zone were 287 from the moist-low montane forest, 128 from moist-pre montane forest, 253 from very moist-low montane forest, 209 from very moist-pre montane forest, and 54 from rainy-low montane forest. A total of 438 pairs were available for analysis. Exclusion of animals and pairs was due to culling (17 animals), missing pair mate (30 animals) or administrative mistakes (four pairs). The number of matched pairs per farm varied between 2 and 49 (average = 18, median = 16).

The incidence of abortion among all cows in the trial was 16.0% (140/876). The treatment specific incidence was 11.2% (49/438) in the vaccinated group and 20.8% (91/438) in the placebo group (Table 1). The incidence of abortion for Holstein and Jersey were 15.8% (38/240) and 15.7% (92/587), respectively. The incidence per parity number and ecological zones are shown in Table 2.

There were no reports of weak calves born in the farms during the study. Regarding to the safety of the vaccine, there were also no reports of adverse effects at the place of the application of the vaccine, or other side effects like decreased milk yield or changes in the behaviour of the cows.

Table 1
Contingency (2 × 2) table for calculation of the prevented fraction and the cumulative incidence ratio for abortion in placebo and vaccine treated groups

End of gestation	Exposure		Total
	Placebo	Vaccine	
Abortion	91	49	140
No abortion	347	389	736
Total	438	438	876

The prevented fraction by vaccination amounted 0.46 (95% CI: 0.26, 0.61). The cumulative incidence ratio for the vaccinated group compared to the placebo group was 0.54 (95% CI: 0.39, 0.74). The effect of the vaccine was not uniform in all herds, a positive effect was observed in 15 herds, no effect in four herds and a (small) negative effect in six herds. There is not a large correlation between the efficacy (prevented fraction) of the vaccine and the global abortion rate within the herd during the previous 2 years (corr. = 0.31, $P = 0.14$).

The Kaplan–Meier failure graph for both groups shows that the difference between both groups arises in the period between day 150 and 200 of gestation (Fig. 1). After day 200, the number of abortions in both groups is equal (23). The Wilcoxon test resulted in a chi-square value of 16.32 ($P < 0.001$), which indicates that both survival functions are different.

A further analysis of the time until abortion using the Cox proportional hazard regression showed that there was no effect of parity, breed or herd on abortion, which was expected due to the matched design. Also, in a more design-based analysis, the effect of pairs within-herds was insignificant, reducing the statistical model to a model with treatment only. The Cox hazard ratio, was 0.51 (95% CI: 0.36, 0.72), meaning that the force of abortion in the vaccinated group is half of that in the placebo group.

Table 2
Overall frequencies of abortion by treatment, breed, parity and ecological zone ($n = 876$)

Variable	Level of variable	% of abortions
Vaccination	Vaccine	11.2 (49/438)
	Placebo	20.9 (91/438)
Breed	Holstein	15.8 (38/240)
	Jersey	15.7 (92/587)
	Cross-breeds	19.6 (10/49)
Parity number	0	14.8 (28/188)
	1	17.4 (46/269)
	2 and 3	16.2 (35/220)
	>3	15.5 (31/199)
Ecological zone	Moist-low montane forest	18.8 (51/270)
	Moist-pre montane forest.	20.6 (25/121)
	Very moist-low montane forest	15.0 (35/238)
	Very moist-pre montane forest	10.2 (20/196)
	Rainy-low montane forest	18.9 (9/51)

