Control measures to reduce Neospora caninum *abortions in dairy farms: a pilot study in Iran*

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Summary
Neospora caninum plays an important role in abortion and economic losses in dairy industry worldwide. The main target of this work was to detect the infection rate of *N. caninum* in various hosts in dairy farms for identifying the risk factors and applying appropriate control programs to reduce the number of abortions. The study was conducted in dairy farms with high incidence of abortion in Hamedan province, West of Iran. After the primary assessment, we conducted a controlling program for reducing the *Neospora*-infection rate and associated abortions. Before implementing the control program, the seropositivity was 24.8% in cows (N = 476 distributed in 10 farms) and 8.6% in dogs (N = 185). Abortion occurred in 3.57% of pregnant cows. 94.1% of aborted cows were positive for *Neospora*-infection. Based on molecular technique, the infection rate was detected in 7.3% of cats (N = 41), 25% of pigeons (N = 19) and 11.8% of rodents (N = 51). After the implementation of neosporosis control programs in the farms, the seropositivity of *N. caninum* decreased to 8.2% in cows and 2.9% in dogs. After the one-year follow-up, no cases of abortions were reported in the farms. This was the first parallel evaluation of *Neospora*-infection and controlling programs in Iranian dairy farms. Regular control of rodents, retesting of seronegative animals and farm biosecurity measures are recommended for reducing the abortion incidence. The access of dogs to the herd and to aborted materials should be restricted.

Introduction

Neospora caninum (Apicomplexa: *Toxoplasmatidae*) is the cause of neosporosis, a serious parasitic disease in dairy cattle and dogs, with global distribution (Dubey 2003). *N. caninum* was detected in puppies with congenital encephalitis and myositis in Norway at the first time (Bjerkas *et al.* 1984). In Iran, the first report of *Neospora*-infection was in dairy cattle from Mashhad region by Sadrebazzaz and colleagues (Sadrebazzaz *et al.* 2004). Different species of animals are definitive and intermediate hosts for *N. caninum*; but the parasite life cycle occurs between dogs and cattle, commonly (Dubey *et al.* 2005). Interestingly, dogs can simultaneously play the role of both definitive and intermediate hosts (Dubey *et al.* 2007).

Neospora caninum has been reported as a cause of abortion and other reproduction disorders in cattle (Dubey *et al.* 2005). Farm dogs have a significant figure in the horizontal transmission of *N. caninum* and incidence of stormy abortions (Dubey 2003). In addition, transplacental transmission of the parasite is a major infection route with rate up to 93.7% in dairy cattle (Dubey and Schares 2011, Nicolino *et al.* 2017). Serological and molecular tests are used for detecting the abortions associated to neosporosis routinely. Enzyme-linked immunosorbent assay (ELISA) is an appropriate and reliable immunoserology technique due to high sensitivity and specificity (Guido *et al.* 2016).

Dairy cattle are more susceptible to neosporosis compared to beef cattle (Ribeiro *et al.* 2019). *N. caninum* infection in Iranian dairy cattle is reported in wide range of 3.8-76.2% (Gharekhani *et al.* 2020a). In the previous works from Hamedan (studied area), *Neospora*-infection was reported 4.9-18% and 12.8-24.8% in different type of dogs and cattle, respectively (Gharekhani *et al.* 2012, 2013, 2014, 2019, 2020b, Gharekhani and Tavoosidana 2013, Gharekhani and Yakhchali 2019). There are no published data on *Neospora*-infection in cats, rodents, and birds in the region. Also, the impact of that infection rate in the transmission of disease in the farms is unclear (Gharekhani *et al.* 2020a). The main aim of the current study was to detect *N. caninum* infection in cats, rodents, and birds in dairy farms from Hamedan province, West part of Iran for identifying the risk factors and appropriate control programs related to abortion. In addition, we evaluated a program for controlling and reducing the frequency of *Neospora*-infection. This project was a continuation of our previous work in which the prevalence and risk factors associated to *N. caninum* infection in dairy farms have been investigated (Gharekhani and Yakhchali 2019).

Materials and methods

Study area

Hamedan province (34.77° N and 48.58° E; an area of 19,546 km^2) is located in the vicinity of the Alvand Mountain, West of Iran, with dry summer, cold semi-arid climate and snowy winters (Figure 1). The mean temperature is 11.3 °C which may fall below - 30 °C in winter. Due to the existence of flat and rich lands, agriculture and animal farming play an important role in the region's economy. There are 149 dairy farms with animal population of ~ 25,000 (minimum and maximum herd size was 10 and 4,500, respectively) in Hamedan province. Daily milk production in dairy farms is 540 tons in the region.

