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*Research paper*

**Using beef-breed semen in seropositive dams for the control of bovine neosporosis**

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## SUMMARY

A program for controlling bovine neosporosis based only on the use of beef semen, without culling seropositive animals, was evaluated in a closed dairy cattle herd over a 5-year period (2013-2017). The program was based on individual and periodic serological screenings to identify seropositive breeders. Seropositive cows were inseminated with beef-breed semen, thus excluding their descendants from the remount in order to prevent the vertical transmission of the disease. Seronegative animals, as well as heifers at first insemination, were tested before each insemination.

Sera of 1097 cattle were examined by a commercial indirect ELISA for the detection of antibodies anti-*Neospora caninum*. To verify the difference in seropositivity values among years of sampling, statistical analysis through generalized estimation equations (GEEs) was performed, also considering the effects of age, lineages, and occurrence of abortion. A seroprevalence of 33.8% was found in the first screening. The prevalence and incidence of the infection within the herd decreased significantly in 2017 (P=28.9%, I=1.4%) (p-value = 0.0001). The family line investigation detected a higher risk of being seropositive for a cow born to a seropositive dam (p-value=0.0001) than to a seronegative dam, decreasing both the apparently vertical and horizontal transmissions. The number of spontaneous abortions decreased after the first year of the study (23 in 2013 to 6 in 2017). Seropositive animals were associated with abortion events (p-value = 0.0001).

Although an eradication of *N. caninum* was not achieved at the end of the study period, a significant reduction in prevalence and incidence of neosporosis in the herd and a reduction

of the abortion rate was achieved with the application of this control plan in five years, without culling a high number of seropositive potential milk-producing animals.

**Key words:** Cattle, *Neospora caninum*, Neosporosis, Abortion, Control program

## INTRODUCTION

*Neospora caninum* is an intracellular protozoan parasite of domestic and wild canids, ruminants and horses (McAllister et al., 1998). It is one of the most common abortifacients in both dairy and beef cattle and leads to substantial economic losses worldwide (Reichel et al., 2013).

*N. caninum* has a heterogeneous life cycle with two distinct methods of reproduction: sexual stages occur in the intestine of a definitive host, while asexual reproduction takes place both in definitive and intermediate hosts (Goodswen et al., 2013). In cattle, transmission of *N. caninum* can occur postnatally (horizontal transmission) by ingestion of food or drinking water contaminated by sporulated oocysts, or transplacentally (vertical transmission) from an infected dam to her fetus during pregnancy (Dubey et al., 2007; Almería and López-Gatius, 2013).

The vertical transmission of *N. caninum* is classified as exogenous, if a dam contracts a horizontal infection during pregnancy. It is classified as endogenous, if transplacental transmission occurs in a persistently infected dam after reactivation of the infection during pregnancy, triggered by a downregulation of cell-mediated immunity that occurs around mid-gestation. Transplacental transmission may cause abortion, but in most cases it leads to the birth of a healthy, seropositive calf. Congenital transmission thus contributes significantly to

the maintenance of *N. caninum* infection in a herd, by propagating the infection to successive generations (Dubey et al., 2006).

After the first infection, cattle remain infected for life and may transmit the infection to their offspring in several consecutive pregnancies (Piergili Fioretti et al., 2003; Almería and López-Gatius, 2013) or intermittently (Boulton et al., 1995; Guy et al., 2001), with rates varying from 65% to 95% (Pare et al., 1996; Dijkstra et al., 2003). The risk of abortion is directly related to the level of *Neospora caninum*-specific antibodies. A high antibody titre could reflect a high infection dose and/or an effective multiplication or, in the case of a latent infection, the reactivation of the parasite in the host (Quintanilla-Gozalo et al., 2000).

The likelihood of cow-to-cow horizontal transmission of *N. caninum* is still an open issue. Although seroconversion has been reported after experimental infection of cattle with fetal membranes (placentophagia), semen, milk or colostrum spiked with *N. caninum* tachyzoites (Davison et al., 2001; Modrý et al., 2001), to date there is no conclusive evidence on the relevance of horizontal transmission between intermediate hosts in field conditions.

Despite several studies, no specific chemotherapy for bovine neosporosis has proved to be fully effective or applicable throughout a farm. Moreover, studies on the immunization of dams have shown that although vaccination reduces the risk of abortion it does not prevent vertical transmission (Weston et al., 2012). Prevention programs at national, regional, and farm levels have been developed in several countries. These programs should be based on a cost-benefit calculation, considering the costs of testing and control measures, and leading to a reduction of the economic losses due to *N. caninum* infection or abortion. In *N. caninum*-free herds, prevention through standard biosecurity measures is the primary goal (Dubey et al., 2007). On the other hand, in *N. caninum*-infected herds, control programs are based on decreasing the risk of the potential horizontal transmission of pathogens, principally by

controlling the definitive host population as a source of oocyst contamination, and on decreasing the vertical transmission (Dubey et al., 2007).

