Effects of crossbreed pregnancies on the abortion risk of *Neospora caninum*-infected dairy cows

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A B S T R A C T

Previous studies have shown that the use of beef bull semen significantly reduces the rate of abortions due to *Neospora caninum* in artificially inseminated (AI) seropositive dairy cows. In addition, certain beef breeds could be more resistant to *N. caninum* infection and abortion than others. The aim of the present study was to determine whether different crossbreed pregnancies, those derived from Limousin, Charolais, Piedmontese or Belgian Blue semen, carry different risks of abortion in *Neospora*-infected dairy cows. The effects of possible interactions between maternal levels of *N. caninum* antibodies and the different breed crosses were also evaluated. The study was performed on five commercial Holstein–Friesian dairy herds in Northeast Spain with previously confirmed diagnoses of *N. caninum* infection in aborted foetuses. The study population was comprised of 1115 pregnancies: 633 pregnancies recorded after AI using Holstein–Friesian semen from 18 bulls and 482 after AI using beef semen from 27 bulls (304 inseminations using semen from Limousin bulls, 191 from Belgian Blue bulls, 89 from Piedmontese bulls and 49 from Charolais bulls). Abortion rates were 32.2% (155/482) and 15.2% (96/633) for seropositive cows inseminated with Holstein–Friesian and beef breed semen, respectively. Logistic regression analysis revealed the herd and the interaction between maternal *N. caninum* antibody titre and the different crossbreds as significant factors affecting the abortion rate. Lowest abortion rates, similar to that shown by seronegative animals in the analysed herds (3.2%, 239/7432), were observed in dams AI using Limousin semen that had low (<30 relative index (RI) units) *N. caninum* antibody titres (2.1% abortion, 3/145) and these cows were used as reference. Compared to the cows used as reference, cows with low *N. caninum* antibody titres (<30 RI units) showed a similar risk of abortion when inseminated with Piedmontese or Charolais bull semen, but higher risk of abortion when inseminated with Holstein (17.9 times) or Belgian Blue (7.2 times) bull semen. All cows with high *N. caninum* antibody titres (≥30 RI units) had a higher risk of abortion, ranging from 8.9 times (cows inseminated with Limousine semen) to 37.8 times (cows inseminated with Piedmontese semen), compared to the cows used as reference. In conclusion, different crossbreed pregnancies carried different abortion risks in *Neospora*-infected dairy cows. The use of beef bull semen dramatically reduced the risk of abortion in dairy cows, especially if Limousin breed semen was used. Moreover, this reduction was found to be dependent on the *N. caninum* antibody titre such that the lowest incidence of abortions...

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1. Introduction

*Neospora caninum* is an obligate, intracellular, protozoan parasite that can infect domestic and wild canids, ruminants and horses (Dubey, 2003). Abortion and stillbirth due to neosporosis, especially in dairy cattle, have been reported worldwide and *N. caninum* infection is now considered one of the most important causes of abortion in cattle (Dubey et al., 2006).

The incidence of *N. caninum*-associated abortion peaks in 5th to 7th month of gestation (Anderson et al., 1991; Thornton et al., 1991; Wouda et al., 1997), and ranges from 3 months to term (Anderson et al., 1991; Barr et al., 1991; Thornton et al., 1991; Otter et al., 1995; Wouda et al., 1997; Hattel et al., 1998). A *Neospora*-seropositive cow is more likely to abort than a seronegative cow (Anderson et al., 1995; Moo et al., 1998; Hietala and Thurmond, 1999; Davison et al., 1999) to the extent that prospective serological tests in dairy herds have been used to predict abortion (Paré et al., 1997). In previous studies performed in northeast Spain, we found that seropositive cows in infected herds had a 12- to 19-fold greater risk of abortion than seronegative cows (López-Gatius et al., 2004a,b).