Study design

In brief, we evaluated the infection rate of *N. caninum* in the various hosts in the farms. Then, we removed some of the risk factors to infection that have been previously recognized (Dubey *et al.* 2007, Dubey and Schares 2011) for reducing the infection rate and occurrence of abortion subsequently. To achieve this goal, ten dairy farms (A-J) with the highest incidence of abortion were selected in the studied area. According to Regional Veterinary Service (IVO 2017) reports, all selected farms had a history of artificial insemination and also were free from tuberculosis, paratuberculosis and brucellosis. *Neospora*-bulk tank milk test was positive for all the sampled farms. All farms had dogs and also free-roaming wild canids (coyote, jackal and fox) were reported in some of them.

Between June and November 2017, blood samples were taken from all available dogs (n = 185) in and around the farms. All dogs were sampled to increase the sampling power. The sample size for determining the seroprevalence of *N. caninum* in pregnant cows was 426. It was calculated by using Cochran formula (expected prevalence 20%, level of confidence 95%, and precision 10%) (Gharekhani and Tavoosidana 2013, Thrusfield 2018). To achieve a desired precision sampling in each farm (at least a significant sample), we increased the sample size to 476 (animal population in 10 farms = 4,760) (Thrusfield 2018). Animals were randomly selected for sample collection in the farms. An ear tag was installed for identifying the animals and also for further investigations. The level of antibodies to *N. caninum* was determined using ELISA. The seropositive cows were monitored during the pregnancy until abortion or full-term delivery. The brain tissue of

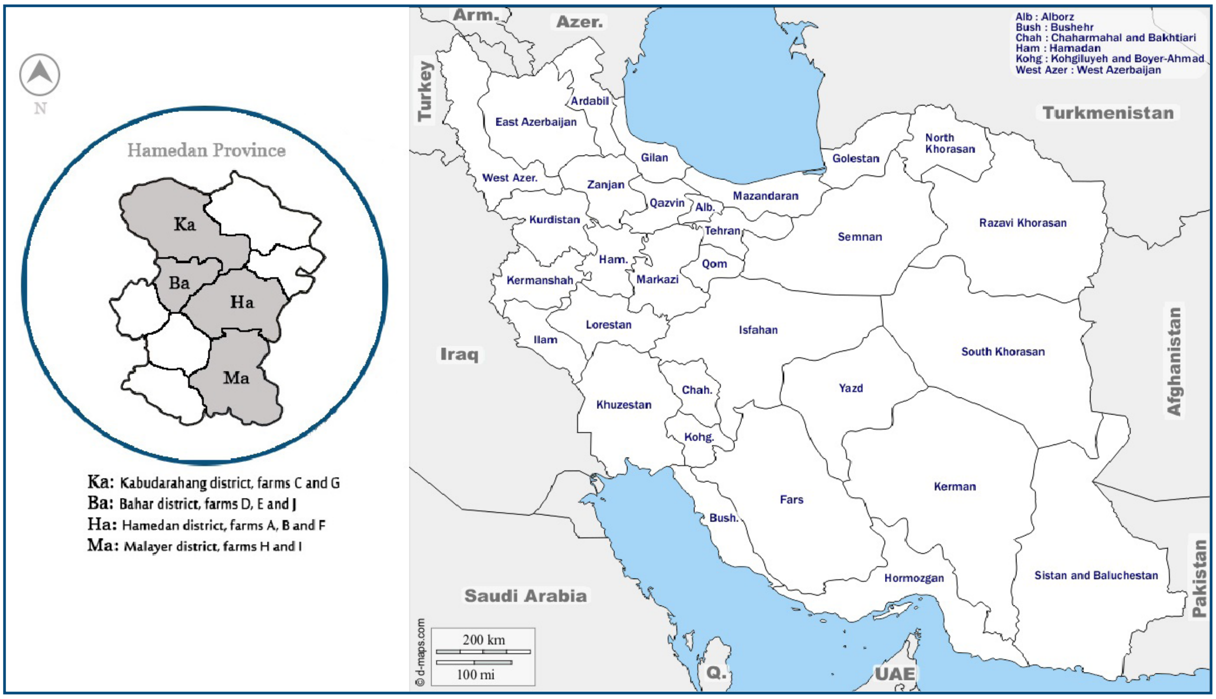

Figure 1. *The map of studied area (Hamedan province, Iran) and the location of farms.*

