

# The Relationship of Abortion, Dog Management and *Neospora caninum* in Dairy Herds in Taiwan

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## Abstract

*Neospora caninum* (*N. caninum*) has caused an abortion storm in dairy herds for two decades in Taiwan. There is no effective medicine for *N. caninum* infection for livestock, therefore, prevention by farm management is very important. The risk factors for *N. caninum* infection in Taiwan is still unclear. Dogs are the common definitive hosts of *N. caninum* on farms. This study aims to estimate the sero-prevalence and detection of *N. caninum* DNA in 361 pregnant dairy cows and establish an effective strategy to prevent abortion caused by *N. caninum* in dairy farms in Taiwan by logistic regression. The sero-prevalence of *N. caninum* was 26.3% (95/361) and 75% (24/32) in cows and farms, respectively. The positive rate of *N. caninum* DNA detection in blood by semi-nested polymerase chain reaction (PCR) and real-time PCR was 2.2% and 17.2%, respectively. There were twelve dairy herds with an abortion rate >5%. Seven farmers claimed to have had severe abortion issues on their farms, of which, six had an abortion rate >5%. Sero-positivity was significantly higher in cows with an abortion history. The presence of domesticated dogs was negatively associated with sero-positivity of *N. caninum*, whereas free-roaming dogs in the farm area were positively associated with sero-positivity of *N. caninum*. In conclusion, managing free-roaming dogs is an efficient approach to reducing the sero-prevalence of *N. caninum* infection in dairy herds and *N. caninum* infection is one of the major factors causing abortion in dairy herds in Taiwan.

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**Keywords:** Abortion, Dairy cows, Dog management, *Neospora caninum*, Sero-prevalence

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## Introduction

Dairy farms in Taiwan are mostly of small field size, housed in an open house system. According to year-end statistics in 2017, dairy farms in Taiwan have reached a total number of 553, raising 130,413 cows, with 236 cows per farm. The average size of dairy a farm was 6,607 m<sup>2</sup>. In this kind of intensive farming, pathogens are easily spread from animal to animal. Therefore, disease control in intensive livestock management is very important. Abortion in dairy farms is not only a health problem but also a significant welfare issue.

Repeated abortions have been reported in some cases due to *N. caninum* infection, with global studies indicating that 8–42% of aborted fetuses in dairy cows are caused by *N. caninum* (Dubey, 2003; Ribeiro, 2019). Also, the presence and number of dogs on a dairy farm is a known risk factor for increased infections or abortion in cows due to *N. caninum* (Dubey et al., 2007). According to Wouda et al., the seropositivity of *N. caninum* in cattle strongly correlates with the high seropositivity of domesticated dogs and on farms without dogs seroprevalence of *N. caninum* infection in cattle is significantly lower ( $P = 0.0002$ ) than farms with dogs (Wouda et al., 1999). Domesticated dogs can ingest body fluids, dead fetuses or the placenta of cows that have undergone an abortion due to *N. caninum* infection, leading to the transmission of *N. caninum* to dogs, which can then contaminate the water or feed of cows, increasing the risk of horizontal transmission, hence the potential for an abortion storm in dairy farms (Frossling et al., 2005).

The first study of *N. caninum* infection in Taiwan was reported in 2000, when antibodies against *N. caninum* in sera, milk, vaginal secretions and saliva of infected cows, as well as in farm dogs were analyzed (Ooi et al., 2000). Abortion in dairy cows caused by *N. caninum* in Taiwan was also investigated during an abortion storm, indicating that more than 65% of dairy cows with an abortion history tested positive for IgG

against *N. caninum*. When the abortion storm passed, 57% of cows remained IgG positive even after one year (Huang et al., 2004).

There are no safe and effective medicinal treatments or vaccines for *N. caninum* infection in livestock (Reichel et al., 2014). Therefore, prevention by farm management is very important. To establish an effective strategy to prevent abortion caused by *N. caninum* in dairy farms, the present study was conducted 1) to determine the current situation of *N. caninum* infection in dairy herds in Taiwan using serological analysis and detection of *N. caninum* DNA 2) to clarify the relationship of abortion, dog management and seroprevalence of *N. caninum* in dairy herds.