There is now evidence to suggest that the epidemiology of neosporosis varies in dairy and beef cattle. Several studies have shown a lower prevalence of infection in beef cattle compared to dairy herds (Quintanilla-Goñalo et al., 1999; Moore et al., 2002; Bartels et al., 2006; Dubey et al., 2007). However, these differences could mainly be attributable to management differences between dairy and beef cattle. Effectively, intensive herd management has been associated with an increased seroprevalence of *N. caninum* (Sanderson et al., 2000; Otranto et al., 2003). Nonetheless, a lower risk of abortion has also been noted in infected beef cows compared to dairy cows (De Meerschman et al., 2000). These last authors detected significantly more prominent and extensive intra-cerebral lesions in dairy than in beef foetuses (De Meerschman et al., 2002).

Differences in susceptibility to infection have been observed among breeds in recent surveys. Cattle breeds in Spain, mainly native breeds pasturing in highlands at very low stocking densities were found to be significantly less likely to test *N. caninum* positive compared to other breeds, while in Sweden, Swedish Red and White breed cattle were more likely to test *N. caninum* positive compared to other breeds (Bartels et al., 2006). Similarly, the Limousin breed showed a seroprevalence of the protozoan 6.7 times lower than that noted in other dual-purpose, or beef cattle breeds under the same extensive management conditions (Armengol et al., 2007). These results could be an indication that certain breeds, e.g., Limousin, are less susceptible to *N. caninum* infection than others.

In previous studies (López-Gatius et al., 2005a,b), we demonstrated that the use of beef bull semen reduces the risk of abortion in *N. caninum*-seropositive dairy cows. Insemination of seropositive dairy cows with semen from beef cattle has been observed to significantly reduce the infection prevalence (hybrid calves are logically not used as replacements) and, more importantly, to dramatically reduce abortions in dairy cattle (López-Gatius et al., 2005a,b). In a recent study, the use of the Limousin breed was found to lower the risk of abortion in greater measure than the use of the Belgian Blue bulls, when compared to the Friesian bulls (Yániz et al., in press). These results reinforce the idea that differences exist between breeds of beef cattle in terms of their susceptibility to both *N. caninum* infection and abortion.

Another factor that has been noted to affect the *N. caninum* abortion risk is the antibody titre of the *N. caninum*-infected dam. Several studies have linked an increased risk of abortion to increased antibody titres (Kashiwazaki et al., 2004; López-Gatius et al., 2005c) and to the presence of lesions in aborted foetuses (De Meerschman et al., 2002). In the study by Yániz et al. (in press), a higher titre of antibodies against *N. caninum* increased the abortion risk in seropositive parous dairy cows. The likelihood of abortion was 3.2 times (1/0.312) lower for parous cows with low titres of antibodies against *N. caninum* (<30 RI units) compared to those with high antibody titres (≥30 RI units).

The aim of the present study was to determine whether different crossbreed pregnancies, those derived from Limousin, Charolais, Piedmontese or Belgian Blue (BB) semen, carry different risks of abortion in *Neospora*-infected dairy cows. The effects of possible interactions between maternal levels of *N. caninum* antibodies and the different breed crosses were also evaluated.

2. Materials and methods

2.1. Cattle and herd management

This study was performed over a 6-year period (2002–2007) on 5 commercial dairy herds in Northeast Spain with prior confirmed diagnoses of *N. caninum* infection in aborted foetuses. The data included in the analysis were derived from 1115 pregnancies recorded in *N. caninum* seropositive lactating Holstein–Friesian cows: 482 pregnancies monitored after AI using semen from 18 Holstein–Friesian bulls, and 633 after AI using semen from 27 beef bulls (10 BB bulls, 9 Limousin bulls, 4 Charolais bulls and 4 Piedmontese bulls) (Table 1).