aborted fetuses was tested for the presence of DNA of *N. caninum* using molecular methods (polymerase chain reaction: PCR and nested-PCR). Parallel to this work, 51 rodents (10 of *Mus musculus* and 41 of *Rattus rattus*), 16 owner pigeons and 41 stray cats were trapped in the farms. Whole blood of cats and also brain tissue of rodents and pigeons were tested to detect DNA of *N. caninum* using nested-PCR. After reviewing the results and control measures, the level of antibodies to *N. caninum* infection were reevaluated in the samples of cattle, dogs and also bulk tank milk (Dubey *et al.* 2007, Lagomarsino *et al.* 2019). All control measures between the two stages of sampling included:

1. Culling the seropositive cows with history of two or more successive abortion.
2. Prevention for introducing cats, wild canids, and new seropositive cattle to the farms.
3. Test sperm/semen for the presence of *N. caninum* before artificial insemination and mating of bulls.
4. Regular control of rodents' population in all of the farms using trapping.
5. Treatment and chemoprophylaxis of all dogs with Sulfonamides and Pyrimethamine compounds (Vetasul® 24%, Aburaihan Company, Iran; 1 ml sub-cutaneous per 10 kg of body weight for 5 day and repeat this protocol after one-month).
6. After the chemotherapy and reevaluations, the seropositive dogs were relocated.
7. For the improvement of shelters sanitation, walls, ceiling, and resting areas were furnished with cement; daily removal of the feces was performed; the shelter and its surroundings were disinfected daily with 1/200 dilution of TH5® (Sogevol, France) and 10% formaldehyde solution.
8. Prevention of dogs to access the farm animals as well as their water and food materials.
9. Obliterating the aborted materials in hygienic status such as burning in the kiln.
10. Installing of nets around the shelters of dogs to prevent birds' access (sparrow and pigeon) to dog feces and food materials test.

Serology examination

Sera were collected after the blood centrifugation (1,000 × g for 10 minutes) and stored at - 18 °C until serology analysis. The serum samples of cattle and dogs and also bulk tank milks were examined for detecting the presence of *N. caninum* antibodies using a commercial ELISA kit (ID Screen® ID-Vet Company, France). According to user guideline, the antibodies level was calculated using S/P% (sample to positive ≥ 30 % and ≥ 50 % was positive for milk and serum samples, respectively).

Molecular methods

DNA extraction

Genomic DNA of *N. caninum* was extracted from brain of aborted fetuses, pigeons and rodents and also whole blood samples of cats using Dyna-BioTM Blood & Tissue Kit (Takapouzist Company, Iran).

PCR

A pair of Np21 plus and Np6 plus primers (sense: 5'-CCCAGTGCGTCCAATCCTGTAAC-3', anti-sense: 5'-CTCGCCAGTCAACCTACGTCTTCT-3') were used to amplify a 330 bp-fragment-length of NC5 gene of *N. caninum* (Muller *et al.* 1996). PCR reaction was carried out in 25 µl of reaction mixture containing 7.5 µL of D/W (Nuclease-Free water, Qiagen, USA), 12.5 µL (2×) of Red load Tag Master (Jena Bioscience, Korea), 4 µL of (100 ng) of genomic DNA, and 1 µL of (20 pmol) each specific primers with positive and negative controls. The reaction was performed in thermal cycler (Primus gradient, MWG Biotech, Germany). The samples were subjected to a primary denaturation step at 94 ˚C for 10 min, followed by 38 cycles in 45 s at 94 ˚C, 45 s at 55 ˚C, 45 s at 72 ˚C, a last extension step at 72 ˚C for 10 min. A volume of 10 µl of each PCR product was evaluated by electrophoresis on 2% (w/v) agarose gel for 90 min at 85 V. The gels were visualized by staining with ethidium bromide (1 µg/ml). All of PCR positive products were confirmed using nested-PCR.

Nested-PCR

For nested-PCR, a pair of primers (sense: 5'-GTGTTGCTCTGCTGACGTGT-3', anti-sense: 5'-TACCAACTCCCTCGGTTCAC-3') were applied to amplify a 100-bp-fragment-length using Barbosa de Macedo and colleagues protocol (Barbosa de Macedo *et al.* 2013).

