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Review

Neospora caninum, A potential cause of reproductive failure in dairy cows from Northern Greece

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ABSTRACT

Neospora caninum infection has been reported in a large number of intermediate hosts, such as ruminants, rabbits, mice, etc. but neosporosis has emerged as a serious disease in cattle and dogs worldwide. Abortions and other infertility issues have been reported in the infected cows, leading to great economic losses in farmers. The aim of our study was to assess *N. caninum* seroprevalence in dairy cattle from Northern Greece (region of Xanthi) by using the indirect fluorescent antibody technique. Blood samples were collected from 875 Holstein – Friesian dairy cows and tested for *Neospora caninum* antibodies. Among the cows that were studied, 184 (21.03%) were positive for *N. caninum* antibodies and concurrently their farms had a known previous history of infertility problems, such as abortions, increased number of artificial inseminations needed for conception, increased rate of returning to estrus and retention of fetal membranes.

1. Introduction

Neospora caninum (Apicomplexa: Coccidia) is an obligate intracellular parasite that is the etiologic agent of the polysystemic disease neosporosis (Donahoe et al., 2015; Dubey et al., 2013). This apicomplexan parasite was initially recognized in 1984 in dogs in Norway.

(Bjerkas et al., 1984) and described as a new genus and species in 1988 (Dubey et al., 1988). In the *N. caninum* life cycle, dogs and other related canids are the only definitive hosts that shed through their feces the unsporulated oocysts into the environment, beside their role of intermediate host (Dubey and Schares, 2011; King et al., 2010; Gondim et al., 2004; Dubey et al., 2002; Basso et al., 2001; Lindsay et al., 2001; Lindsay et al., 1999a; McAllister et al., 1998). Dogs can acquire infection by ingestion of the infected tissues from the intermediate hosts, by vertical transmission or by consumption of the sporulated oocysts from the environment (Gondim et al., 2002; Dijkstra et al., 2001; Schares et al., 2001; Lindsay et al., 1999a; Lindsay et al., 1999b; McAllister et al., 1998). Thus, dogs play an important role in the horizontal transmission and maintenance of *N. caninum* infection in dairy cattle (Dubey and Schares, 2011; King et al., 2010; Gondim et al., 2004; McAllister et al., 1998).

N. caninum has been reported in a large number of intermediate hosts, such as ruminants, rabbits, mice, etc. (Dubey et al., 2007), but neosporosis has emerged as a serious disease in cattle and dogs worldwide (Dubey and Schares, 2011; Dubey et al., 2007). While this disease has a considerable impact on reproduction in cattle, in adult and older dogs appears to be asymptomatic (Silva and Machado, 2016; Kul et al., 2015; Lindsay et al., 1999a). It has been shown that 12–42% of the aborted bovine fetuses worldwide are infected with *N. caninum* (Piagentini et al., 2012; Xu et al., 2012; Dubey et al., 2007; Hall et al., 2005; Jenkins et al., 2002).

N. caninum causes abortions in both dairy and beef cattle. The abortions can occur starting with month three of gestation until delivery (Dubey et al., 2013; Reiterová et al., 2009; Dubey et al., 2007) in an epidemic or endemic manner (Wouda et al., 1999). *N. caninum* can also cause fetal viability disorders or neurological birth defects in newborn calves (Lassen et al., 2012; Malaguti et al., 2012) and those younger than 2 months of age (Dubey, 2003). The *N. caninum*-infected young calves may present neurologic signs, low birth weight (Dubey and Schares, 2011), difficulties to rise and stand, flexed or hyperextended hind and/or forelimbs, and in some cases exophthalmia or asymmetrical appearance of the eyes. However, most of the calves born congenitally-infected remain clinically healthy animals (Dubey, 2003; Bielanski et al., 2002).

Natural *N. caninum* infections can occur via horizontal (lateral) or transplacental (vertical, congenital) transmission (Dubey et al., 2007). In cattle and other domesticated bovine species, the transplacental transmission is the most frequent route of infection, being observed in up to 93.7% of cases (Dubey et al., 2007; Schares et al., 1998). In the definitive canid hosts, the horizontal transmission through ingestion of

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tissues infected with tachyzoites, tissue cysts or food and water contaminated with sporulated oocyst is the predominant infection route (Donahoe et al., 2015; Dubey et al., 2007). The lactogenic transmission of *N. caninum* has been demonstrated experimentally in newborn calves fed with colostrum infected with tachyzoites, but there is an ongoing debate regarding whether or not this occurs naturally (Davison et al., 2001). It has been shown that dogs fed with milk infected with *N. caninum* tachyzoites do not shed oocysts (Dijkstra et al., 2001).