Reared within the herds and kept in open stalls, the cows calved all year round and were milked three times daily. Mean annual milk production for these herds was 10,300 kg per cow, with a mean culling rate of 28% for the study period. All animals were tuberculosis- and brucellosis-free, as shown by yearly tests from 1985 to 2008.
Table 1

Possible variables affecting the risk of *N. caninum*-associated abortion in seropositive pregnant dams artificially inseminated with semen from different breed bulls.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>N classes</th>
<th>Class descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen-providing breed</td>
<td>5</td>
<td>Holstein–Friesian (482), Limousin (304), Piedmontese (89), Charolais (49), BB (191)</td>
</tr>
<tr>
<td>Herd</td>
<td>5</td>
<td>1 (111), 2 (716), 3 (210), 4 (63), 5 (17)</td>
</tr>
<tr>
<td>Parity</td>
<td>2</td>
<td>Primiaparous (197), multiparous (918)</td>
</tr>
<tr>
<td><em>N. caninum</em> antibody titre</td>
<td>2</td>
<td>Low titre (&lt;30) (476), high titre (&gt;30) (639)</td>
</tr>
</tbody>
</table>

Vaccination programmes were undertaken for the prevention of bovine virus diarrhoea (BVD) and infectious bovine rhinotracheitis (IBR). All animals were bred by artificial insemination (AI).

Pregnancy diagnoses were performed on days 28–42 post-insemination by transrectal ultrasound, and by palpation per rectum on days 90 and 180. As from day 90, animals were visually inspected daily for signs of abortion until parturition. Only lactating cows diagnosed pregnant on day 90, within the period 0–365 days after serological analysis for *N. caninum* infection, were included in the study. Data from seronegative animals (7432 pregnancies) were recorded and used as reference data. The final study population only included *Neospora*-seropositive cows.

2.2. Serological diagnosis

Serological analysis for *Neospora* infection was performed during annual screening for brucellosis. Blood samples were centrifuged and sera stored at −20°C until the time of analysis. Sera were tested for antibodies against *N. caninum* using a commercial enzyme-linked immunosorbent assay (ELISA) kit (CIVTEST\* anti-*Neospora*, Hipra, Girona, Spain), based on detecting the whole tachyzoite lystate of *Neospora* NC-1. This test, previously validated by the present authors (López-Gatius et al., 2004a), was performed according to the manufacturer’s instructions and a value of >6.0 relative index (RI) units taken to denote seropositivity. *N. caninum* antibody titers were used to classify the cows as showing a high (≥30 RI units) or low (<30 RI units) antibody titre (Becch-Sábat et al., 2007).

2.3. Statistical analysis

Logistic regression analyses were performed on data from each animal, considering abortion as the dependent variable, and herd, parity (primiparous versus multiparous), semen-providing breed bulls (Holstein–Friesian, Limousin, BB, Piedmontese or Charolais) and *N. caninum* titre (low <30 units, high ≥30 units) at the last screen prior to 90 days pregnancy diagnosis, as independent factors (class variables). All values are expressed as the mean ± standard deviation (S.D.). Possible interactions of herd × breed of bull and of breed of bull × parity and breed of bull × antibody titre were also analysed. Values of the variables possibly affecting the abortion risk in *N. caninum* seropositive dairy cows are listed in Table 1. Regression analyses were conducted according to the method of Hosmer and Lemeshow (1989) by the logistic procedure (SAS software, 2001). Basically, this method involves five steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all the variables found to be significant in the univariate analysis; stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding factors; evaluation of interactions among variables; and assessment of model fit using Hosmer–Lemeshow statistics. Variables with univariate associations showing P values <0.25 were included in the initial model. We continued modelling until all the main effects or interaction terms were significant according to the Wald statistic at P < 0.05.

3. Results

Of 7432 pregnancies recorded in seronegative animals, 239 (3.2%) ended in abortion, whereas abortion occurred in 251 (22.6%) of the 1115 pregnancies recorded in seropositive animals. Abortion rates were 32.2% (155/482) and 15.2% (96/633) for seropositive cows inseminated with Holstein–Friesian and beef breed semen, respectively. The mean period in which abortion was recorded was within 160 ± 29 days of pregnancy, ranging from 101 to 259 days. Abortion rates in response to AI using semen from different breeds of beef bulls were 9.9% (30/304), 19.9% (38/191), 22.4% (11/49) and 19.1% (17/89) for Limousin, BB, Charolais and Piedmontese bulls, respectively.