Statistical analysis of data

The non-parametric Chi-square (χ^2) test was used to evaluate association between *Neospora*-infection and sex and age in dogs and cats as well as abortion risk in cows (SPSS 16.0, Chicago, IL, USA). A probability score of $P \leq 0.05$ was regarded as significant.

Ethical approval

During the blood collection, animals (cows, dogs, and cats) were restrained manually or using a suitable physical restrainer. But, for the collection of rodents and pigeons brain specimens we had to euthanatize them by CO_2 (Charbonneau *et al.* 2010). This project was approved by the ethical committee of Urmia University, Iran (IranDoc: 1453018). Ethical standards have been carefully observed during the sample collection, preparation of results and submission the manuscript.

Results

First stage of sampling

Overall seroprevalence of *N. caninum* infection was 24.8% in pregnant cows (ranging from 2.5-42.3% in different farms) and 8.6% in dogs. Abortion occurred in 3.57% (17/476, CI 95% = 1.97-5.17%) of cows. The seroprevalence rate of *N. caninum* in aborted cows (94.1%, 16 out of 17 cases) was 56-times higher than animals which did not abort (OR = 56); a high significant difference was seen (χ^2= 45.447, P < 0.0001) between the two groups of animals. No abortion occurred in seronegative cows. In addition, abortion occurred in 42.4% (14/33) of seropositive cows with history of previous abortion (χ^2 = 32.566, P < 0.0001, OR = 30.6). Based of molecular technique, infection was detected in 7.3% (3/41) of cats, in 25% (4/16) of pigeons and in 11.8% of rodents (6/51; all of positive samples were from *Rattus rattus*) (Table I). No statistically significant correlation was seen between infection rate, age and sex in cats (Table II).

Second stage of sampling: after the run of controlling programs

During one-year follow-up after implementing the controlling procedures, seroprevalence decreased to 8.2% (ranging from 0-12.9% in different farms) in cows and 2.9% in dogs (0-16.7%) (Table I and II). No abortion was recorded in pregnant cows in this period. Bulk milk test was positive in 4 out of 10 farms. DNA of parasite was not detected in the seropositive cows. There was no statistically significant association between the seroprevalence of *N. caninum* in dogs and their sex and as well as different age (Table II).

In this stage, 81 seropositive dogs and 14 seropositive cows with history of abortion were relocated with the consent of herd owners. In the monitoring time, the blood sera of 35 cows (to get a certificate for entering the farm), 2 bulls (before mating) and also 22 samples of semen (tested only by PCR before using for artificial insemination) were tested using ELISA and PCR techniques; and all of them were negative for *Neospora*-infection except a sample of semen (controlling procedures of 2 and 3). The positive semen was not used for artificial insemination.

Discussion

Knowledge on risk factors related to neosporosis in dairy herds is helpful for selecting the appropriate control options and then reducing the economic losses (Dubey *et al.* 2007, Santos *et al.* 2016). There is no report on radical treatment of neosporosis in cattle. Interrupting the parasite-life cycle is an effective way for controlling the infection; this target could be achieved by using regularly a "test-and-cull" strategy for reducing the rodents' population and removing the immunodeficiency options such as viral infections, mycotoxins, and some of fungal pathogens. Stressful factors can accelerate the reactivation of chronic infections and vertical transmission (Dubey *et al.* 2007).

One of the most important control measures

Table I. *Infection rate of* Neospora caninum *in different dairy farms (n = 10) from Hamedan, Iran.*

