

## Plasma pregnancy-associated glycoprotein-1 (PAG-1) concentrations during gestation in *Neospora*-infected dairy cows<sup>☆</sup>

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### Abstract

The aim of this study was to determine whether plasma pregnancy-associated glycoprotein-1 (PAG-1) concentrations in pregnancy are affected by persistent *Neospora caninum* infection in dairy cows. The data analyzed were derived from 22 multiparous cows: 16 *N. caninum*-seropositive and 6 *N. caninum*-seronegative animals (used as controls). Three of the 16 seropositive cows aborted during the study period and the corresponding data were analyzed separately. Pregnancy diagnoses were performed on day 40 post-insemination by transrectal ultrasound, and by palpation per rectum on days 90, 120, 150, 180 and 210. Blood samples were collected from each animal immediately before each pregnancy diagnosis, and then at parturition or at the time of abortion detection. Plasma was tested for antibodies against *N. caninum* and PAG-1 concentrations were determined by radioimmunoassay. In non-aborting animals, the effects of neosporosis (seropositive versus seronegative), *N. caninum* antibody levels, semen providing bull, sex of the newborn, and day of gestation on PAG-1 concentrations were evaluated by GLM repeated measures analysis of variance. The effect of the gestation period (first half versus second half) on the *N. caninum* antibody titer was established by the Student's *t*-test in seropositive cows. A significant positive effect of gestation day on PAG-1 concentrations was observed (d.f. = 6;  $F = 12.6$ ;  $P < 0.0001$ ). For all cows, PAG-1 concentrations increased steadily during the course of gestation, with peak concentrations recorded at parturition. Neosporosis ( $P = 0.493$ ), *N. caninum* antibody levels ( $P = 0.921$ ), sex of the newborn ( $P = 0.856$ ) and semen providing bull ( $P = 0.087$ ) had no effect on plasma PAG-1 concentration. There was a significant 52% increase ( $P < 0.0001$ ) in *N. caninum* antibody titers during the second half of gestation compared to the first half. The fates of the three aborting cows were abortion on gestation day 215 in one, and fetus mummification diagnosed on gestation days 180 and 210, respectively, in the remaining two cows. A luteolytic dose of prostaglandin was applied 30 days after mummification diagnosis in these last two cows, and fetus expulsion was detected on days 215 and 250, respectively. Two of the aborted fetuses were submitted to laboratory analysis and the presence of *N. caninum* was confirmed by specific PCR. In the cows with a mummified fetus, PAG-1 concentrations were low or undetectable when the diagnosis was made. These findings suggest that *N. caninum*

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infection has no effect on placental function in chronically infected, cows not suffering abortion, while PAG-1 measurements in aborting animals provide a useful indication of feto-placental status.

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## 1. Introduction

*Neospora caninum*, an obligate intracellular protozoon that infects domestic and wild canids, ruminant and horses, is distributed worldwide and causes substantial economic losses, inducing abortion and stillbirth in cattle [1,2]. In Spain, bovine neosporosis was first diagnosed by Fondevila et al. [3] and has been since recognized as a leading cause of abortion in cattle. Antibodies to *N. caninum* have been detected in approximately one third of all dairy cattle tested [4], and the parasite identified in 32–57% of all aborted bovine fetuses in Northern Spain [5]. In previous studies performed in Northeast Spain, we detected a high incidence of *N. caninum*-associated abortions in high producing dairy cows, irrespective of seroprevalence at the farm level. However, seropositive cows had a 12–19-fold greater risk of abortion than seronegative cows, and abortion rates ranged from 30% to 44% in seropositive animals [6,7].

*N. caninum*-infected cows show a very high rate of transplacental transmission [7–9]. This incidence has been estimated to be as high as 95% [10]. The majority of calves born to infected mothers are clinically normal, but are infected for life. In non-aborting cows, mean titers of antibodies to *N. caninum* increase in mid-pregnancy and diminish as from 2 months before parturition [8,11–13]. This antibody rise during the mid-gestation period has been associated with spontaneous transplacental infection [13].