## Materials and Methods

**Animal population and sampling:** A survey was conducted from April 2017 to February 2018, when 361 multiparous pregnant cows from 32 dairy herds in North, Middle, South and East Taiwan were selected by random sampling and stratified based on dairy cattle numbers from a list of 60,165 primiparous and multiparous dairy cattle in 2017 (Table 1). A sample of pregnant cows was randomly selected for testing from each herd, which was sufficient to estimate the 33.1% sero-prevalence rate of a previous study (Cheng et al., 2000) with a 95% confidence interval. In this study, at least 340 blood samples were required with a 5% error and 95% confidence interval and the recruited samples satisfactorily represented all of the dairy cattle in Taiwan. All owners agreed samples for collection in this study. Ethical review and approval were waived due to this study being based on field clinical diagnosis for farm animal health. All sampling procedures were executed by veterinarians under the Animal Protection Act from the Council of Agriculture in Taiwan. No extra animals were used or purchased for this study.

**Table 1** The number of dairy cattle in different regions in Taiwan.

Regions	Numbers of dairy cattle in Taiwan in 2017 (%)	Numbers of dairy cattle recruited for this study (%)	Areas included in this region
North	4,797(8.0%)	25(6.9%)	Yilan, New Taipei, Keelung, Taipei, Taoyuan, and Hsinchu
Middle	23,712(39.4%)	140(38.8%)	Miaoli, Taichung, Nantou, Changhua, and Yunlin
South	30,175(50.2%)	153(42.4%)	Chiayi, Tainan, Kaohsiung, Penghu, and Pingtung
East	1,481(2.5%)	43(11.9%)	Hualien and Taitung

A questionnaire was filled out after personal interviews with the farm owners to collect information regarding the average annual abortion rate on the farm, if farmers considered their farm to have severe abortion issues, the presence of domesticated dogs or presence of free-roaming dogs in the farm area. Abortion was defined as the dams delivering their fetuses from 45 to 260 days in the pregnancy period. The average farm abortion rate was separated into two groups, higher than 5% and

lower than 5%, based on United States Department of Agriculture data. The US National Animal Health Monitoring Service considered an average of a 5% abortion rate each year in dairy farms as normal (United, 2008). An abortion rate higher than 5% meant the farm was undergoing large scale abortion.

Domesticated dogs could be caged, leashed or free-roaming. All of the domesticated dogs were under surveillance and fed by the owners of the farms. Free-roaming dogs included both stray dogs

and unleashed domesticated dogs (caged/leashed but having a particular time to be uncaged/unleashed and walking in the farm). The sampled cows were in various stages of pregnancy. The abortion history of each sampled cow was also obtained.

#### **Enzyme-linked immunosorbent assay (ELISA):**

Plasma samples were tested for antibodies against *N. caninum* using an indirect ELISA kit and the *N. caninum* Antibody Test Kit (IDEXX, USA). The kit used anti-ruminant IgG conjugated to the ruminant antibodies with *N. caninum* antigen, which interpreted S/P% <30 as negative, S/P% ≥40 as positive, and  $30 \leq \text{S/P\%} < 40$  as suspicious.

**Semi-nested PCR:** Blood samples were collected into a 10 ml EDTA tube (BD Vacutainer®, USA) from the coccygeal vessel of 361 cows. The DNA was isolated from 200 µl of the buffer coat and extracted using the Genemark Plus Blood Genomic DNA Purification Kit (GMBiolab, Taiwan) according to the manufacturer's instructions. The extracted sample DNA was used as a template to amplify NC-5 (Genbank: HM031966.1), one of the most highly sensitive and specific for the detection of neosporosis (Hughes *et al.*, 2006), by semi-nested PCR. The first step of semi-nested PCR was performed using 5 µl of extracted sample DNA, 10 µl of 2X Taq PCR MasterMix (Genomics Bioscience and Technology, Taiwan), 0.5 µl (10 µM) of each forward and reverse primer and 4 µl pure water in a 0.2 ml Eppendorf. The PCR conditions were as follows: 94°C for 5 mins, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 2 mins and 72°C for 10 mins. The second step of semi-nested PCR was performed using 5 µl of the PCR product from the first reaction rather than the extracted DNA and the same cycling conditions as the first step. The primers for the first step were Np21+ and Np6+, and the primers for the second step were Np7 and Np6+, a pair of semi-nested internal primers to amplify a 232bp fragment to enhance the sensitivity of the first step (Ishigaki *et al.*, 2012). The positive control was sequenced (Genomics Bioscience and Technology, Taiwan) and BLAST analysis demonstrated a 100% match for the NC-5 (Genbank: HM031966.1) sequence.