To date, culling positive animals and purchasing replacement cattle from disease-free herds or herds with records of excellent reproductive performance and to test all potential replacements is the only way to prevent vertical transmission from cows to heifers (Conraths and Ortega-Mora, 2005; Dubey et al., 2007). However, test and cull strategies against *N. caninum* in cattle are not always financially feasible (Dubey et al., 2007).

Without the culling of seropositive animals, a program to reduce the risk of abortion in seropositive cows, is based on active testing and on the insemination of seropositive breeders with beef semen (López-Gatius et al., 2005; Almería and López-Gatius, 2013, Almeria et al., 2009, Yaniz et al., 2010). Indeed, the likelihood of abortion is reported to be 2.8 times lower for pregnant cows inseminated with beef bull semen rather than Holstein–Friesian semen (López-Gatius et al., 2005). In addition, differences in the epidemiology of *N. caninum* infections in beef and dairy cattle have been highlighted. Lower prevalence values and lower risk of abortion have been recorded in beef cattle compared to dairy cattle, with values differing according to the geographical origin of sampled animals (Hornok et al., 2006, Armengol et al., 2007; Moore et al., 2002, Bartels et al., 2006; Fort et al., 2015). Moreover, differences in immune response against *N. caninum* and related abortions were recorded among dairy and beef purebreeds and dairy/beef crossbreed cattle (Santolaria et al., 2011), with beef purebreed and crossbreed cattle less susceptible to *N. caninum* infections compared to Holstein Friesian cows. Finally, the protective effect of insemination with beef bull semen might also be due to better placenta functions in crossbreed pregnancies (López-Gatius et al., 2005). Pregnancy-associated glycoproteins (PAG), abundantly expressed in the outer-layer of the artiodactyl placenta (Garbayo et al., 2000), have been used for pregnancy diagnosis and

as a marker for placental/fetal well-being (Skinner et al., 1996; Zarrouk et al., 1999b; Zarrouk et al., 1999a). In crossbreed pregnancies, PAG levels were found to be higher than those in cows bearing fetuses of their own breed (Zoli et al., 1992).

In dairy farms, the control program of neosporosis, based on insemination of seropositive breeders with beef semen could reduce the impact or even eradicate the protozoal infection of herds avoiding the perpetuation of the infection to lineage; indeed, these cross-bred calves would not be used as internal remount but sold for slaughtering. However, despite the importance of control strategies against *N. caninum*, there are few published data about the long-term effects and the economic benefits of control strategies on the reduction of seroprevalence of *N. caninum* infection in cattle herds.

The aim of this study was thus to analyze the long-term effects of a control program against *N. caninum* applied in a commercial dairy herd in northern Italy, based on individual and periodic serological screenings and on the use of the beef-breed semen in seropositive cows. The epidemiology of the infection within the herd was explored by checking the prevalence and incidence of infection once a year, and analyzing data concerning apparently vertical transmission and the abortion rate over the study period.

## **MATERIALS AND METHODS**

### **Herd selection**

A dairy herd in northern Italy was selected as a case-study since it had experienced recurrent and increasing abortion events from 2007 up to 14 abortions of both milking cows and heifers in the first semester of 2013. Dams that aborted in the first semester of 2013 were blood-sampled in June and serologically tested (as described below) for *N. caninum* and for other abortifacient pathogens: Bovine Viral Diarrhea Virus (BVDV), Bovine Herpesvirus 1 (BHV-1),

*Clamidia psittaci* and *Leptospira* spp.). A definitive and unique etiological diagnosis could not be completely ruled out serologically. Indeed, out of 14 examined, antibodies against *N. caninum*, BVD and *C. psittaci* were found in 11, 8 and 8 animals, respectively. Furthermore, two new aborted fetuses (from cows not included in this first sampling) were collected in June 2013 and submitted to pathological and molecular examination. They were tested in an external laboratory for the same panel of abortifacient pathogens: only *N. caninum* DNA was detected.