Pregnancy-associated glycoproteins (PAG) are a multigene family belonging to the aspartic proteinase superfamily [14]. These proteins are abundantly expressed in the outer cell layer of the placenta of artiodactylids such as pigs and ruminants, from the time the placenta attaches to parturition [15]. Despite their unknown function, most are thought not to be proteolytically active. They can be separated empirically into two main groups [15]: one of ancient origin (PAG-2 subgroup), largely localized at the placental fetomaternal interface, and one produced by a more recent series of gene duplications (PAG-1 subgroup), expressed primarily in trophoblastic binucleate cells. In cows, some PAG in the PAG-1 subgroup are released into the maternal circulation at the time of implantation (i.e., approximately day 25), rises steadily as gestation

proceeds and peaks just before parturition. PAG-1 levels in maternal blood have been used for pregnancy diagnosis and as markers of placental/fetal well-being [16,17]. However, to our knowledge there are no data in the literature on the possible effect of neosporosis infection on plasma PAG-1 concentrations. The present study was designed to determine whether plasma PAG-1 concentrations during pregnancy are affected by *N. caninum* infection in persistently infected dairy cows. Plasma PAG-1 values were also examined in three aborting infected animals.

## 2. Materials and methods

### 2.1. Cattle and herd management

The study was performed over a 24-month period (1 January 2003 to 31 December 2004) on a commercial Holstein-Friesian dairy herd in Northeast Spain. In this herd, *N. caninum* had been previously confirmed in aborted fetuses, and over a 1-year period 44% of seropositive pregnant animals had aborted [7]. The seroprevalence rate of *N. caninum* infection for the herd was 26% during the study period. All the animals were bred by artificial insemination. The seminal doses used in the study population were provided by six bulls.

The data analyzed were obtained from 22 multiparous cows in their second to fourth lactation period. Sixteen cows were *N. caninum*-seropositive on day of AI, 14 of which had undergone at least one abortion over the last two years, and the remaining 6 *N. caninum*-seronegative animals were used as controls. Three of the 16 seropositive cows aborted during the study period and their data were analyzed separately.

Pregnancy diagnosis was performed on day 40 post-insemination by transrectal ultrasound and by palpation per rectum on days 90, 120, 150, 180 and 210. Only cows carrying a single fetus on the ultrasound diagnosis were included in the study. Animals were subjected to daily observation for signs of abortion from 120 days of gestation. The outcome of pregnancy was recorded for all non-aborting animals.

Blood samples were collected from each animal immediately before each pregnancy diagnosis, and then at parturition or at the time of abortion detection. Blood

was collected into a tube with lithium heparin (BD Vacutainer™, Becton, Dickenson and Company, Plymouth, UK). The blood samples were centrifuged (10 min,  $1600 \times g$ ) and the plasma was stored in two tubes at  $-20^\circ\text{C}$  until analysis.

## 2.2. Serological diagnosis of neosporosis infection

Plasma was tested for antibodies against *N. caninum* using a commercial enzyme-linked immunosorbent assay (ELISA) kit (CIVTEST® anti-*Neospora*, Hipra, Girona, Spain) based on the whole tachyzoite lysate of *Neospora* NC-1. This test, previously validated by the present authors [6], was performed according to the manufacturer's instructions and a value of more than 6.0 units was taken as the threshold for seropositivity.

## 2.3. Histopathology of aborted fetuses

Two aborted fetuses (one mummified) from seropositive animals were submitted for laboratory analysis. We looked for evidence of protozoal lesions in haematoxylin-eosin stained tissue consistent with multifocal necrosis indicating mononuclear inflammation. The presence of *N. caninum* was established by a specific immuno-histochemical procedure [18] and specific Nc5 PCR performed on brain tissue [19]. In the mummified fetus, lesions could not be examined and only PCR was performed.