Neosporosis is recognized as one of the most important cause of reproductive issues and abortion in cattle worldwide (Reichel et al., 2013; Dubey et al., 2007; Haddad et al., 2005). The abortions and neonatal mortality can cause severe financial loss, especially when the disease is endemic or epidemic. The economic impact is directly related with the costs associated with abortion and indirectly with the cost of veterinary services, rebreeding, loss of milk yield and replacement if cows that aborted are culled (Ansari-Lari et al., 2017).

Knowledge of the infected and non-infected cows in a region would increase our understanding of the economic impact due to *N. caninum* infection and would help us eradicate the disease.

The aim of this study was to assess *N. caninum* seroprevalence in dairy cattle from Northern Greece (region of Xanthi) by using the indirect fluorescent antibody technique (IFAT).

2. Materials and methods

2.1. Cattle and herd management

This was a prospective study conducted between March 2016 and May 2018 in 5 Holstein–Friesian dairy farms located in the prefecture of Xanthi (Northern Greece). All farms reported low fertility rates and high rates of miscarriage and provided us with the reproductive history of their cows. A number of 875 Holstein–Friesian dairy cows (mean age 4.28 years) were included in the study. The herds were kept in free-stall housing and were divided according to the stage of reproduction cycle and milk production. All cows received a balanced feed ratio and were vaccinated against infectious bovine rhino tracheitis (IBR), bovine viral diarrhea (BVD), Coronavirus, Rotavirus and *Escherichia coli*, according to the national vaccination program. There was no seasonal breeding management in the herd, but artificial insemination was performed throughout the year.

2.2. Indirect fluorescent antibody technique

Blood samples were collected from the jugular vein of each cow at the time of early pregnancy detection through rectal examination (around 28 days after insemination in heifers and around 34 days in multiparous). Within 30 min after collection, the samples were centrifugated at 2000 rpm for 10 min and the serum was separated and stored in labelled testing tubes at −4 °C, pending examination for the presence of *N. caninum* antibodies.

Many serological tests are available for the diagnosis of bovine *N.caninum* infection (Wapenaar et al., 2007; Dubey and Schares, 2006). We used IFAT, based on the protocol described by Dubey et al., 1988. This technique is commonly used for diagnosis of protozoan infections and is based on the labeling of antibodies with a fluorescent stain (fluorescein) that gives a green-yellow color in ultraviolet light. An antibody titer of 1:200 or higher was considered as positive, and a titer < 1:200 was considered as negative (Silva et al., 2007; Dubey, 2003; Reichel and Drake, 1996).

2.3. Herd follow-up during the study period

The number of artificial inseminations, birth interval and pregnancy outcomes were assessed. In case of abortion, the aborted fetuses were submitted to necropsy. Calves born of *N. caninum* positive dams were clinically examined.

2.4. Statistical analysis

Statistical analyses were carried out with SPSS software (IBM DPDD Statistics®, Version 19.0). Data were analysed by applying the Chi – Square test. The difference between groups (*N. caninum* seropositive dams vs *N. caninum* seronegative dams) was considered statistically significant if $p \leq .05$.

3. Results

3.1. Serological status of the herds

Among the 875 cows that were included in the study, 184 (21.03%) were seropositive for the detection of *N. caninum* antibodies by IFAT and 691 were seronegative (78.97%).

3.2. Number of artificial inseminations

The total number of artificial inseminations used in the group of seropositive cows was 721 dosages, while in the group of seronegative cows was 1651 dosages. The mean number of artificial inseminations performed until conception was 2.39 in seronegative cows, whereas in seropositive cows increased to 3.92 ($p < .05$) (Table 1). In the group of seropositive cows 170 of 184 (92%) returned to estrus, while in the group of seronegative cows 206 of 691 (30%) returned to estrus.