**Real-time PCR:** The step of real-time PCR was performed using 2.5 µl of extracted sample DNA same as semi-nested PCR, 10 µl of 2X SYBR® Green Master Mix (Bio-rad, USA), 0.25 µl (10 µM) of each forward and reverse primer and 7 µl pure water in a 0.2 ml Eppendorf. The primers of real-time PCR were NeoF (5'-GTGAGAGGTGGGATACG) and NeoR (5'-GTCCGCTTGCTCCCTA), following the research in 2005, which amplified 188bp of NC-5 fragment of *N. caninum* (Okeoma *et al.*, 2005). Real-time PCR was carried out using the CFX Connect™ Real-time PCR Detection System (Bio-rad, USA) and the standard curves were generated by 10-fold serial dilutions of positive control *N. caninum* genomic DNA and were used to compute the relative concentration of parasite DNA and the cycle threshold in the test samples in CFX Maestro™ software (Bio-rad, USA). The thermal

cycles of real-time PCR were as follows: denaturation at 95°C for 10 mins, followed by 45 cycles of 95°C for 10 secs, 59°C for 10 secs, 72°C for 20 secs and the amplification reaction was followed by a melting curve (from 65°C to 95°C by 0.5°C). The standard curve was used to calculate the efficiency of the real-time PCR from the given slopes in CFX Maestro™ software (Bio-rad, USA). The efficiency of a real-time PCR cycle is calculated using the equation  $E = [1 - 10^{-1/\text{slope}}] \times 100\%$ .

**Statistical analysis:** The statistical analysis between regions in the results of ELISA, nested PCR, and real-time PCR was conducted by Fisher's exact test using the computing environment R (R Development Core Team, 2010).

Logistic regression was performed to construct two models to analyze the management-related factors of abortion in each cow. The association between the occurrence of abortion in a pregnant cow (abortion history "Yes") and the ELISA (two suspicious samples were regarded as negative) or PCR results of *N. caninum* was first analyzed to evaluate whether *N. caninum* infection was associated with abortion in cows during pregnancy. Then, it was determined whether the environmental variables were associated with *N. caninum* infection.

First, all the categorical explanatory variables were transformed into dummy variables, and the numerical variables were centered and standardized. The logistic regression was fitted according to the maximum likelihood using the computing environment R (R Development Core Team, 2010). For statistical analysis, the correlation with ELISA or PCR results of *N. caninum* and abortion risk factors in the farm were analyzed for all sampled farms. For model construction, the model was first fitted with each explanatory variable alone, retaining the variables with a p-value less than 0.1 for further multivariable model construction.

Multicollinearity between explanatory variables was evaluated using the variance inflation factor (VIF) (Graham, 2003), which was set at 10 to avoid the problematic effect of multicollinearity on parameter estimations (Allison, 1999). A variable was discarded from the model construction if the VIF value was larger than 10.

Explanatory variables selected in the models were based on the Wald test with a *p* threshold value set to 0.05 (Bolker *et al.*, 2009; Dohoo *et al.*, 2009). The backward stepwise method was used for variable selection. Values of the Akaike information criterion (AIC) and Akaike weights were used to assess the model fit and the best-fitting model was selected (Bolker *et al.*, 2009; Burnham and Anderson, 2002; Dohoo *et al.*, 2009).

## **Results**

**Sero-prevalence analysis of *N. caninum* in cattle blood samples by ELISA:** The sero-prevalence of *N. caninum* infection was 26.3% (95/361) in the 361 pregnant cows. The percentage of *N. caninum*-antibody positive herds was 75.0% (24/32) and seropositivity in different herds varied from 0% to 100%.

Sero-prevalence in North, Middle, South and East Taiwan are shown in Table 2. There was no significant difference between each region.