## 2.4. PAG radioimmunoassay

Pure bovine PAG (boPAG-67), purified according to the protocol of Zoli et al. [20], was used as the standard and tracer in each assay. Iodination ( $\text{Na-I}^{125}$ , Amersham Pharmacia Biotech, Uppsala, Sweden) was carried out according to the chloramine T method [21]. Rabbit polyclonal antiserum (R#497) was raised against boPAG-67 (anti-boPAG-67) according to the Vaitukaitis procedure [22]. The first anti-boPAG-67 titer was determined to obtain a tracer binding ratio for the zero standard of approximately 20–30% (final dilution 1:200,000).

The secondary antibody precipitation system was a mixture of sheep anti-rabbit immunoglobulin (0.83%, v/v), normal rabbit serum (0.17%, v/v), polyethylene glycol 6000 (20 mg/ml; Vel, Leuven, Belgium), microcrystalline cellulose (0.05 mg/ml; Merck, Darmstadt, Germany) and BSA (2 mg/ml; INC Biochemicals, Aurora, OH) diluted in Tris-HCl buffer (25 mM Tris, 10 mM  $\text{MgCl}_2$ , and 0.02%, w/v  $\text{NaN}_3$ , pH 7.5).

Plasma PAG-1 concentrations were determined according to the method reported by Zoli et al. [23]

with some modifications. The minimal detectable concentration of our assay was 0.2 ng/ml, and the standard curve ranged from 0.2 ng/ml to 25 ng/ml. Standard and plasma samples (0.1 ml) were diluted in 0.2 ml and 0.3 ml of Tris-BSA buffer (1 mg/ml of BSA; pH 7.5), respectively. Samples and standard tubes were incubated with 0.1 ml of the diluted antiserum (1:200,000). On the next day, 0.1 ml of  $^{125}\text{I}$ -PAG was added and the tubes incubated for a further 4 h. After the 4 h incubation step, 1.0 ml of the double antibody precipitation system was added to all tubes except those reserved for total counts (Tc) and incubation continued for a further 30 min. Bound and free PAG were separated by centrifugation (20 min,  $1500 \times g$ ) after washing with 2.0 ml of Tris-BSA buffer. Once the supernatant had been aspirated, the pellet containing the  $^{125}\text{I}$ -PAG was counted using a gamma counter (LKB Wallac 126 Multigamma counter, Turku, Finland) of counting efficiency 75%. All incubations were performed at room temperature ( $20\text{--}22^\circ\text{C}$ ). The assay volume was 0.6 ml. The intra-assay variation was assessed as the coefficient of variation (CV) of 20 replicate determinations in the same assay. The inter-assay variation was assessed as the CV of 10 duplication determinations in different assays. Intra-assay and inter-assay CV were 3.5% and 6.8%, respectively.

## 2.5. Statistical analysis

For the non-aborting animals, the effect of neosporosis infection (seropositive versus seronegative), *N. caninum* antibody levels, semen providing bull, outcome of gestation (male versus female newborn), day of gestation (40, 90, 120, 150, 180, 210 and parturition), and possible interactions of paired factors on PAG-1 concentrations were evaluated by GLM repeated measures analysis of variance using the SPSS computer package, version 11.5 (SPSS Inc., Chicago, IL, USA). In seropositive cows, the effect of the gestation period (first half: blood samples taken on days 40, 90 and 120;

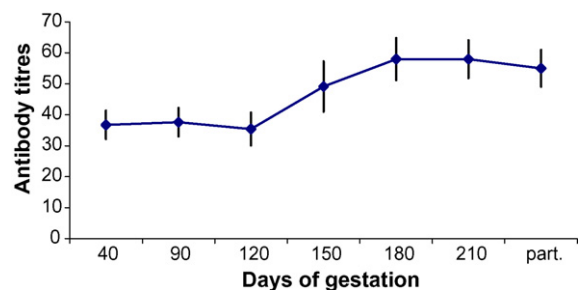


Fig. 1. Mean (units  $\pm$  S.E.M.) anti-*N. caninum* antibody titres recorded during gestation in 13 non-aborting seropositive cows.

