Effects of *Neospora caninum* on reproductive performance and the efficacy of treatment with a combination of sulphadiazine-trimethoprim and toltrazuril: a longitudinal field study

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ABSTRACT: This study was designed to determine the prevalence of *Neospora caninum*, the effect of infection on abortion and fertility parameters and the efficacy and outcomes with combination treatment in a dairy farm with high abortion rates and low fertility parameters. Four hundred and eighty-six cows were tested using the immunofluorescence antibody test (IFAT). The seroprevalence of *N. caninum* was 19%. Sulphadiazine-trimethoprim and toltrazuril were administered to the seropositive animals. The risk of abortion increased 19-fold in animals infected with *N. caninum* ($P < 0.05$), and *N. caninum*-induced abortions occurred more often between the fourth and the sixth months of gestation. *N. caninum* infection also had an adverse influence on the number of inseminations to conception ($P < 0.05$) and calving to conception interval ($P < 0.05$). The treatment protocol improved the fertility parameters. Although, it is not a radical approach, this combination therapy may be recommended as the primary treatment in neosporosis.

Keywords: neosporosis; dairy cow; abortion; fertility; treatment

Neospora caninum is an infectious protozoan with increasing significance in cow reproduction. It has been reported that 12–42% of aborted foetuses are infected with *N. caninum* and that this protozoan is one of the most important abortion agents in cows worldwide (Dubey 1999, 2003; Jenkins et al. 2002; Hall et al. 2005; Dubey et al. 2007; Piagentini et al. 2012; Xu et al. 2012). In addition to abortions, *N. caninum* causes foetal viability disorders, neurological symptoms in newborns (Lassen et al. 2012; Malaguti et al. 2012), an inability to grow, low average birth weight (Dubey and Schares 2011) and decreases in milk production (Tiwari et al. 2007), and reproductive performance (VanLeeuwen et al. 2010b). It has also been reported that *N. caninum*-infected cows require more inseminations per pregnancy, have a longer time of open days, and have a higher culling rate than seronegative cows (Hall et al. 2005; Tiwari et al. 2005; Kamga-Waladjo et al. 2010). Abortion due to *N. caninum* can occur from three months to term of pregnancy, especially in the fifth and sixth months (Anderson et al. 2000; Dubey 2003; Dubey and Schares 2006; Ghanem et al. 2009). Transitory immunosuppression of T lymphocytes begins around the second trimester of pregnancy, and this handicap could cause the animals to be hypersensitive to parasitaemia during this trimester. According to Almeria et al. (2009), the highest numbers of abortions occur in this trimester. Foetuses may die *in utero* and be resorbed or mummified, likewise *N. caninum*-infected cows can give birth to stillborn or live calves. The calf may be chronically infected, with or without clinical symptoms (Anderson et al. 2000; Dubey and Schares 2006) or *Neospora* free (Orozco et al. 2012). Some experimental studies have investigated the importance of chemotherapeutic agents for the control of *Neospora* infections, such as toltrazuril and ponazuril (Kritzner et al. 2002; Strohbusch et al. 2009), monensin (VanLeeuwen et al. 2010a; 2011), and a combination of sulphadiazine-trimethoprim and toltrazuril (Cuteri et al. 2005).

During preliminary investigations on this farm, we observed high abortion rates, a long calving to conception interval, a high insemination index, and decreased milk yields. Serological tests ruled out a role for common abortifacient agents. Therefore, this study was carried out in two steps and was aimed (i) at determining the seropositivity and the clinical course of *N. caninum*, the effect of neosporosis on abortion and some fertility parameters, (ii) at evaluating the possible efficacy on fertility outcomes of treatment with sulphadiazine-trimethoprim and toltrazuril.

MATERIAL AND METHODS

Study materials

This study was performed in a biparous Holstein-Friesian dairy cow herd in Western Turkey between October 2009 and May 2012. The herd was characterised by high abortion rates and fertility problems and were housed in a free stall resting barn and fed a balanced ratio. In total, the study comprised 486 cows with a mean age of 4.12 years. Serological checks (*Brucella*, *Toxoplasma*, *Clostridium*, IgG, IgA, liver enzyme tests) and vaccinations (IBR, BVD, *Coronavirus*, *Rotavirus* and *Escherichia coli*) were performed routinely. The fertility parameters of the herd are shown in Table 1.

Serological sampling

Blood samples were collected from the jugular vein to identify the seroprevalence of *N. caninum* in the herd. The immunofluorescence antibody test (IFAT) was applied to the sera samples using the method previously described by Dubey et al. (1988). A serological titre of ≥ 1 : 200 constituted seropositivity (Reichel and Drake 1996).