### **Description of herd**

At the beginning of the study period (July 2013), the herd was composed of 827 Holstein Frisian cattle (272 lactating cows, 58 dry cows, 125 pregnant heifers, 139 non-pregnant heifers and female calves, 232 males). The farm had been managed as a closed herd since 2000. Based on a managerial strategy of expansion, the number of dairy animals was increased, especially between 2013 and 2014. In 2016, although the number of cows continued to grow, the total herd size was decreased mainly by selling bulls and veal male calves. In 2017, there was an increase in the number of heifers and female calves. Table 1 summarizes the average herd size and average size of the various animal groups per year.

Milking cows were housed separately from the rest of the herd, in a large free stall with a slatted concrete floor and cubicles covered with soft mattresses. Newborn calves were fed with colostrum from the bank, kept in single calf pens until they were two weeks old and then moved to a large collective shed with automatic calf feeders. Heifers were housed on another side of the farm.

Pregnancy diagnoses were performed five weeks post-insemination (PI) via transrectal ultrasonography and confirmed by palpation per rectum on the 90th day PI. Pregnant animals



were inspected daily to detect signs of abortion until calving by the farmer and the farmhand, who recorded all abortions. Four weeks before the expected date of calving, pregnant heifers were moved to the calving pen, and housed with dry cows until calving. Lactating cows were then artificially inseminated directly inside the principal barn. The herd was accredited IBR-free, immunized for neonatal diarrhea agents and sporadically for BVDV. The farm was digitally managed using AfiFarm software (AfiMilk Ltd., Israel).

### **Control program**

Seropositive cattle were retained, because culling a large number of seropositive animals would have caused a serious financial burden, especially in this farm with a managerial strategy of expansion in productive dairy animals. Seropositive animals were culled only when there were additional reasons for them to be culled (e.g. mastitis, abortion, poor milk production, abomasal displacement). Once seropositive animals and the lineage were identified, the control program was developed: seropositive animals were excluded from breeding remounts by artificial insemination with beef semen. Male and female crossbreeds, born from seropositive animals, were considered beef animals and sold as veal calves or bred until slaughtered.

### **Herd sampling and serology**

An initial screening of the herd was carried out in July 2013. Seropositive animals were considered infected and never re-tested. Seronegative cows were tested before each insemination as well as heifers when ready for the first artificial insemination. For the first screening, only females over six months of age were enrolled in the study, to avoid false positives related to colostrum immunity interference (Pare et al., 1996; Alvarez-García et al.,

2013). Blood samples were collected from 565 animals in July 2013, from 419 in 2014, 574 in 2015, 518 in 2016 and 369 in 2017. An overall 1097 animals were enrolled in the study, with 2445 recorded observations.

Blood samples, obtained from the coccygeal vein using 18-gauge needles and vacutainer tubes without anticoagulants, were transported to the laboratory within a few hours. Blood was centrifuged (15 min, 2120×g), and serum stored at -20°C until serological analysis. Samples were analyzed by a commercial indirect multi-species ELISA kit for the detection of anti-*N. caninum* antibodies (ID Screen®, *N. caninum* Indirect Multi-species, ID Vet, Grabel, France), with a 99.6% sensibility and 98.9% specificity (Alvarez-García et al., 2013), according to the manufacturer's instructions.

Blood samples and data were collected during the voluntary application of the control program against *N. caninum*. Publication of data was approved by the ethical committee of the University of Milan (approval number 47/2017, November 28th 2017).

### **Data analysis**

For each animal examined, the following data were collected: date of birth, results of *N. caninum* serology, and abortions, along with the serological results and abortions regarding the ancestors and offspring.

To assess the herd size and the number of animals in each herd group per year, the number of cattle was checked monthly throughout the year, and the average values were calculated. In addition, the herd size was checked on December 31 of each year.

The following data were calculated each year: number of lactating cows, dry cows and pregnant heifers; number of seropositive and seronegative female breeders; number of new seropositive animals detected every year. Period prevalence (P) and incidence (I) of

seropositive animals were calculated per year of study. Furthermore, the incidence of abortion was calculated in each year in seropositive and seronegative dams (Thrusfield, 2018). Subsequently, data were statistically analyzed through generalized estimating equations (GEEs), the animal ID was entered as the subject and the year of sampling as the within-subject variable.

Firstly, differences in seropositivity values among years of sampling were verified. Three models were run, entering the year of sampling as the independent variable, and the serological status as dependent variable (dichotomous variable, binomial distribution with Logit link function) considering a) animals on the farm at December 31 of each year of sampling; b) new cases of infection, including only tested animals and excluding seropositive animals thus not re-tested in the following years; c) seroconverting animals, including only animals that had a negative score the previous year. The estimated means were then compared through pairwise comparisons.