Table 1

Mean (±S.D.) PAG-1 concentrations (ng/ml) recorded during gestation in non-aborting *N. caninum* seropositive and in seronegative cows

Days of gestation	Seropositive (n = 13)	Seronegative (n = 6)	Total (n = 19)
40	1.87 ± 1.27	3.95 ± 2.28	2.53 ± 1.87
90	4.84 ± 3.24	5.59 ± 2.36	5.08 ± 2.95
120	17.44 ± 14.67	18.84 ± 17.52	17.88 ± 15.14
150	29.52 ± 14.59	34.89 ± 15.91	31.21 ± 14.79
180	47.77 ± 20.38	61.73 ± 26.57	52.18 ± 22.75
210	75.03 ± 57.48	83.86 ± 40.1	77.82 ± 51.65
Parturition	852.92 ± 676.34	963.42 ± 474.17	887.82 ± 608.44

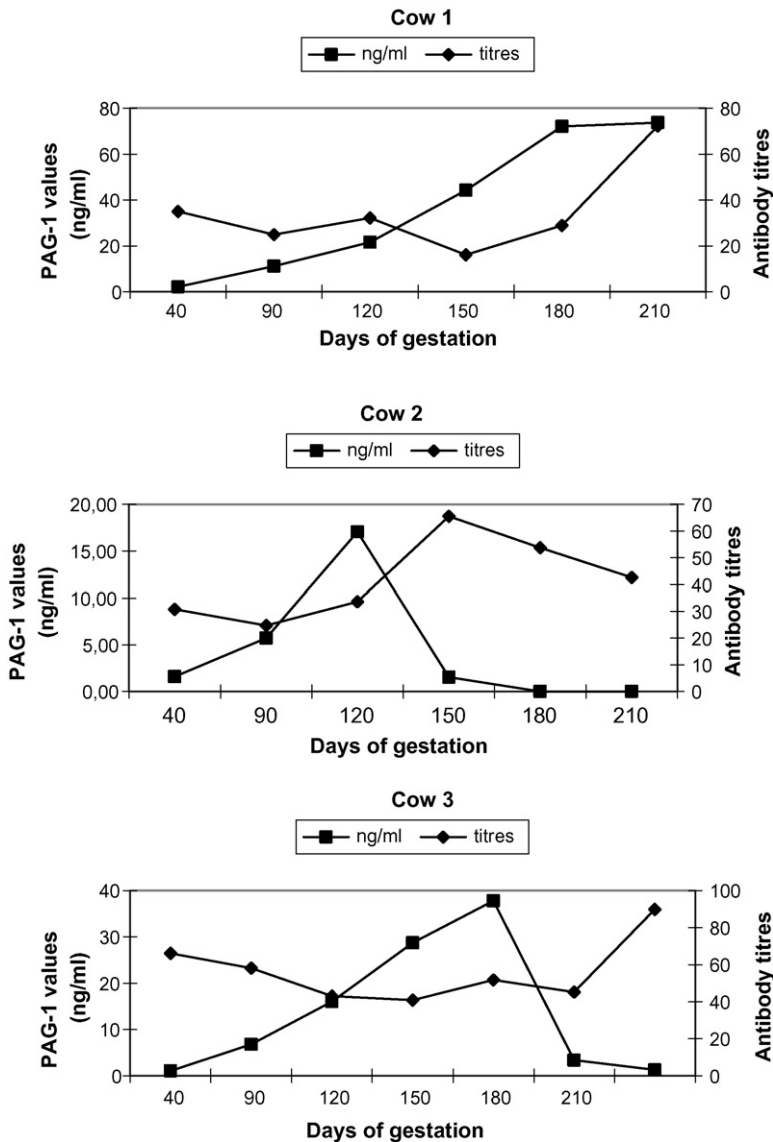


Fig. 2. Plasma PAG-1 concentrations and anti-*N. caninum* antibody titres for the three aborting cows. Cow 1 aborted on gestation day 215. A mummified fetus was diagnosed on days 180 and 210 of gestation in Cows 2 and 3, and fetal expulsion was detected on days 215 and 250, respectively, after treatment with PGF2 $\alpha$ .

versus second half: samples taken on days 150, 180 and 210) on *N. caninum* antibody levels was examined by the Student's *t*-test.