High (≥30 RI units) and low (<30 RI units) *N. caninum* antibody titres were recorded in 639 and 476 cows, respectively. The mean titre was 63.1 ± 78.1 RI units (range 30–140.4 RI units) and 17 ± 16.9 RI units (range 6–29.9 RI units) for the high and low antibody titre groups, respectively.

Logistic regression analysis revealed no significant effects of parity, antibody titre or semen-providing breed nor of herd × breed of bull and of breed of bull × parity on the abortion rate. Table 2 shows the odds ratios and abortion rates of variables included in the final model. An abortion rate of 37.3% was recorded in one of the five herds. Using the herd with the lowest abortion rate as reference, one single herd (Herd 1) had a higher risk of abortion (8.1 times), compared to the reference herd. Clear interaction was detected between maternal *N. caninum* antibody levels and the different crossbreeds on the abortion rate. Lowest abortion rates were observed in dams AI using Limousin semen that had low (<30 RI units) *N. caninum* antibody titres (2.1% abortion, 3/145) and these cows were used as reference. Compared to the cows used as reference, cows with low *N. caninum* antibody titres (<30 RI units) showed a similar risk of abortion when inseminated with Piedmontese or Charolais bull semen, and a higher risk of abortion when inseminated with Holstein (17.9 times) or BB (7.2 times) bull semen. All cows with high *N. caninum*
Table 2
Abortion rates and odds ratios for the variables selected by the logistic regression procedure in Neospora-seropositive lactating pregnant cows artificially inseminated with semen from bulls of different breeds.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Class</th>
<th>N</th>
<th>%</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd</td>
<td>1</td>
<td>111</td>
<td>37.3</td>
<td>8.1</td>
<td>1.7-38.7</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>716</td>
<td>19.7</td>
<td>3.2</td>
<td>0.66-12.4</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>210</td>
<td>24.8</td>
<td>3.1</td>
<td>0.7-13.0</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>63</td>
<td>23.8</td>
<td>2.1</td>
<td>0.66-17.6</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>17</td>
<td>12.5</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Interaction semen-providing bull × N. caninum Ab titre

- LM-low: 145, 21, Reference
- HO-low: 226, 27.0, 17.9, 5.5-59.0
- BB-low: 42, 11.9, 7.2, 1.6-32.1
- CH-low: 9, 11.1, 7.9, 0.5-11.1
- PM-low: 54, 5.6, 3.3, 0.6-17.0
- PM-high: 35, 40, 37.8, 9.9-144.0
- HO-high: 256, 36.7, 30.0, 9.2-97.0
- CH-high: 40, 25, 21.1, 3.5-127.2
- BB-high: 149, 22.1, 15.3, 4.5-52.1
- LM-high: 159, 17.0, 8.9, 2.6-10.1

Likelihood ratio test = 125.2, 13 df, P < 0.0001.

Neyman and Lemeshow Goodness-of-fit test = 1064; 7 df, P = 0.94 (the model fits).

HO: Holstein–Friesian, LM: Limousin, BB: Belgian Blue, PM: Piedmontese, CH: Charolais; high: high N. caninum antibody titre (≥30 relative index units), low: low N. caninum antibody titre (<30 relative index units).

antibody titres (≥30 RI units) had a higher risk of abortion, ranging from 8.9 times (cows inseminated with Limousine semen) to 37.8 times (cows inseminated with Piedmontese semen), compared to the cows used as reference (Table 2).

4. Discussion

The present study was designed to analyse the effects of different crossbreed pregnancies on the abortion risk shown by Neospora-infected dairy cows.

Limousin cross-pregnancies consistently rendered the lowest abortion rates in N. caninum seropositive cows with both low and high antibody titres. In fact, the lowest abortion rate of 2.1% achieved in cows with low antibody titres inseminated with Limousin beef bull semen was similar to the 3.2% rate observed in N. caninum seronegative herdmates. This finding is especially significant if we consider the 22.6% abortion rate of all seropositive animals in the herds.