Table 1. Average fertility parameters of the herd before commencement of the study

Parameter	Value
Calving to oestrous interval (days)	43–46
Calving to first insemination interval (days)	72
Calving interval (days)	468
Pregnancy rate after first insemination (%)	19.75
Insemination index	3.06
Milk yield (l)	9925
Number of abortion	39 (8.02%)

Experiment design

Assessment of fertility parameters in seropositive and seronegative cows. Before the serological sampling, the insemination index, milk yield, calving to conception interval and the prior abortion rates in the seropositive and the seronegative cows were recorded retrospectively. Foetuses, stillborn, and live calves were evaluated macroscopically.

Therapeutic protocols for seropositive cows and data collection. Sulphadiazine-trimethoprim (20 mg/kg b.w., q 12 h, im for four days) and toltrazuril (q 24 h, 20 mg/kg, b.w. *per os* for two days) were administered to non-pregnant cows (aborted or normal delivery) simultaneously after the determination of seropositivity. After the therapeutic approach, the insemination index, milk yield, calving to conception interval, and the abortion rates in the seropositive cows were recorded and compared with previous data.

Statistical analysis

The Kolmogorov-Smirnov test was used to control for the distribution of normality of the data. The seroprevalence of *N. caninum* and the abortion rate were examined using the chi-squared test, and the days of calving to conception interval were examined using ANOVA. The insemination index was quantified using the Kruskal-Wallis test.

RESULTS

Ninety-one of the 486 cows were seropositive, and 395 cows were seronegative. Thus, the seroprevalance of *N. caninum* in this dairy herd was 19%. Interestingly, the seropositive cows (10.190 l) produced more milk than the seronegatives (9.970 l), but this difference among the two groups was not significant ($P > 0.05$). The insemination index was 3.84, and the calving to conception interval was 197 days in the seropositive cows. In the seronegative cows, the insemination index was 2.29, and the calving to conception interval was 120 days ($P < 0.01$). A total of 35.16% (32/91) of the seropositive cows and 1.77% (7/395) of the

Table 2. Fertility parameters of *N. caninum* seropositive and seronegative cows (mean)

Fertility parameters	*N. caninum*-negative (n = 395)	*N. caninum*-positive (n = 91)	P-value
Milk yield (l)	9970[a]	10190[a]	> 0.05
Insemination index	2.29[a]	3.84[b]	< 0.05
Number of abortions	7[a]	32[b]	< 0.01
Calving to conception interval (days)	120[a]	197[b]	< 0.01

Values with different superscripts (a, b) on the same line are significantly different

seronegatives aborted ($P < 0.01$) (Table 2). The risk of abortion with *N. caninum* infection showed a 19-fold (35.16/1.77) increase.

In the seropositive cows, abortion occurred at 90 to 120 days of gestation in four cows, at 120 to 150 days of gestation in 10 cows, at 150 to 180 days of gestation in 13 cows, and at 180 to 210 days of gestation in five cows. In the seronegative animals, abortions occurred at 91, 99, 110, 123, 145, 192, and 213 days of gestation in seven cows. Abortions occurred predominantly at 144 to 187 days (mean 135 days) of gestation in the seropositive cows and throughout gestation (mean 123 days) in the seronegatives. Retention of the placenta was observed in only one seropositive cow and in four seronegative cows. Neurological disorders such as exophthalmia, ataxia, lack of coordination, hydrocephalus, and hyper-extension of the hind limbs were observed in four calves from the seropositive cows. Autolysis was the dominant symptom in the aborted foetuses born from the seropositive cows. The macroscopic analyses of the aborted foetuses and stillborns revealed inflammatory lesions in several internal organs, especially in the brain and skeletal muscle. Pale white foci were noted in cardiac and skeletal muscle and in the brain, together with hydrocephalus.

Following treatment of the seropositive cows with toltrazuril and sulphadiazine-trimethoprim, the insemination index and the calving to conception interval decreased to 2.46 and 126 days, respectively. The number of abortions decreased from 32 to 5 (Table 3).