Secondly, any differences were recorded in seroprevalence values between productive categories (productive cows vs. heifers) throughout the study period. A GEE was run entering the year of sampling, the productive category and their interaction as independent variables and the serological status (considering animals on the farm at December 31 of each year of sampling) as the dependent variable, with a pairwise comparison of the estimated means of the interaction.

Thirdly, to verify whether the risk of *N. caninum* infection could be enhanced by the seropositivity of the dams, three models were run, entering the serological status of the dam (considering the last available testing), the year of sampling and their interaction as independent variables and the serological status as the dependent variable (dichotomous variable, distribution binomial with logit link function) considering a) animals on the farm at

December 31 of each year of sampling; b) new cases of infection; c) seroconverting animals.

The estimated means were then compared with pairwise comparisons.

Finally, to verify whether the risk of abortion associated with *N. caninum* differed among years, a GEE was run entering the abortion (presence/absence, dichotomous variable, binomial distribution with logit link function) as the dependent variable and the serological status (considering animals on the farm at December 31 of each year of sampling), the year of sampling, and their interaction as independent variables, with a pairwise comparison of the estimated means of the interaction. Only those abortions that had occurred in the second and third trimesters of gestation were considered.

For all the analyses, the level of significance was set at  $p$ -value  $< 0.05$ . Statistical analysis was performed using SPSS (version 19.0; SPSS, IBM, Chicago, IL).

## RESULTS

In July 2013, 191 out of 565 (33.8%) tested cattle had anti-*N. caninum* antibodies as determined by indirect ELISA. During the following years, the period prevalence and the incidence had decreased down to 28.9% and 1.7%, respectively. Likewise, the number of new seropositive animals (i.e., seroconverting animals and heifers at their first insemination) decreased from 127 in 2014 to 57 in 2017 (Table 2).

Differences in the serological status of the herd throughout the study period were found. The seroprevalence at December 31 of each year was associated with the year of sampling ( $p$ -value=0.0001): in fact, seroprevalence decreased significantly in the last year of sampling (28.9%). Similarly, differences among years of sampling in the results obtained considering only new cases of infection were recorded ( $p$ -value=0.0001), decreasing the number of new cases throughout the study period, particularly from the third year of study (16.5% of

seropositive animals out of 574 tested). Finally, data of animals with negative scores the previous years and having more than one test (1529 observations from 656 animals) were analyzed: the percentage of seroconverting animals decreased during sampling years ( $p$ -value=0.001), from 6.6% in 2014 to 1.4% in 2017 (Table 3).

Subsequently, it was verified whether the seropositivity differed between the productive categories (cows and heifers). All variables in the model (productive category, year of sampling and their interaction) were associated with *N. caninum* seropositivity ( $p$ -values=0.0001), with cows at higher risk of infection [ $\beta \pm s.e.$ :  $2.889 \pm 0.5926$ ; OR (95% CI): 17.982 (5.629-57.448)] than heifers. Considering only cows, seroprevalence values slightly differed during the study period, while in heifers values began to decrease significantly from 2015 (from 26.9% in 2013, to 14.2% in 2015 up to 2.8% in 2017) (Table 4).

Data on the serological status of dams of 745 animals were known. Considering animals on the farm at December 31 of each year of sampling (2278 observations), the serological status of the dams [ $p$ -value=0.0001;  $\beta \pm s.e.$ = $2.567 \pm 0.2675$ ; OR (95%CI) = 13.023 (7.709-22.001)], the year of sampling ( $p$ -value=0.011) and their interaction ( $p$ -value=0.035) were associated with seropositivity.

The serological status of animals born to positive dams did not differ among year of sampling, while pairwise comparisons highlighted differences between results obtained on the serological status of animals born to seronegative dams tested in 2017 and all the previous years of study (Table 5). Also considering only tested animals (excluding animals that had tested positive the previous year of sampling and thus not retested) (1590 observations from 745 animals), the serological status of the dams [ $p$ -value=0.0001;  $\beta \pm s.e.$ = $2.560 \pm 0.2676$ ; OR (95%CI) = 12.937 (7.657-21.859)], the year of sampling ( $p$ -value=0.0001) and their interaction

( $p$ -value=0.034) were associated with seropositivity, since seropositivity decreased in animals born to both seropositive and seronegative dams (Table 5).