Several studies have shown that the Limousin breed shows a lower prevalence of N. caninum infection (Hornok et al., 2006; Armengol et al., 2007) and lower N. caninum abortion rates in Limousin cross-pregnancies have been previously observed (García-Ispierto et al., 2005; Vániz et al., in press). Since the BB breed is genetically closer to the Holstein–Friesian than the Limousin, Charolais or Piedmontese breeds (Blott et al., 1998; Roughsedge et al., 2001), the use of BB semen also led to a significantly increased abortion risk in cows showing low N. caninum antibody levels compared to the use of Limousin semen. In contrast, as expected based on the genetic distances noted above, we found a similar abortion risk associated with the use of Limousin, Charolais or Piedmontese bull semen in cows with low N. caninum antibody levels. However, abortion rates were similar when comparing the use of Holstein–Friesian and Piedmontese semen if the inseminated dams showed high N. caninum antibody levels. An explanation for this finding could be the reduced number of animals with high antibody titres inseminated with Piedmontese breed semen.

Our results, based on a large number of animals, confirm those of early studies that indicated a significant reduction in the incidence of abortion in N. caninum seropositive dairy cows using beef bull semen compared to Holstein–Friesian semen (López-Gatius et al., 2005a,b; García-Ispierto et al., 2005; Vániz et al., in press). The possible reasons for the reduced abortion risk observed in crossbreed pregnancies have been previously discussed (López-Gatius et al., 2005a).

De Meerschman et al. (2000) proposed that transplacental transfer of the parasite during pregnancy is reduced in beef cattle, compared to dairy cattle. Moreover, placental function might be improved in crossbreed pregnancies. A protective mechanism against rejection has been suggested based on the finding that peripartum pregnancy associated glycoproteins-1 (PAG-1) levels (used for pregnancy diagnosis and as a marker of placental/foetal well-being) (Skinner et al., 1996; Zarrour et al., 1999a,b; López-Gatius et al., 2007) in cows carrying foetuses of a different breed were higher than levels in cows bearing foetuses of their own breed (Zoli et al., 1992). Finally, higher plasma PAG concentrations were described in a recent study on dairy cows inseminated with Limousin versus Holstein–Friesian bull semen and this difference increased significantly as gestation progressed (Serrano et al., in press). High plasma PAG-1 concentrations could reflect placental mechanisms needed to avoid maternal rejection and thus compensate for the genetic distance from the foetus. This process could have a particularly relevant role in reinforcing the barrier effect of the placenta against transplacental infectious agents such as N. caninum.

An important finding of our study was that N. caninum-associated abortion rates were dependant on the antibody titre in the crossbreed pregnancies. Crossbreed pregnancies achieved a greater reduction in the abortion risk if the inseminated dams showed low N. caninum antibody titres. In fact, even Limousin crossbred animals showed significantly
higher abortion rates when seropositive AI dams had high antibody titres. Several studies have been able to link an increased risk of abortion with increased antibody titres (Kashiwazaki et al., 2004; López-Gatius et al., 2005c; Waldner, 2005; Yániz et al., in press) as well as with the occurrence of lesions in aborted foetuses (De Meerschman et al., 2002). Our results support the proposal by Quintana-Gozalo et al. (2000), that the antibody titre (and not only seropositivity) could be used as a cost-effective predictive tool to identify animals carrying a high risk of abortion in herds with a high seroprevalence of Neospora.