DISCUSSION

Recently, *N. caninum* has become an important cause of infectious abortion in dairy cows (Anderson et al. 2000; Jenkins et al. 2002; Hall et al. 2005; Dubey and Schares 2006; Orozco et al. 2012). The seroprevalence of *N. caninum* varies, depending on the country, the type of serological tests and sampling methods that are used (Dubey 2003; Hurkova et al. 2005; Dubey et al. 2007). In Europe, the seroprevalence of *N. caninum* has been reported to be 16% in Sweden, 49% in Germany, 63% in Spain, 76% in the Netherlands (Bartels et al. 2006), 3% in Ireland (McNamee et al. 1996), 2.9–39.4% in Slovak Republic (Reiterova et al. 2009), and 2.5% ± 0.7 in Estonia (Lassen et al. 2012). In Turkey, the seroprevalence of *N. caninum* has been reported to range from six to 33% (Ica et al. 2006; Kul et al. 2009). In this study, we found a prevalence of 19% in Holstein-Friesian cows, revealing that *N. caninum* is prevalent in Western Turkey in common with many other regions. Romero et al. (2005) reported that oocytes could persist for a long time in humid regions and that they could be dispersed over long distances via rainfall. Thus, climate can influence the seroprevalence. One potential reason for the lower seroprevalence (19%) in this study might be the geographical location of Western Turkey.

Many authors reported that the abortion rate in seropositive cows is higher than in seronegatives (Thurmond and Hietala 1997; Anderson et al. 2000; Lassen et al. 2012). Gonzalez-Warleta et al. (2011) demonstrated that the odds ratio for abortion in

Table 3. Fertility parameters of seropositive cows (n = 91)

Fertility parameters	Pre-treatment	Post-treatment	P-value
Insemination index	3.84[a]	2.46[b]	< 0.01
Aborting cow	32[a]	5[b]	< 0.01
Calving to conception interval (days)	197[a]	126[b]	< 0.05

Values with different superscripts (a, b) on the same line are significantly different seropositive cows was 9.1 times higher than in seronegatives. In two other studies, there was a 13-fold and a 23-fold increase, respectively, in the risk of abortion in seropositive cows (Hall et al. 2005; Weston et al. 2005). In the present study, the number of abortions in the seropositive and the seronegative cows was 35.16% (32/91) and 1.77% (7/395), respectively ($P < 0.01$). Therefore, seropositive cows had a 19-fold (35.16/1.77) greater risk of abortion than the seronegative cows. These results match those of previous studies. We also demonstrated that there was a very strong association between the serostatus for *N. caninum* and the risk of abortion. Anderson et al. (1991) declared that seropositive cows aborted mostly at five to seven months of gestation. *N. caninum*-induced abortions occurred more often between the fourth and sixth and between the third and seventh months of gestation in the seropositive and the seronegative cows, respectively, in this study. Weston et al. (2005) determined that the median survival time of foetuses in seropositive and seronegative cows was 122 and 120 days, respectively. Pare et al. (1997) found that the median survival time of foetuses was 147 days in seropositive cows and 117.5 days in seronegative cows. In the present study, the median intrauterine survival time of the foetuses was 135 days in the seropositive cows and 123 days in the seronegative cows.

Aborted foetuses and stillborns rarely show macroscopic lesions; however, pale white foci have been reported in the brain, heart, and skeletal muscles (Dubey and Schares 2006). Fioretti et al. (2003) reported that foetuses were usually autolysed and mummified. In the present study, it was observed that most of the foetuses were autolysed and had inflammatory lesions in all their internal organs, especially in the brain and the skeletal muscle. These macroscopic findings are in accordance with those of a previous study (Dubey 2003). Encephalomyelitis was the predominant type of lesion in the neonatal calves. Other symptoms included flexion or hyperextension of the hind limbs and forelimbs, ataxia, decreased patellar reflex, exophthalmia or asymmetry in the eyes, scoliosis, hydrocephalus, and narrowing of the spinal cord (Dubey et al. 2006, 2007; Innes, 2007; Malaguti et al. 2012). Four neonatal calves from seropositive cows had neurological symptoms, such as exophthalmia, ataxia, and lack of coordination. We suggest that these neurological symptoms could be related to *N. caninum.*

In *N. caninum*-seropositive cows, the retention of placenta and metritis can occur on rare occasions following abortion (Dubey and Lindsay, 1996; Dubey 2003; Georgieva et al. 2006). In the present study, the retention of the placenta occurred in four seronegative and in one seropositive cow. These results correlate with those of previous studies where the placenta was retained after *N. caninum*-associated abortion.

The effect of *N. caninum* infection on milk yield remains unclear. Some studies reported that *N. caninum*-seropositive cows produce less milk (Thurmond and Hietala 1997; Hernandez et al. 2001; Romero et al. 2005), whereas others concluded that seropositives produce 0.4–0.6 kg more milk/day/cow (Hobson et al. 2002; Pfeiffer et al. 2002). Hall et al. (2005), also demonstrated that seropositives produce 0.4 kg more milk/cow/day ($P > 0.05$). In this study, the seropositive cows produced more milk (0.7 kg/day/cow) than the seronegative group, but the difference among the two groups was not considered statically significant ($P > 0.05$) due to the inadequate number of cows and the small difference in the milk yield.