Considering only animals with negative scores the previous year (923 observations from 400 animals), the year of sampling was not statistically associated with seropositivity ( $p$ -value>0.05), as opposed to the serological status of the dams [ $p$ -value=0.0001;  $\beta \pm s.e.$ =2.443 $\pm$ 0.6380; OR (95%CI) =11.510 (3.2986-40.194)] and its interaction with the year of sampling ( $p$ -value=0.004). No difference in seropositivity was found among years of sampling considering animals born to positive dams, while seropositivity of animals born to negative dams decreased during the study period (Table 5).

Finally, the risk of abortions was associated with seropositivity to *N. caninum* and it differed among years of sampling. Data concerning heifers were not considered. During the four years of study, we recorded 56 abortions. Gestational ages at abortion were higher in the second trimester for the first two years (7/19 in second trimester, 8/19 in third trimester in 2013; 9/12 in second trimester, 1/12 in third trimester in 2014), whereas for 2015-2017 the number of abortions in the second and third trimesters were similar (2/7 in second trimester, 5/7 in third trimester in 2015; 5/12 in second trimester, 7/12 in third trimester in 2016; 4/6 in second trimester, 1/6 in third trimester in 2017). Excluding from the analysis abortions occurred in first trimester of gestation, GEE revealed that the risk of abortion was enhanced by seropositivity to *N. caninum* [ $p$ -value: 0.0001;  $\beta \pm s.e.$ : 1.481 $\pm$ 0.2993; OR (95% CI): 4.396 (2.445-7.902)] and the year of sampling ( $p$ -value=0.04), as opposed to their interaction ( $p$ -value>0.05). The risk of abortion decreased during the study period, with the lowest percentage of abortions in the last year of sampling (Table 6).

## DISCUSSION

A control program of bovine neosporosis, based exclusively on the use of beef semen, was tested in a dairy cattle herd with previously reported cases of abortions attributable to *N. caninum* and with a seroprevalence of 33.8% at the beginning of the 5-year study period.

Previous studies have shown that the use of beef semen significantly reduces the risk of *N. caninum* abortions in seropositive dairy cows (López-Gatius et al., 2005; Almería and López-Gatius, 2013). We hypothesized that long-term systematic use of beef semen in seropositive breeders, may reduce *N. caninum* incidence and prevalence, due to seropositive descendant exclusion from remount. Considering the paucity of research on the role of a long-term-cross-breeding-based control strategy, our results provide novel information on this disease in dairy cattle.

The application of this long-term control program significantly reduced the prevalence and incidence of *N. caninum* infection over the five years, although it was not fully eradicated.

At the first serological screening in July 2013, the animal seroprevalence was 33.8%, which was higher than the prevalence values reported in the literature. Magnino et al.(1999) reported that neosporosis had a seroprevalence of 24.4% in 5912 sera collected from aborting cows. In a following study, a cross-sectional serological survey for *N. caninum* was carried out on beef and dairy cattle in southern and northern Italy. The seroprevalence within the herds ranged from 10 to 50% in southern Italy (median 20%), and from 6.3 to 61.1% in northern Italy (median 18%) (Otranto et al., 2003). The median-prevalence of *N. caninum* worldwide was reported at 16.1% (range: 3.8-89.2%) (Reichel et al., 2013).

Our control program began after the first serological screening in 2013, and consisted in removing female breeders from the reproductive dairy line, without culling them and without replacing them with purchased seronegative pregnant heifers. Seropositive breeders were artificially inseminated with beef semen. Seroprevalence remained almost the same until

2015 (33.8-35%), whereas it started decreasing in 2016 and in 2017 (34.4% and 28.9%, respectively). There were significant differences between the seropositivity in 2016 and 2017 and the other three years of the study (for each comparison:  $p$ -value=0.0001). Likewise, the incidence remained similar in 2014 (15.9%) and 2015 (10.6%), and then decreased in 2016 (5.1%) and 2017 (1.7%).

Our control strategy, in a period of five years, did not bring the seroprevalence to zero, compared to the test-and-cull method reported in the literature (Hall et al., 2005). In fact, we did not cull seropositive animals unless they presented additional reasons to be culled. Therefore, the removal of seropositive animals from the herd was slower, thus the decrease in seropositive prevalence requires more time. A similar eradication program, applied in a dairy goat herd, consisted in removing all positive animals and all female offspring from perpetuating lines from the herd (Altbuch et al., 2012).