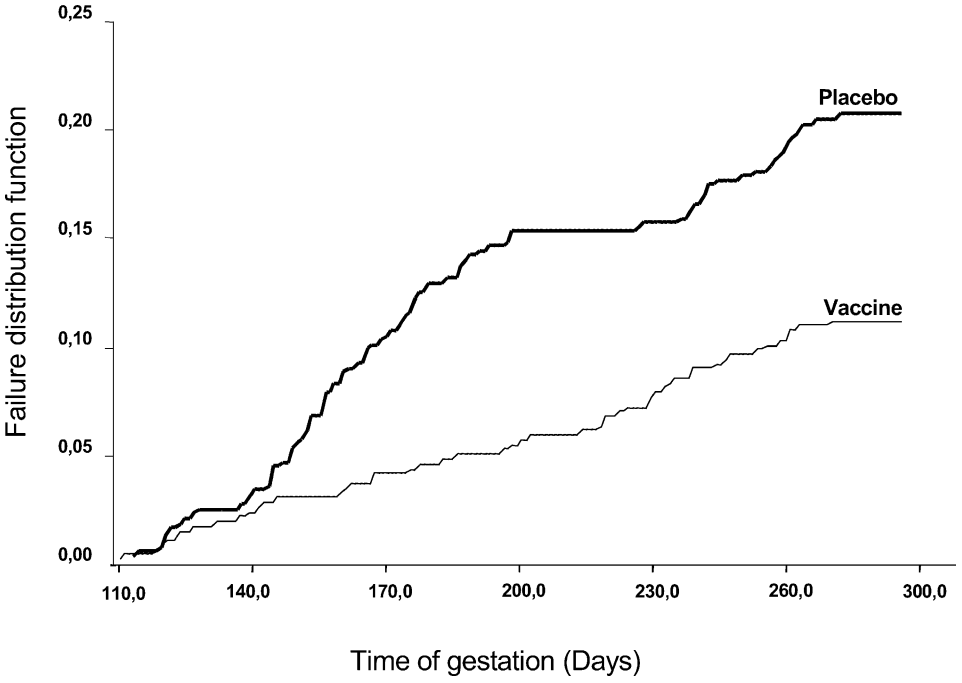

Fig. 1. Kaplan–Meier abortion curves in the placebo and vaccine treated groups.

4. Discussion

This is the first report of a large multi-herd comparative trial of a *N. caninum* vaccine under field conditions, carried out in cattle of specialised dairy farms. Only one paper has been found that reported a decreasing number of abortions after a year of vaccination using this vaccine in a Minnesota dairy herd (reducing from 27 abortions in the 2 previous years to 4 during the year of study, Choromanski et al., 2001). This result should be interpreted carefully as no simultaneous control group was present and considering that bovine abortion is multi-causal in origin. Another vaccination field trial was carried out in five seasonally calving, commercial dairy farms in New Zeeland (Heuer et al., 2003). The efficacy (prevented fraction) to prevent abortion was variable between farms (0.7%, 39.0%, 54.2%, 31.4%, and −5.2%, respectively). These authors suggest that *N. caninum* may induce abortion not only in about mid gestation but also at an earlier stage of gestation when the foetus is exceptionally vulnerable to *N. caninum* infection, and is unlikely to survive. In our study, this period was excluded as the treatments were administered during pregnancy and hence an underestimation of abortion rates and of efficacy may have occurred. Concerning the time of vaccination, Williams et al. (2000) and Innes et al. (2001) reported that experimental inoculation with tachyzoites before pregnancy did not result in transmission of infection to their calves. So, it is probable that immunization before insemination or in earlier stages of gestation than used in our study could provide an even

higher effect on the observed crude abortion rate; however, the effect of neosporosis during the first trimester of pregnancy is still unclear.

The methodology followed in this trial allowed assessing the effect of the vaccine on the crude abortion rate under field conditions. The systematic error was minimised by using a paired design where a pair consisted of two animals of the same parity and breed and originating from the same herd. Sample size calculations indicated that over 400 animals per group were to be included. Due to these large numbers, other potential sources that may affect the abortion rate, like abortion due to other abortive diseases are assumed to be equally distributed over both treatment groups. Factors such as herd, parity and breed were controlled for in the design. Therefore, a difference between both treatment groups can be assigned to the vaccine.