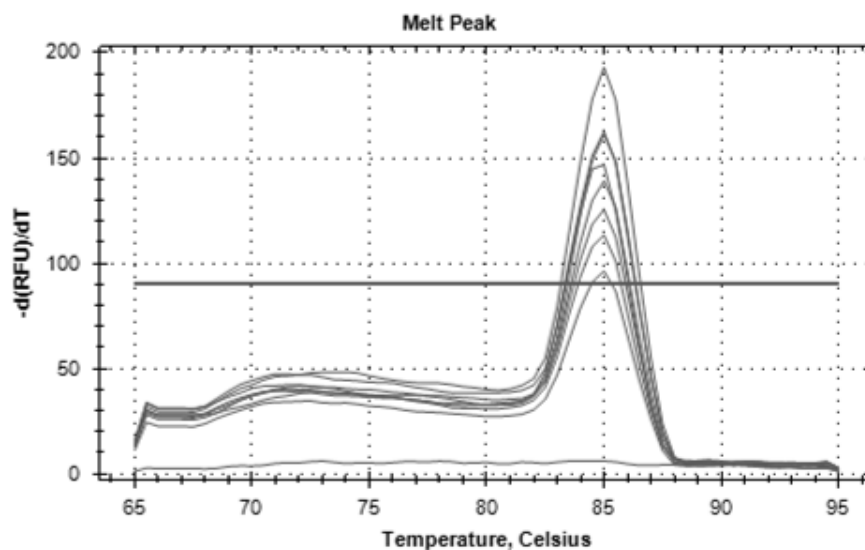
**Detection of *N. caninum* DNA in cattle blood samples using semi-nested PCR and real-time PCR:**

The positive rate of detection of the NC-5 fragment of *N. caninum* by semi-nested PCR and real-time PCR was 2.2% (8/361) and 17.2% (62/361), respectively. The peak of melting temperature was 85°C (Fig. 1) and

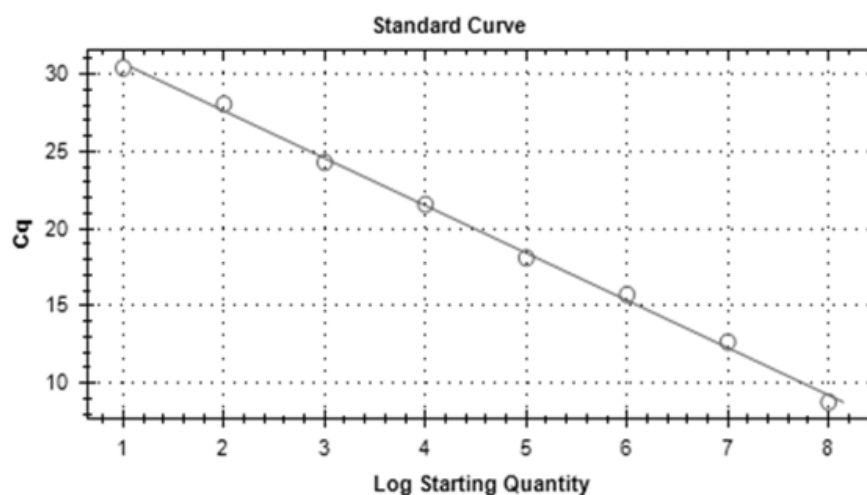
the correlation coefficient  $R^2$  of standard curve was 0.998 in real-time PCR (Fig. 2). The cycle threshold of positive samples in real-time PCR was from 30.12 to 44.59. The *N. caninum* DNA-positive percentage in North, Middle, South and East Taiwan is in Table 2. There was no significant difference between each region in semi-nested PCR results but in real-time PCR results, the positive rate in Middle Taiwan was significantly higher than South and East Taiwan ( $p < 0.05$ ).

**Table 2** Seroprevalence of *N. caninum* infection and positive number of *N. caninum* DNA detected by semi-nested PCR in dairy cows in different regions in Taiwan with a 95% confidence interval (CI).