3.3. Birth interval and gestation outcomes

The birth interval was 199 days in the *N. caninum* seropositive cows, whereas in the seronegative cows was significantly lower 120 days ($p < .05$). The number of abortions was significantly higher in seropositive cows compared to the seronegative cows: 52 versus 18, respectively ($p < .05$) (Table 1). Following abortion, retention of fetal membranes was recorded in 11 seropositive dams. Necropsy of the aborted fetuses revealed inflammatory lesions in the internal organs, mostly in the brain and skeletal muscles. Autolysis was also observed in the fetal tissues harvested from the aborted fetuses. Six neonatal calves

Table 1
Artifical insemination, birth interval and gestation outcomes.

Category	Study groups		Statistical outcome *p*-value
	N. caninum seropositive dams $N = 184$	*N. caninum* seronegative dams $N = 691$	
Number of artificial inseminations, mean ± SD	3.92 ± 1.56	2.39 ± 1.1	< 0.05
Birth interval (number of days), n	199	120	< 0.05
Number (percentage) of abortions, n (%)	52 (61.9%)	18 (2.6%)	< 0.05
Number (percentage) of calves born with clinical signs of neosporosis, n (%)	6 (3.3%)	0 (0.0%)	< 0.05

N, number of dams; SD, standard deviation.

born of seropositive dams presented clinical signs of neosporosis, including exophthalmia, ataxia, incoordination and weakness, hydrocephalus and hyperextension of the hind limbs.

4. Discussion

This is the first report in Greece when *N. caninum* seroprevalence was assessed in dairy cows by IFAT. To date, there was only a previous report in which *N. caninum* infection was evaluated in milk samples by the enzyme-linked immunosorbent assay (ELISA) (Sotiraki et al., 2008). IFAT is the first serological test used for diagnosis of neosporosis (Dubey et al., 1988) and is the accepted technique in *N. caninum*-antibody research, being extensively used for the diagnosis of *N. caninum* infection in epidemiological studies conducted in other domestic and wild animals (Björkman and Uggla, 1999).

In the present study, the percentage of abortions in *N. caninum* seropositive dams was 10 times higher than in the seronegative ones (61.9% versus 2.6%, respectively). These findings are in line with those from a study in Iran, which also found a percentage of abortions 3 times higher in seropositive dams compared to the seronegative ones (21.6% versus 7.3%, respectively) (Mazuz et al., 2014). Spilovska et al. showed a correlation between the presence of *N. caninum* specific antibodies and occurrence of abortions (Spilovska et al., 2015). It has also been shown that < 5% of cows may have repeated abortions due to neosporosis (Dubey et al., 2007; Pabon et al., 2007). In addition, a cohort study showed that the *N. caninum* infected dams with abortion history had a 5.6-fold-higher abortion risk than the congenitally infected cows that had not experienced an abortion before (Thurmond and Hietala, 1997). In our study, the proportion of seropositive cows with a history of abortion (28.26%) was significantly higher than the proportion of cows with the same clinical signs but seronegative (2.60%). This finding provides an indirect evidence that *N. caninum* may be involved in abortions of cows from the studied region.

N. caninum seroprevalence we found by IFAT in dairy cattle was 21.03%, being higher than the seroprevalence reported in Hungary (3.4%) (Hornok et al., 2006), Germany (4.1%) (Conraths et al., 1996), Ireland (12.6%) (McNamee et al., 1996), Spain (11.2%) (Caetano-da-Silva et al., 2004) and in the Czech Republic (3.2%) (Václavek et al., 2003), and lower than in Belgium (29%) (De Meerschman et al., 2002), Germany (27%) (Schares et al., 1998), Italy (24.4%) (Magnino et al., 1999), Slovakia (24.1) (Spilovska et al., 2015) and Denmark (22%) (Jensen et al., 1999). In some European countries (Germany, The Netherlands, Spain and Sweden) the prevalence of *N. caninum* ranges between 16% and 76% (Bartels et al., 2006). Bartels et al. performed a study in The Netherlands and the average prevalence in random herds was 10.8% (range: 1.0% to 48.7%), while in epidemic-abortion herds, the average was 31.2% (range: 11.3% to 61.6%) (Bartels et al., 2006). In the western Romania, neosporosis prevalence range was shown to be 10% to 52.2% in different regions (Imre et al., 2012).