Farms	First stage of sampling						Second stage of sampling		
	Bulk tank	No. of sample (positive)					Bulk tank	No. of sample (positive)	
		cattle	dog	cat	rodent	pigeon		cattle	dog
A	+	25 (24%)	39 (12.8%)	6 (16.7%)	11 (18.2%)	10 (30%)	+	25 (12%)	20 (5%)
B	+	22 (18.2%)	21 (14.3%)	4 (0%)	2 (0%)	6 (16.7%)	-	21 (0%)	10 (0%)
C	+	50 (18%)	27 (3.7%)	8 (12.5%)	4 (25%)	0 (0%)	+	50 (10%)	13 (0%)
D	+	40 (2.5%)	8 (0%)	11 (0%)	3 (0%)	0 (0%)	-	38 (0%)	5 (0%)
E	+	19 (10.5%)	10 (10%)	2 (0%)	10 (10%)	0 (0%)	-	18 (0%)	7 (0%)
F	+	26 (42.3%)	11 (27.3%)	5 (20%)	14 (14.3%)	0 (0%)	+	26 (11.5%)	5 (0%)
G	+	25 (32%)	29 (6.9%)	2 (0%)	1 (0%)	0 (0%)	-	25 (8%)	18 (0%)
H	+	54 (7.4%)	11 (0%)	3 (0%)	3 (0%)	0 (0%)	-	52 (0%)	8 (0%)
I	+	200 (36%)	17 (5.9%)	0 (0%)	1 (0%)	0 (0%)	+	193 (12.9%)	12 (16.7%)
J	+	15 (6.7%)	12 (0%)	0 (0%)	2 (0%)	0 (0%)	-	14 (0%)	6 (0%)
Total	10/10	476 (24.8%)	185 (8.6%)	41 (7.3%)	51 (11.8%)	16 (25%)	4/10	462 (8.2%)	104 (2.9%)

Table II. *Infection rate of* Neospora caninum *in farm dogs and cats in different age groups and gender from Hamedan, Iran.*

Animals		Sex			Age groups (year)			
		Male	Female	Statistical analysis	<1	1-2	>2	Statistical analysis
Dog ≠ (n = 104)	No. of sample	36 (34.6%)	68 (65.4%)	$\chi^2 = 1.339$ $P = 0.247$ $df = 1$	52 (50%)	22 (21.2%)	30 (28.8%)	$\chi^2 = 0.184$ $P = 0.911$ $df = 2$
	No. of positive	0 (0%)	3 (4.4%)		0 (0%)	1 (4.5%)	2 (6.7%)	
Cat (n = 41)	No. of sample	12 (29.3%)	29 (70.7%)	$\chi^2 = 0.025$ $P = 0.872$ $df = 1$	19 (46.3%)	17 (41.5%)	5 (12.2%)	$\chi^2 = 0.433$ $P = 0.805$ $df = 2$
	No. of positive	1 (8.3%)	2 (6.9%)		1 (5.3%)	2 (11.8%)	0 (0%)	

applied in this study was the assessment of infection in purchased animals before entering the farms. This allows us to reduce the seroprevalence rate of *N. caninum* in cows from 24.8% to 8.2%, after implementing the control programs. The prevalence of *N. caninum* in cattle was calculated 20% in global scale by Ribeiro and colleagues (Ribeiro *et al.* 2019); additionally, the seropositive cows were 1.6 times more likely to abort than seronegative ones. Overall seroprevalence Iranian dairy cattle was estimated to be 23.6% with a significant higher seroprevalence in animals with history of abortion (OR = 2.5) (Ansari-Lari 2020). Gharekhani and Yakhchali (Gharekhani and Yakhchali 2019) reported a significant connection between seropositive animals and history of abortion. In Iranian cows, the rate of abortion associated to neosporosis ranges from 7.8% to 66.7% (Sadrebazzaz *et al.* 2004, Razmi *et al.* 2006, 2013, Salehi *et al.* 2010, Nematollahi *et al.* 2011, Gharekhani and Tavoosidana 2013, Gharekhani *et al.* 2014, Ansari-Lari *et al.* 2017). This rate was estimated as 10% in Brazil and 26.5% in New Zealand with 4.2% of relative risk (Weston *et al.* 2012, Nicolino *et al.* 2015). Cows can abort repeatedly in consecutive pregnancies; this rate has been reported up to 5% (Anderson *et al.* 1995). In Gharekhani and Heidari study (Gharekhani and Heidari 2014), 26% of animals with history of abortion were seropositive to *N. caninum* ($P < 0.0001$ and OR = 2.9). We removed all of the fetal/placental materials in sanitary status for cutting the parasite life-cycle. Regarding to our findings, no abortion occurred after the run of the control programs. Ansari-Lari and colleagues (Ansari-Lari *et al.* 2017) believe that 30% of abortions may be managed by controlling of *Neospora*-infection. The differences may be due to the type of control methods. The seropositive cows with a history of abortion in the herds have a high risk of abortion and vertical transmission of *N. caninum* (Dubey *et al.* 2007). So, we drove them out of the herds for reducing the rate of vertical transmission. Regarding to previous reports (Snak *et al.* 2018, Gharekhani and Yakhchali 2019, Okumu *et al.* 2019), the infection rate and occurrence of abortion were significantly high in animals with history of artificial insemination. We detected *N. caninum* infection in bulls and also in semen samples. Screening of semen for *Neospora*-infection before artificial insemination and using of beef semen are appropriate options for control programs (Lagomarsino *et al.* 2019).