Regions	Seroprevalence of <i>N. caninum</i> infection (%; 95% CI)	Positive number of <i>N. caninum</i> DNA detection by semi-nested PCR (%; 95% CI)	Positive number of <i>N. caninum</i> DNA detection by real-time PCR (%; 95% CI)
North	5/25 (20.0%, 4.3-35.7%)	1/25 (4.0%, 0-11.7%)	3/25 (12.0%, 0-24.7%)
Middle	36/140 (25.7%, 18.5-33.0%)	4/140 (2.9%, 0.1-5.6%)	35/140 (25.0%, 17.8-32.2%)
South	48/153 (31.4%, 24.0-38.7%)	2/153 (1.3%, 0-3.1%)	20/153 (13.1%, 7.8-18.4%)
East	6/43 (14.0%, 3.6-24.3%)	1/43 (2.3%, 0-6.8%)	4/43 (9.3%, 0.6-18.0%)
Total	95/361 (26.3%, 21.8-30.9%)	8/361 (2.2%, 0.7-3.7%)	62/361 (17.1%, 13.2-21.0%)



**Figure 1** The melting curve for 10 times serial diluted positive control in real-time PCR. Curve shows one single peak at 85°C with no primer dimers.



**Figure 2** The standard curve of real-time PCR. The log concentration of positive control *N. caninum* DNA (X axial) and the crossing point of different concentrations of positive control (Y axial) are in linear correlation. The efficiency of a PCR cycle is 112% and the correlation coefficient  $R^2$  of standard curve is 0.998 in this experiment.

**Abortion issues in herds:** There were twelve dairy herds with an abortion rate >5%, with seven farmers claiming to have severe abortion issues on their farms, six of which had a high abortion rate (average abortion rate >5%).

**Statistical analysis of the management-related risk factors with abortion history of each cow:** The first model evaluated the relationship between the abortion history of each cow and *N. caninum* infection based on the results of ELISA and PCR screening (as explanatory variables) for the plasma antibody and *N. caninum* DNA, respectively. The abortion history in 13 of the 361 cows was not provided by the farmers in the questionnaire. ELISA screening was significantly and positively associated with abortion, whereas the PCR results revealed no positive correlation ( $p = 0.108$ ). The AIC value and Akaike weight also indicated that the model including the

ELISA screening variable had higher proportional support compared to that of the PCR screening variable (Table 3).

The possible environmental variables associated with *N. caninum* infections in cows using ELISA screening as the dependent variable were analyzed using the second model. For the final fitted model with explanatory variables of farms located in East Taiwan with domesticated dogs, the presence of free-roaming dogs in the farm area had the lowest AIC value and Akaike weight of 0.579 (Table 4). The presence of domesticated dogs was negatively associated with the sero-positivity of *N. caninum*, whereas the presence of free-roaming dogs in the farm area was positively associated with the sero-positivity of *N. caninum* (Table 4). The odds ratios of explanatory variables significantly associated with the dependent variable are listed in Table 4.

**Table 3** Comparison of Akaike information criteria and Akaike weights between different model fits for abortion history and *N. caninum* infection in dairy cows. For both models with abortion history or ELISA result as the dependent variable, we list two fitted models with lower AIC values and null model to compare the AIC value and Akaike weight.

Dependent variable	Explanatory Variables included	AIC	$\Delta AIC^a$	Akaike weight
Abortion history	ELISA result	199.7	-1.94	0.565
Abortion history	PCR result	201.58	-0.06	0.22
Abortion history	Null model <sup>b</sup>	201.64	0	0.21
ELISA result	East area	388.55	-7.02	0.408
	South area			
	Domesticated dogs			
	Free roaming dogs			
ELISA result	East area	387.85	-7.72	0.579
	Domesticated dogs			
	Free roaming dogs			
ELISA result	Null model	395.57	0	0.012

<sup>a</sup>  $\Delta AIC$ , difference in AIC value from final fitted model.

<sup>b</sup> Null model denotes that there are no explanatory variables included in the logistic regression model.

**Table 4** Explanatory variable statistics and Akaike weight of final fitted abortion and ELISA result model estimated by logistic regression. The explanatory variable of abortion history model was the ELISA result only; for ELISA result model, explanatory variables include east area of Taiwan, the presence of domesticated dogs and the presence of free-roaming dogs in the farm area.