Descriptive statistics was performed for the three aborting cows.

### 3. Results

#### 3.1. Non-aborting cows

All 13 *N. caninum*-seropositive cows remained seropositive throughout the gestation period (Fig. 1). A significant 52% increase ( $P < 0.0001$ ) in *N. caninum* antibody levels was recorded during the second half of gestation ( $55 \pm 3.6$  units; mean  $\pm$  S.E.M.) compared to the first half ( $36.2 \pm 2.8$  units). The seropositive cows delivered four males and nine females, all live. Two of the newborn females, which remained in the herd, showed positive blood tests for *N. caninum* when tested 10 months later. The remaining newborn calves from seropositive animals were culled and not screened for neosporosis. All six *N. caninum*-seronegative cows were seronegative throughout pregnancy and delivered four males (one stillborn) and two females. The two newborn females remained in the herd and were diagnosed negative for *N. caninum* 10 months later.

Table 1 shows the mean PAG-1 concentrations recorded during the course of gestation for *N. caninum* seropositive and seronegative cows. A significant positive effect of gestation day on PAG-1 concentrations was observed (d.f. = 6;  $F = 12.6$ ;  $P < 0.0001$ ). In all cows, PAG-1 concentrations increased rapidly during the first trimester of gestation, continued to increase but at a slower rate from days 120 to 210 of gestation, and peaked at parturition. Neosporosis ( $P = 0.493$ ), *N. caninum* antibody levels ( $P = 0.921$ ), the sex of the newborn ( $P = 0.856$ ) and semen providing bull ( $P = 0.087$ ) had no effect on plasma PAG concentrations. No significant interaction of paired factors on PAG-1 concentration was detected.

#### 3.2. Aborting cows

One aborted on day 215 of gestation. In the aborted fetus, lesions indicative of *N. caninum* infection were observed and the presence of the parasite was confirmed by immunohistochemistry and specific PCR.

Fetus mummification was diagnosed by palpation per rectum on gestation days 180 and 210 in two further cows. A non-mobile fetus with no associated fluids present within the uterus was considered as mummified. A luteolytic dose of prostaglandin was applied 30 days

later, and fetus expulsion was detected on days 215 and 250, respectively. One of these fetuses was submitted for laboratory analysis (the other one was lost) and specific PCR analysis revealed the presence of the parasite.

Fig. 2 shows plasma PAG-1 levels and *N. caninum* antibody titers for the three cows. In the cows with mummified fetuses, PAG-1 concentrations were low (Cow 3) or even undetectable (Cow 2) when fetal mummification was detected. In the case of Cow 2, PAG-1 concentration was already low 30 days before mummification had been detected.

### 4. Discussion

To the best of our knowledge, this study is the first to evaluate plasma PAG-1 concentrations during gestation in cows persistently infected with *N. caninum*. Non-aborting animals had PAG profiles correlated to the stage of gestation, as previously reported [16,23]. Mean PAG-1 concentrations similar to those described previously increased steadily from day 40 of gestation to parturition, and neosporosis did not affect plasma profile. Thus, *N. caninum* infection does not affect PAG-1 concentrations in chronically infected cows, not suffering from abortion. The following should be highlighted: (a) no effects of neosporosis on placental function were detected and (b) the increased humoral immune response observed during the second half of gestation in seropositive cows could not be related to variations in PAG-1 concentrations.