The crude analysis indicated that vaccination was associated with a 46.2% decrease of the abortion rate. In other words, almost half of the abortions in the unvaccinated group could have been prevented using the vaccine. The design-based analysis using survival techniques showed that the force of abortion in the vaccinated group was half that of the placebo treated group indicating that the design (pairs and pairs within-herd effects) had no significant effect on the crude outcome. This reduction is very significant when considering that abortion can be induced by several infectious and non-infectious causes, not only being neosporosis. Between 30% and 50% of abortions are attributable to a non-infectious cause (Kirkbride, 1992), meaning that infectious causes might contribute for 50% to 70% to the abortion rate. The level of *Neospora* infections in Costa Rica is higher than measured elsewhere, and therefore the vaccine effect on the crude abortion rate might be considerably high. Romero et al. (2002) found within-herd seroprevalences between 25% and 70% in a specific dairy area in Costa Rica. Another preliminary study (Romero et al., unpublished) showed seroprevalences at herd level close to 95% and within-herd between 20% and 90%. Besides, a case-control study carried out by Pérez et al. (1998) estimated that cows seropositive to neosporosis had 12 times increased odds to abort compared with seronegative cows, while the odds were not increased for seropositives to brucella, BHV-1, leptospires and BVDV.

Until now, most of studies carried out with preparations based on killed tachyzoites showed variable effects in the humoral and cell-mediated response in the hosts. However, most of them showed increasing Ab titers (especially of IgG) from 2 weeks after the application of the formulation. Besides, it was shown that IFN and IL were produced after administration of different doses of preparation based on tachyzoites (Andrianarivo et al., 2000, 2001). This multiple way of reaction of the immune system of the host could lead to a clearance of parasites in early stages of infection or protection against the intracellular infection at the late stage of infection (Nishikawa et al., 2001b).

A study carried out by Innes et al. (2001) showed that experimental infection of cows with *N. caninum* tachyzoites prior to mating was protective against vertical transmission when the animals were challenged with *N. caninum* at mid gestation. In their study, the cell-mediate and humoral responses were higher in the group infected and/or challenged at mid gestation compared with the non-infected and not challenged group. This finding suggests that in dairy herds with high seroprevalences towards *N. caninum*, which may indicate high levels of natural infection, an administration of a preparation based on tachyzoites with adjuvant might elicit an immune response to prevent the *Neospora*-

induced abortion. Paré et al. (1997) and Piergili-Fioretti et al. (2000) showed that seropositive cows with high antibody levels at the third trimester of gestation were less likely to abort than cows with low antibody levels at that time. This might be due to a combined effect of the antibody level in the dam and the immune-competence of the foetus at this stage of pregnancy (Osburn, 1986; Piergili-Fioretti et al., 2000). However, maternal antibodies to *N. caninum* per se do not prevent foetal infection (Barr et al., 1993). The fate of the foetus depends on the timing of parasitaemia, the parasitic load and the gestational age of the foetus (Williams et al., 2000). Therefore, it is possible that the vaccine we investigated sufficiently promotes the immune response so that the minimal dose to cause foetal death is not reached. Unfortunately, we do not have evidence of reduced in uterus transmission because pre-colostral samples from the calves were not taken as in general calves are born in the field without any assistance.

A criticism on this trial might be that that the placebo did not consist of adjuvant, which might have had some effect on the immune status. Andrianarivo et al. (2000) have shown that the adjuvant was able to induce the production of considerable amounts of interferon, which most likely plays a role in the defence to this parasite, together with the humoral defence. However, this study assessed the efficacy of the preparation—in the formulation as used in practice—under field conditions, regardless whether this effect was due to the tachyzoites preparation or due to the adjuvant. Another criticism might be that animals were not tested for other abortive diseases but due to the large sample size we believe that such effects have levelled out. Also no testing of aborted foetuses has been done, as in most cases the aborted foetus will either be absorbed or will not be found due to the year round pasturing.

In conclusion, our study shows that the killed whole *N. caninum* tachyzoite preparation had a reasonable efficacy and can be used as one of the tools (next to improved management towards other abortifacients) to reduce the abortion rate in herds with a high abortion incidence. Also, this study gives more evidence of the importance of neosporosis as abortifacient in Costa Rican dairy herds.

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