As part of this study, the lineage of new seropositive animals was investigated to highlight vertical transmission. Transplacental transmission contributes significantly to the maintenance of *N. caninum* infection in a herd, by propagating the infection to subsequent generations (Dubey et al., 2006). In herds in which neosporosis is endemic, vertical transmission is the dominating route of infection, and thus seropositivity of *N. caninum* infection follows family lines (Wouda et al., 1998; Dijkstra et al., 2001). In our study, the number of new seropositive animals with a positive lineage, within animals never tested before, decreased from 66.1% in 2014 to 55.4% in 2017. This may be imputable to animals that were younger than six months in 2013 or not yet born, and therefore never tested before. On the other hand, among new cases in animals previously tested as negative but born from seropositive ancestors, seroconversion may be attributed to antibody fluctuations during the



lifetime of the animal (Wouda et al., 1998), or to a post-natal transmission through the horizontal way of infection (Dubey et al., 2007).

Another interesting finding was that abortion frequency decreased from 19 in 2013 to 6 in 2017. There was a statistically significant difference in the number of abortions during the years of study; furthermore, seropositive animals were associated with the risk of abortion ( $p$ -value=0.0001). In fact, the percentage of aborting seropositive cows (8.3% in 2013) decreased to under the acceptable abortion rate of 5% (Hopper, 2014) already from the second year of study (3%) up to 2.2% at the end of the study period. These results are supported by the observation that insemination with beef-breed semen halves the abortion rate (López-Gatius et al., 2005; Almería and López-Gatius, 2013).

The results of our study demonstrated that *N. caninum* infection can be controlled without using a test and cull strategy. Although the prevalence slightly decreased from the beginning of the study, the incidence of seropositivity in the herd was 15.9% in 2014 and had dropped to 1.7% in 2017. The removal of seropositive animals from the dairy reproductive line reduced the number of new seropositive animals in the herd by the insemination with beef semen, thus blocking vertical transmission. Furthermore, the abortion rate decreased.

A limitation is that more than five years would be required to eradicate *N. caninum* infection in the herd, depending on the longevity and productivity of seropositive animals. Further studies are necessary to investigate prevalence and incidence in a longer time period. Ideally, we need to know the time required to eradicate *N. caninum*, or alternatively to understand exactly when this control program should be terminated, and a test and cull strategy might become more economically advantageous. In addition, the financial costs of the test and cull strategy versus our long-term control plan need to be compared. A problem with this control plan is that it may lead to a lack of remount dairy heifers. In the present study it did not

happen. To prevent this situation, it is useful to pay attention to the reproduction and to the health of dairy calves. If this is not enough, dairy sexed semen on seronegative dams can be employed to increase the number of dairy heifers.

In conclusion, the reduction of the prevalence and incidence of seropositivity in a *N. caninum*-affected herd can be obtained through the serological monitoring of the herd and the exclusion of seropositive animals from breeding, without culling and subsequently with negligible economic losses.

ACCEPTED MANUSCRIPT

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## TABLES

**Table 1.** Summary of the average herd size per year. The number of cows per year was calculated as the average of the monthly number of animals during the year.

	<b>2013</b>	<b>2014</b>	<b>2015</b>	<b>2016</b>	<b>2017</b>
<b>Lactating cows</b>	285	335	349	394	386
<b>Dry cows</b>	41	43	47	47	52
<b>Pregnant heifers</b>	108	100	140	103	86
<b>Other heifers + female calves</b>	183	283	239	243	264
<b>Male calves/Steers</b>	231	269	268	179	455
<b>Average herd size</b>	<b>852</b>	<b>1030</b>	<b>1044</b>	<b>966</b>	<b>1243</b>



**Table 2.** Summary of seropositive animals to *Neospora caninum*.

Year	Period prevalence: Seropositive/Examined <sup>1</sup> (%)	Period incidence: new seropositive animals <sup>2</sup> /population at risk (%) <sup>3</sup>	New seropositive animals/ (re- tested animals <sup>4</sup> (%)	Seroconverting cows/previously negative cattle <sup>5</sup> (%)
2013	191/565 (33.8)	-	-	-
2014	266/723 (36.8)	87/544 (15.9)	127/419 (30.3)	22/332 (6.6)
2015	267/762 (35.0)	65/612 (10.6)	95/574 (16.5)	36/416 (8.7)
2016	242/703 (34.4)	24/473 (5.1)	86/518 (16.6)	23/425 (5.4)
2017	189/653 (28.9)	8/462 (1.7)	57/369 (15.4)	5/356 (1.4)

<sup>1</sup>Number of tested animals on the farm on the 31<sup>st</sup> of December of each year of sampling; <sup>2</sup>new seropositive animals on the farm on the 31<sup>st</sup> of December; <sup>3</sup>animals scoring seronegative to previous samplings and heifers never tested before were considered as an at risk population; <sup>4</sup>tested animals during each year of sampling (i.e., animals scoring seronegative to previous sampling and heifers never tested before; animals scoring positive to previous sampling were excluded); <sup>5</sup>only animals with negative scores during the previous year were considered (heifers never tested before were therefore excluded).