A clear increase in anti-*N. caninum* antibodies was detected during the mid-gestation period, in agreement with the results of previous studies [8,11–13]. Although the transplacental transmission of neosporosis was only confirmed in two newborn calves, it is reasonable to assume that vertical infection occurred in most of the fetuses during the mid-gestation rise in antibody titers [13]. Effectively, in a previous study on the same herd, it was shown that 91% of seropositive dams had seropositive offspring [7]. Tachyzoites have to cross the placental barrier to reach the fetus, and placental lesions, such as focal necrosis, serum leakage and inflammation that have been observed where experimental infections seemed to regenerate [24]. Severe lesions related to increased IFN-gamma levels have also been described in a pregnant cow with a mummified fetus six weeks after experimental infection [25]. The factors known to affect the outcome of *N. caninum* infection are the quantity and duration of parasitaemia, the timing of parasitaemia and the effectiveness of the maternal immune response and ability of the fetus to

respond to infection [26,27]. However, since plasma PAG-1 levels are markers of placental well-being [16,17], our results suggest that placental damage in *N. caninum* infection is probably related to the number of tachyzoites present. In non-aborting animals, the number of parasites would be small and thus not affect PAG-1 concentrations and placental function, yet if this number rose, this could trigger abortion affecting PAG-1 concentrations as observed in the present study.

During the evolution of eutherian mammals, preventing the rejection of the conceptus was a mandatory step [28]. The mother can be exposed to “foreign” paternal antigens, because mammalian fetuses inherit almost half of their genes from their father. Given PAGs have been attributed immunosuppressive properties [15], they could participate in the placental mechanisms needed to avoid maternal rejection. Consistent with this theory is, for example, the plasma PAG-1 peak that immediately precedes the peripartum inhibition of polymorphonuclear leukocyte function [29]. However, the question of how the conceptus avoids maternal immune attack has not yet been fully resolved, and the conflict between reproductive and immune systems is even more difficult to understand in the case of parasitaemia. The factors that determine whether or not a *N. caninum* infected cow will transmit the infection to its calf during the gestation period remain unclear [2]. The immune response in cows, primarily cell-mediated immunity, is diminished during the mid-gestation period [26]. A bias towards humoral immunity during gestation, associated with the successful implantation of the conceptus and the maintenance of gestation, has also been suggested [30,31]. This immunomodulation is associated with the control of gestation but not with control of neosporosis [32–34], and is a possible cause of the high sensitivity of infected cows to neosporosis recrudescence during the second trimester of gestation [35,36]. The rising antibody levels observed during this period support this rationale. Our results revealed similar PAG concentrations in seropositive and seronegative cows during the second half of gestation, the period in which the humoral immune response to *N. caninum* is enhanced. Hence, if PAGs are maternal immunomodulators, PAG immunomodulation is probably more important at the fetomaternal and/or cell-mediated immunity levels than at the level of the general humoral immune system. Relationships between PAGs and the maternal immune response during gestation remain to be established.

The small size of our study population probably prevented the detection of a possible effect of the sex of the newborn, as previously noted [23], or of the semen

providing bull on PAG concentrations. However, the tendency observed in the case of the bull ( $P = 0.087$ ) was confirmed in a more recent study [37].

All three aborting cows were seropositive for *N. caninum* throughout gestation and at the time of abortion, consistent with previous data [8,12]. Although our findings are based only on three cases (one spontaneous abortion and two prostaglandin induced abortions after mummification diagnosis), they point to the possible use of PAG-1 measurements to indicate fetoplacental status, as already suggested by others in animals with failing pregnancies [16,17]. The similar PAG levels noted in the cow undergoing spontaneous abortion (Cow 1) to those in non-aborting cows could indicate either a long half life of PAG or that fetal death occurred a few days before abortion. The *N. caninum* antibody peak recorded at the time of abortion supports the latter assertion. Infected cows show an antibody titer increase before abortion [2]. In the cows carrying mummified fetuses, low (Cow 3) or undetectable (Cow 2) PAG-1 levels were recorded on diagnosis of fetal mummification. Probably, fetal death and subsequent mummification are secondary to the failure of placental function. More extensive studies are needed to evaluate relationships between PAG values and the process of fetal mummification.

In conclusion, the present findings suggest that *N. caninum* infection fails to affect placental function in chronically infected cows not suffering abortion, while in aborting animals, PAG-1 values may be useful indicators of fetus-placental status.

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