The *N. caninum*-seropositive cows required a greater number of inseminations for conception than the seronegatives. Hall et al. (2005) reported that the number of inseminations to conception was 3.7 in seropositive cows and 2.4 in seronegative cows. Kamga-Waladjo et al. (2010) reported that it was 3.9 in seropositive and 2.1 in seronegative cows. In contrast, another study found no significant difference in the number of inseminations to conception in seropositive and seronegative cows (Bjorkman et al. 1996). In the current study, the number of inseminations to conception was 3.84 and 2.29 in the seropositive and the seronegative cows, respectively ($P < 0.05$), demonstrating that the seropositive cows required a higher number of inseminations for conception than their seronegative mates. Thus, as seen in similar studies, neosporosis had a negative impact on the insemination index.

Many authors have shown that the time from calving to conception is longer in *N. caninum*-seropositive cows and have therefore speculated that *N. caninum* could be a cause of early foetal death (Trees et al. 1999; Waldner et al. 2001; Reichel and Ellis 2002; Dubey et al. 2007). Kamga-Waladjo et al. (2010) found that the interval from calving to conception in *N. caninum*-infected cows was 28.9 days than in seronegative cows. Hall et al. (2005) reported that this interval was 22.5 days longer in *N. caninum*-seropositive cows than in seronegatives. Nevertheless, these increases have not always reached the level of statistical significance (Hall et al. 2005; Romero et al. 2005). In this study, the interval from calving to conception was 77 days longer in the seropositive cows compared with the seronegatives ($P < 0.01$). It is therefore possible that infection with *N. caninum* prolonged the calving to conception interval.

Many other factors such as animal care and nutrition, efficacy of artificial insemination or bull effects and diseases also influence reproductive parameters. Therefore, it is difficult to determine the actual effect of *N. caninum* on fertility. Nevertheless, in this study, in so far as was possible, the cows in both groups were exposed to equal conditions; further all of the cows on the farm were included in the study. Therefore, it is possible that *N. caninum* might be responsible for the prolonged calving to conception interval and increased insemination index.

In vivo and *in vitro* experimental studies with chemotherapeutic agents for the control of *Neospora* infections have been previously performed (Lindsay et al. 1994; Darius et al. 2004). Although the efficacy of toltrazuril has been demonstrated in mice (Gottstein et al. 2001; Kritzner et al. 2002), the efficacy and the reliability of chemotherapeutic methods have not been demonstrated in bovine neosporosis (Dubey and Schares 2011; VanLeeuwen et al. 2011).

Cuteri et al. (2005) used sulphadiazine-trimethoprim and/or toltrazuril protocols to reduce *N. caninum*-induced abortion in dairy cows. In addition, this therapeutic protocol was applied to dogs and the farm was also disinfected. They found that the seroprevalence of *N. caninum* decreased from 68.7% to 0% and that the number of abortions fell from 188 to nine. According to some studies, toltrazuril may be a potential therapeutic agent in the treatment of *N. caninum* infection in cows (Gottstein et al. 2001; Kritzner et al. 2002; Dirikolu et al. 2008). In this study, toltrazuril was used for its antiprotozoal effect, and sulphadiazine-trimethoprim was employed to prevent secondary infections and to support immunity. As well as its effects against bacteria, the combination of sulphadiazine-trimethoprim may have an impact on some protozoa. The home range of the dogs was limited, so their contact with the cows (water, forage, placenta, aborted foetuses, etc.) was minimised. The calving to conception interval in the seropositive cows was reduced from 197 days to 126 days ($P < 0.05$), and the numbers of inseminations to conception were decreased from 3.84 to 2.46 ($P < 0.01$). The number of abortions in the seropositive cows decreased from 32 to five ($P < 0.01$). These results demonstrate that the treatment protocol may reduce the number of abortions and improve fertility parameters among *N. caninum*-infected animals. Disinfection of the farm may further reduce the abortion rate.

We found that *N. caninum* increased the risk of abortion and negatively influenced fertility parameters. The *N. caninum*-induced abortions also resulted in the retention of the placenta, and the abortions occurred predominantly between the fourth and the sixth months of gestation. We conclude that toltrazuril and sulphadiazine-trimethoprim can decrease the abortion rate and improve fertility parameters in seropositive cows, demonstrating the usefulness of this treatment protocol. This combination of antibiotic and antiprotozoal drugs is a simple treatment approach for neosporosis, and it can be recommended as an alternative, supportive, and beneficial therapy for *N. caninum*-infected cows.

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