**Table 3.** Values of seropositivity to *Neospora caninum* and results of Generalized Estimating Equations (GEEs).

	Year of sampling	seropositive/examined	% <sup>4</sup>	$\beta \pm s.e.$ <sup>5</sup>	p-value	OR (95% CI)
Seroprevalence at 31 December of each year of sampling <sup>1</sup>	2013	191/565	33.8 <sub>a</sub>	0.226±0.1067	0.034	1.254 (1.017-1.545)
	2014	266/723	36.8 <sub>b</sub>	0.357±0.0903	0.0001	1.429 (1.197-1.706)
	2015	267/762	35.0 <sub>ab</sub>	0.281±0.0719	0.0001	1.324 (1.150-12.525)
	2016	242/703	34.4 <sub>ab</sub>	0.254±0.0525	0.0001	1.289 (1.163-1.428)
	2017 (reference)	189/653	28.9 <sub>c</sub>	0	-	1
New seropositives / (re-)tested animals <sup>2</sup>	2013	191/565	33.8 <sub>a</sub>	1.041±0.1536	0.0001	2.833 (2.097-3.829)
	2014	127/419	30.3 <sub>b</sub>	0.867±0.1674	0.0001	2.381 (1.715-3.305)
	2015	95/574	16.5 <sub>c</sub>	0.082±0.1715	0.632	1.086 (0.776-1.519)
	2016	86/518	16.6 <sub>c</sub>	0.086±0.1747	0.623	1.09 (0.774-1.535)
	2017 (reference)	57/369	15.4 <sub>c</sub>	0	-	1
Seroconverting cows/previously negative cattle <sup>3</sup>	2014	22/332	6.6 <sub>a</sub>	1.606±0.5020	0.001	4.982 (1.862-13.326)
	2015	36/416	8.7 <sub>a</sub>	1.895±0.4853	0.0001	6.651 (2.569-17.218)
	2016	23/425	5.4 <sub>a</sub>	1.390±0.4996	0.005	4.016 (1.509-10.693)
	2017 (reference)	5/356	1.4 <sub>b</sub>	0	-	1

<sup>1</sup>Number of examined animals on the farm on the 31<sup>st</sup> of December of each year of sampling;

<sup>2</sup>tested animals during each year of sampling (i.e., animals scoring seronegative to previous sampling and heifers never tested before; animals scoring positive to previous sampling and therefore not re-tested during the following years were excluded); <sup>3</sup>only animals with negative scores during the previous year were considered (heifers never tested before were therefore excluded) <sup>4</sup>For each GEE, values of seropositivity per each year of sampling with different superscript letters (a, b, c) are statistically different from each other (p-value <0.05, GEE, pairwise comparison), while those with the same superscript letters (a, b, c) are not statistically different from each other (p-value >0.05, GEE, pairwise comparison); <sup>5</sup> $\beta \pm \text{s.e.} = \text{Coefficient} \pm \text{standard error}$ .

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**Table 4.** Seroprevalence values of *Neospora caninum* in examined animals in farm at 31 December of each year of sampling in the considered productive categories and results of pairwise comparisons obtained within the Generalized Estimating Equation (GEE).

Year of sampling	Cows		Heifers	
	seropositive/examined	Period prevalence % <sup>1</sup>	seropositive/examined	Period prevalence % <sup>1</sup>
2013	156/435	35.9% <sup>abcd</sup>	35/130	26.9% <sup>a</sup>
2014	199/517	38.5% <sup>abcd</sup>	67/206	32.5% <sup>a</sup>
2015	238/558	42.7% <sup>e</sup>	29/204	14.2% <sup>b</sup>
2016	241/654	37.6% <sup>abc</sup>	1/49	2.0% <sup>c</sup>
2017	186/547	34.0% <sup>abd</sup>	3/106	2.8% <sup>c</sup>

<sup>1</sup>For each GEE, values of seropositivity per each year of sampling with different superscript letters (a, b, c, d, e) are statistically different from each other (p-value <0.05, GEE, pairwise comparison), while those with the same superscript letters (a, b, c, d, e) are not statistically different from each other (p-value >0.05, GEE, pairwise comparison).

**Table 5.** Data on seropositivity to *Neospora caninum* in individuals born to seropositive and seronegative dams and results of pairwise comparisons obtained within the Generalized Estimating Equations (GEEs).