The presence of free roaming dogs in the herds is a risk factor for distributing the infection and occurring the stormy abortions (Ribeiro *et al.* 2019). The risk of seropositivity increases 1.4-fold for each additional dog in the herd (Macchi *et al.* 2020). In Gharekhani and colleagues (Gharekhani *et al.* 2014) research, the seroprevalence of *N. caninum* was significantly higher in the farms with the presence of dogs and wild canids. In the previous researches, the infection rate in Iranian farm dogs was between 2.1% and 46% (Haddadzadeh *et al.* 2007, Malmasi *et al.* 2007, Dalimi *et al.* 2014). The seropositivity in dogs worldwide was 17.1%; this rate was higher in males (15.8%), as compared to females (15.2%) (Anvari *et al.* 2020). The top of our control process was to reduce the infection rate in dogs. We improved the shelters sanitation of dogs for reducing the sporulation rate of the *Neospora*-oocysts as well as horizontal transmission. In addition, we used a commercial compound for blocking the parasite proliferation in the lumen of dogs at the first time in Iran. Then, the seropositive dogs were relocated after reevaluation. Rodents, an intermediate host for *N. caninum*, plays a significant role in continuing the parasite-life cycle (Dubey *et al.* 2007). In Gharekhani and Yakhchali report (Gharekhani and Yakhchali 2019), the presence of rodents in dairy farms had a positive correlation with seropositivity of cows. In the study of Mosallanejad and colleagues (Mosallanejad *et al.* 2018), antibodies to *N. caninum* were detected in 6% of *Rattus rattus* blood samples. In Nazari and colleagues (Nazari *et al.* 2019) molecular study, the brain tissue of 70 rodents (*Meriones persicus*: 50, *Cricetulus migratorius*: 9, and *Mus musculus*: 11) were evaluated for *N. caninum* infection in Northwest of Iran, and all of them were negative similar to Khani (Khani 2016), who investigated on *N. caninum* infection in rodents in the farms. Discrepancies of infection rate in rodents might be attributed to difference in study design, diagnostic techniques, and also sampling. In our work, all the samples were taken in *Neospora*-positive dairy farms. The level of health and biosecurity management as well as frequency of *Neospora*-positive animals such as dogs and cattle are options for increasing and/or reducing the infection rate in rodents inside the farms.

Cats might play an important role in the life cycle of *N. caninum* in the farms by eating the infected rodents (Dubey and Schares 2011). In Hamidinejat and colleagues (Hamidinejat *et al.* 2011 a, b) study, 19% of feral cats and 14% of domestic Persian cats were seropositive to *N. caninum*. After the control of rodents' population, blocking the presence of cats was effective for reducing the infection maintenance and cutting the parasite-life cycle in the farms. Birds may contribute to parasite transmission in sylvatic cycles either as mechanical vectors or as intermediate hosts (Barros *et al.* 2018). Barros and colleagues (Barros *et al.* 2018) reported a significant association between the presence and number of poultries and the increase of abortion storms related to *N. caninum* in the farms. Based on molecular tools, 25%, 11.8% and 7.3% of exanimated pigeons, rodents and cats were positive for *Neospora*-infection, respectively. In other researches from Iran, this rate has been reported 2.8% and 3.7% in sparrows (Abdoli *et al.* 2015, Bahrami *et al.* 2015), 17.3% in chickens (Sayari *et al.* 2014), 9.8% and 30.4% in pigeons (Bahrami *et al.* 2016), and 9.9% in hooded crows (*Corvus cornix*) (Abdoli *et al.* 2018). According to these findings, for decrease the infection in the birds, we restricted their access to the feces of dogs.

In conclusion, we presented a successful control program for reducing the infection rate of *N. caninum* and associated abortions in the herds. Regular control of rodents' population and retesting seronegative animals are necessary for management of the infection in the farms. Due to the importance of transplacental transmission, culling of seropositive animals with a history of abortion is essential for cutting the chain of transmission and reducing the economic losses. Biosecurity measures and screening of bulk milk are highly recommended in the farms.

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