Model	Variables	Coefficient	SE <sup>a</sup>	p value	Odds ratio	95% CI	Akaike weight
Abortion history	ELISA	0.814	0.399	0.042	2.257	1.01–4.905	0.565
	East area	-0.922	0.465	0.047	0.397	0.145–0.925	
ELISA	Domesticated dogs	-0.675	0.287	0.019	0.509	0.29–0.897	0.579
	Free roaming dogs	0.697	0.262	0.008	2.007	1.207–3.38	

<sup>a</sup> Standard error

## Discussion

According to the questionnaire, 37.5% (12/32) farms in Taiwan had a high abortion rate, with 85.7% (6/7) of farmers claiming to have severe abortion issues and a high abortion rate (average abortion rate >5%). For these two variables, an farm average abortion rate of >5% is the current situation, whereas farmers claimed to have a severe abortion problem.

The results indicate that even when farmers recognized the issue of abortion on their farms, they still did not have effective strategies to control abortion problems and they suggest that the abortion of cows remains an important issue in dairy farms in Taiwan.

A previous study reported that infected pregnant cows have *N. caninum* present in tissues and circulating in the blood (Ooi *et al.*, 2000) but rarely active parasitemia (Yao *et al.*, 2009). Although random

sampling was performed in this study, there were 17.2% of pregnant cows that had active parasitemia of *N. caninum*, indicating that *N. caninum* infection remains a problem in dairy farms in Taiwan.

The seropositivity of *N. caninum* was positively associated with an abortion history in cows, indicating that *N. caninum* infection is a major cause of abortion of dairy cows in Taiwan. The results also showed that farms located in East Taiwan were negatively associated with the seropositivity of *N. caninum* infection. Eastern Taiwan is surrounded by high mountains and is very sparsely populated in comparison with the western part (Chang and Feng, 2007), therefore, the number and accessibility of dairy farms in eastern Taiwan is much lower. Lower accessibility and fewer visitors make biosecurity action easier to conduct comprehensively and makes the transmission of *N. caninum* in East Taiwan is less frequent than in other regions of Taiwan.

In many previous studies, the presence of domesticated dogs was found to increase the risk or prevalence of *N. caninum* infection (Anvari *et al.*, 2020; Huang *et al.*, 2004; Wouda *et al.*, 1999). Interestingly, we found that the presence of free-roaming dogs was positively associated with seropositivity of *N. caninum* but the presence of domesticated dogs was negatively associated with seropositivity. Statistical analysis indicated that unleashed dogs or stray dogs played an important role in increasing the exposure of cows to *N. caninum* but domesticated dogs might deter stray dogs from invading the farm, thereby reducing the horizontal transmission of *N. caninum* in dairy herds (negatively associated with seropositivity of *N. caninum*). Therefore, leashed domesticated dogs positioned at each entrance to the farm and the prevention of stray dogs from entering the farm area are recommended to reduce the spread of *N. caninum* in dairy farms. Regarding domesticated dogs, it is recommended that they should be leashed and not have free access to the barns. Implementation of these dog controlling strategies is expected to significantly reduce the incidence of abortion caused by *N. caninum* in dairy farms in Taiwan. On the other hand, the policy in Taiwan for managing stray dogs is “zero euthanasia”, means most of the government dog shelters have been occupied and there is no room for new stray dogs, resulting in many stray dogs hanging around on the streets. To prevent stray dogs invading farm areas, first, it is needed to figure out if there is an owner of that dog, if no, if the dog could be tame as a domesticated dog, if so, tamed as guardian dog, leashed well and separated from other original domesticated dogs, with all of the dogs kept on the farm never having the chance to access bovine placental materials, dead calves and aborted fetuses.

In conclusion, the current situation of *N. caninum* infection is 26.3% in antibodies detection and there are 17.2% of pregnant cows that had active parasitemia in dairy herds in Taiwan. Kept dogs in dairy farm could decrease the abortion risk to cows but the presence of free-roaming dogs in farm area could increase the abortion risk to cows in Taiwan in this study. *N. caninum* infection remains an important factor associated with dairy cow abortion in Taiwan, hence, effectively managing dogs on the farm is critical to

decreasing the seroprevalence of *N. caninum* in dairy herds.

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