There is some evidence that cattle breed can affect the seroprevalence of *N. caninum* in a herd (Munhoz et al., 2009; Duong et al., 2008; Armengol et al., 2007). A study conducted in Belgium found a higher abortion rate (28.6%) in *N. caninum* seropositive dairy cows compared to seropositive beef cows (14.0%) (De Meerschman et al., 2002).

However, a study conducted Spain found similar rates of abortion between dairy and beef cows (Eiras et al., 2011). Further investigations are needed to determine whether improved breeds of dairy cattle are more susceptible to *N. caninum* infection than the zebu cattle or crossbreeds (Munhoz et al., 2009).

Regarding the number of inseminations applied before each conception, we observed that *N. caninum* seropositive cows have required 1.64 times more inseminations compared to the seronegative ones. The seropositive cows also returned more often to estrus and had a longer period of days open, which is typically associated with reduced profitability. These findings are in line with previous studies (Kamga-Waladjo et al., 2010; Hall et al., 2005; Tiwari et al., 2005).

The association between returning to estrus after pregnancy and occurrence of *N. caninum* in a herd also remains to be further assessed. A Canadian case-control study suggested that there is a positive association between the occurrence of *N. caninum*-related abortions in a herd and the annual rate of cattle returning to estrus after pregnancy (Hobson et al., 2005). In addition, a high rate of early pregnancy losses could increase the chance of definitive hosts to have access to the infectious material and thereby it could also increase the rate of oocyst-mediated horizontal transmission. This assumption is supported by the results of four other studies (Waldner, 2005; Muñoz-Zanzi et al., 2004; Waldner et al., 2001; Waldner et al., 1998). However, other epidemiological studies showed no indication that *N. caninum* is able to cause early pregnancy losses (López-Gatius et al., 2004; López-Gatius et al., 2005; Romero et al., 2005; Jensen et al., 1999).

Retention of fetal membranes and subsequent metritis occur rarely among cows infected with *N. caninum* (Dubey, 2003). In our study, the retention of fetal membranes after abortion was 5.98% in the seropositive cows.

Six neonatal calves born of seropositive dams in our study presented clinical signs of neosporosis, such as exophthalmia, ataxia, incoordination, hydrocephalus and hyperextension of the hind limbs. Similar clinical signs, as well as the inability to rise and low birth weight were previously described by Dubey and Schares (2011) in calves born to *N. caninum* seropositive dams). In addition, different studies suggest that calves born of dams with a previous history of *N. caninum* fetal infection and abortion may have congenital *N. caninum* infections and/or neurological dysfunctions at birth, including mild neurologic limb disorders and mild nonsuppurative encephalomyelitis (Barr et al., 1993; Bryan et al., 1994).

From our experience, the preventive measures for *N. caninum* infection in cattle should be focused on limited consumption of raw meat by the canines rather than chemotherapy. The latter appears to be uneconomical because is mostly used only as a preventive measure and its longterm use leads to unacceptable milk or meat residues, followed by withdrawal periods for their consumption and major economic losses.

5. Conclusion

To the authors' knowledge, this is the first report of a sero-epidemiological approach for assessment of *N. caninum* infection in Greece by using IFAT. Our findings suggest that neosporosis has a high seroprevalence in Northern Greece with a much greater geographical distribution than previously believed. We also noted a significantly increased number of insemination needed for conception in *N. caninum* seropositive dams compared to seronegative dams, as well as a substantial increase in birth interval and abortions in seropositive dams. All these may lead to major financial losses, especially if preventive measures for *N. caninum* spread are not be applied.

We recommend the following preventive measures that could avoid financial losses caused by chemotherapy: quarantine and testing of replacement and other new cows (farmers may decide to remove infected cows or their progeny from the herd), limited contamination of pastures, food and feeding stuff with feces from dogs and other potential definitive hosts, regular control of water supply, regular rodent control to eliminate a potential reservoir of *N. caninum* in livestock and reproductive management control (e.g. the control of embryos transfer from infected dams into uninfected recipients can prevent endogenous transplacental transmission of the parasite, artificial insemination of seropositive dams with semen from beef bulls).

Ethical statement for solid state ionics

None.

Declaration of Competing Interest

None.

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