A higher antibody titre could indicate increased parasite numbers (Stenlund et al., 1999) and foetal infection (Guy et al., 2001) and hence a higher risk of abortion. Another explanation for elevated levels of non-protective antibodies could be non-specific polyclonal B-cell activation, as has been observed in several parasite infections, including those caused by protozoa (Montes et al., 2007). This phenomenon has, nevertheless, not been observed in mice infected with N. caninum (Teixeira et al., 2005). B-cells could play an active role in the protective immune response against neosporosis (Eperon et al., 1999), a likely function being to help reduce the invasion of host cells (Innes et al., 2002; Haldorson et al., 2006). In cattle, however, high maternal antibody production is not protective against neosporosis and, in effect, a sharp increase in the maternal antibody titre has been associated with endogenous transplacental infection (Guy et al., 2001). In cows chronically infected with N. caninum, antibody titres rise from mid to late gestation (Nogareda et al., 2007; González-Warleta et al., 2008), while a diminished cell-mediated immune response has been observed in mid-gestation (Innes et al., 2001). Interestingly, higher antibody titres have been related to a higher risk of abortion in N. caninum-seropositive parous dairy cows, but not in heifers (Yániz et al., in press), results that could indicate substantial differences between primary infections (heifers) versus secondary, or multiple, infections (parous animals).

Collectively, these results could indicate differences in the susceptibility of the different cattle breeds to N. caninum-associated abortion, the Limousin breed being less susceptible to N. caninum infection and abortion compared to the others. Within breed variation is an important factor in susceptibility or resistance to helminth infections in ruminants (Windon, 1996; Gasbarre et al., 2001). Although, we did not observe major variations in abortion rates among the individual Limousin bulls involved in the present study (data not shown), this issue merits further study. Irrespective of the reason for the lower abortion rates observed in the crossbreed pregnancies, the use of Limousin semen is highly recommended for AI N. caninum-seropositive cows in herds with a high prevalence of N. caninum regardless of their antibody titres.

In the present study, significantly different abortion rates were observed among herds. This finding could be related to the different management practices, density of animals or stress conditions on each farm. All animals were high milk yielding, such that among other stress factors, intense metabolic stress could compromise their immune response. The herd showing the highest abortion rate suffered an outbreak of N. caninum-associated abortion and while management practices in the years leading up to the outbreak were similar, over the last 2 years the herd has increased in density.

Among others, the control measures recommended for N. caninum infection in dairy cattle include: testing and culling seropositive animals, discontinued breeding with offspring from seropositive cows, chemotherapy treatment of calves born to seropositive cows, vaccination of susceptible and infected animals and insemination with beef bull semen (Dubey et al., 2007). Mathematical models have shown that annual culling of infected cattle is the most effective means of controlling N. caninum infection, especially in the short-term (French et al., 1999; Häsl er et al., 2006a; Reichel and Ellis, 2006). However, as argued by Reichel and Ellis (2006), the most effective option might not necessarily be the most economically feasible. The control measure of "not breeding replacements from infected cattle" has also proved to be effective in the short-term, particularly in herds with a high turnover of cattle (French et al., 1999; Häsl er et al., 2006a) and has been considered the best control strategy currently available in economic terms (Häsl er et al., 2006b). The practice of artificially inseminating N. caninum-seropositive cows with beef bull semen as a "discontinued breeding with offspring from seropositive cows," measure, besides reducing the prevalence of N. caninum, will achieve lower abortion rates, increase the number of complete lactations in seropositive cows and provide the economic returns of selling crossbreed offspring. This use of beef bull semen in seropositive cows could help offset some of the expenses of yearly serological tests for N. caninum and should be included in mathematical models assessing the costs and benefits of control strategies against N. caninum in cattle. It should of course be taken into account that the long-term effectiveness of these control measures will also depend on the amount and source of horizontal infection (French et al., 1999) and that current control strategies should consider to apply strict dog-management measures as well as to minimize within-herd seroprevalence by monitoring serostatus of animals, as indicated by Bartels et al. (2007).

In conclusion, different crossbreed pregnancies carry different risks of abortion in Neospora-infected dairy cows. The use of beef bull semen, especially semen from Limousin bulls, can significantly reduce the abortion risk. The maternal N. caninum antibody titre also has a clear effect on this risk, with lower risk of abortion if the inseminated N. caninum seropositive dams have low antibody titres. Regardless of antibody titre, the insemination of seropositive cows with Limousin bull semen is highly recommended in herds with a high N. caninum seroprevalence. As an infection control measure, the use of beef bull semen in seropositive cows could help offset some of the costs of Neospora diagnosis and control strategies used to date.

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