Year of sampling	Positive animals born to positive dams						Positive animals born to negative dams					
	Seroprevalence at 31 December of each year of sampling <sup>1</sup>		New cases of infection/new (re-)tested animals <sup>2</sup>		Seroconverting cows/previously negative cattle <sup>3</sup>		Seroprevalence on the 31 <sup>st</sup> of December of each year of sampling <sup>1</sup>		New cases of infection/new (re-)tested animals <sup>2</sup>		Seroconverting cows/previously negative cattle <sup>3</sup>	
	Positive/examined	%*	Positive/examined	%*	Positive/examined	%*	Positive/examined	%*	Positive/examined	%*	Positive/examined	%*
2013	111/150	74 <sup>a</sup>	111/150	74 <sup>a</sup>	-	-	33/84	17.9 <sup>a</sup>	33/84	17.9 <sup>a</sup>	-	-
2014	166/17	76.5 <sup>a</sup>	78/18	66.1 <sup>ab</sup>	9/35	25.7 <sup>a</sup>	37/31	16 <sup>a</sup>	15/47	10.2 <sup>b</sup>	4/7	2.9 <sup>abc</sup>
2015	167/16	77.3 <sup>a</sup>	44/9	48.4 <sup>c</sup>	8/48	16.7 <sup>a</sup>	44/87	15.3 <sup>a</sup>	26/64	9.8 <sup>b</sup>	18/88	9.6 <sup>d</sup>
2016	153/89	81 <sup>a</sup>	54/7	62.1 <sup>b</sup>	10/3	23.3 <sup>a</sup>	40/06	13.1 <sup>a</sup>	16/61	6.1 <sup>bc</sup>	7/22	3.1 <sup>ab</sup>
2017	116/51	76.8 <sup>a</sup>	31/5	55.4 <sup>bc</sup>	8/33	24.2 <sup>a</sup>	32/47	9.2 <sup>b</sup>	11/33	4.7 <sup>c</sup>	1/4	0.5 <sup>ac</sup>

<sup>1</sup>Number of tested animals on the farm on the 31<sup>st</sup> of December of each year of sampling; <sup>2</sup>tested animals during each year of sampling (i.e., animals scoring seronegative to previous sampling and heifers never tested before; animals scoring positive to previous sampling and therefore not re-tested during the following years were excluded); <sup>3</sup>only animals with negative scores the previous year were considered (heifers never tested before were therefore excluded); \*For each GEE, values of seropositivity per each year of sampling with different superscript letters (a, b, c, d) are statistically different from each other (p-value <0.05, GEE, pairwise comparison), while those with the same superscript letters (a, b, c, d) are not statistically different from each other (p-value >0.05, GEE, pairwise comparison).

**Table 6.** Data on abortions occurred within the study period and results of Generalized Estimating Equation (GEE). Only abortions occurred in the second and third trimester of gestation were considered.

Year of sampling	Overall abortions					Abortion in seropositive animals		Abortion in seronegative animals	
	abortions/examined	%	$\beta \pm s.e.^1$	p-value	OR (95% CI)	abortions/examined	% <sup>2</sup>	abortions/examined	% <sup>2</sup>
2013 (reference)	15/435	3.4%	0	-	1	10/156	6.4% <sub>a</sub>	5/279	1.8% <sub>a</sub>
2014	10/517	1.9%	0.642±0.4229	0.129	0.526 (0.235-1.206)	5/199	2.5% <sub>ab</sub>	5/318	1.6% <sub>ab</sub>
2015	7/558	1.3%	-1.144±0.4611	0.013	0.318 (0.129-0.786)	6/238	2.5% <sub>b</sub>	1/320	0.3% <sub>b</sub>
2016	12/654	1.8%	-0.671±0.3944	0.089	0.511 (0.236-1.107)	11/241	4.6% <sub>b</sub>	1/413	0.2% <sub>b</sub>
2017	5/547	0.9%	-1.338±0.5229	0.010	0.262 (0.094-0.731)	3/186	1.6% <sub>b</sub>	2/361	0.6% <sub>b</sub>

<sup>1</sup>  $\beta \pm s.e.$  = Coefficient  $\pm$  standard error; <sup>2</sup> the values of proportion of abortions in the columns per each year of sampling with different superscript letters (a, b) are statistically different from each other (p-value <0.05, GEE, pairwise comparison), while those with the same superscript letters (a, b) are not statistically different from each other (p-value >0.05, GEE, pairwise comparison).