Annals of Parasitology 2020, 66(4), 495–500
doi: 10.17420/ap6604.290

Original paper

Vertical transmission of *Neospora caninum* in Iranian dairy cattle

Jamal GHAREKHANI, Mohammad YAKHCHALI

Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

Corresponding Author: Mohammad YAKHCHALI; e-mail: m.yakhchali@urmia.ac.ir

ABSTRACT. Placentally, transmission of *Neospora caninum* plays significant role on retaining of infection and economic losses in dairy farms. The objective of present study was to assess placental transmission of *N. caninum* in Iranian dairy cattle. A total of 476 blood samples of pregnant dairy cattle were randomly collected for serology test using ELISA technique. Genomic DNA was extracted and subjected for conventional PCR and Nested-PCR to amplify 330bp and 100bp fragment length of NC5 gene, respectively. Positive animals were follow-up during gestation till abortion and/or full term delivery occurred. Molecularly, 25.3% (118/476; 95% CI: 21%–28.6%) of examined animals were seropositive for *N. caninum* infection in Holstein cattle (15.1%) with <2 years-old (75%) and history of abortion. The infection was detected. TT and abortion occurred in 13.6% and 3.57% of animals, respectively. Aborted cattle (94.1%, 16/17) were significantly infected to *N. caninum* and history of abortion was recorded in 42.4% of infected cattle. Placental transmission and abortion in naturally infected dairy cattle due to *N. caninum* was the first molecular evidence in all examined herds of cattle. It was also uncovered *N. caninum* had significant role on cattle abortion. Further studies for culling of seropositive cattle with history of abortion, associated risk factors related to neosporosis and lunch control program for reducing side effects of infection are essential in the region.

Keywords: abortion, dairy cattle, neosporosis, NC5 gene, risk factor, vertical transmission

Introduction

Neosporosis caused by *Neospora caninum* (Apicomplexa: Toxoplasmatidae) is a cosmopolitan and common parasitic disease in cattle and dogs [1]. Canines (especially domestic dogs) and wide-range of herbivores are the definitive and intermediate hosts for *N. caninum*, respectively [2,3]. Different levels of antibodies to *N. caninum* were particularly detected in immunocompromised humans and it has been successfully cultured in human cell lines; because of that zoonotic role is unclear [3]. Definitive hosts have a noteworthy figure in the horizontal transmission and occurring stormy abortions in dairy herds [1]. The disease is transplacentally transmitted from cows to the neonates in gestation and/or lactation periods; this way is the superlative significant infection route in dairy cattle [4].

In cattle, the most of transplacental transmission (TT) of *N. caninum* occurs during the second trimester stage of pregnancy. Which is associated with abortion, neonatal mortalities, stillbirths, genitally tract infection, and decrease of milk production [1]. In affected neonatal calves, DNA of *Neospora* is mainly localized in the brain, and followed by the placenta, blood, serum, milk or colostrum and semen with unknown clinical significance [2,3].

To date, different laboratory methods, *i.e.* histopathology, bioassay, serology, and molecular tools are using for recognition of neosporosis in animals. Serologic methods may obtain useful information of *N. caninum* infection in a farm; because of the specific antibodies to *N. caninum* presence in fetal blood, and also in fluids of pleura, pericardia and abomasa content [5]. For instance, enzyme-linked immunosorbent assay (ELISA) is a suitable technique due to high sensitivity and specificity [6]. With regard to pregnancy role in reactivation of the latent infection and replicating tachyzoites in infected fetus through TT, isolation of

Neospora DNA using molecular tools like polymerase chain reaction (PCR) and Nested-PCR are prior to others in terms of sensitivity [3,7]. Cattle neosporosis is causing considerable economic losses due to culling of infected animals in the farm, abortion and other genitally failures with more than US$1.3 billion losses annually in global scale [4,8]. In Iran, cattle neosporosis may be one of the major risk factor of abortion and varied of 7.8–66.7% [9–12]. Thus the basic role of this research was to determine the prevalence of *N. caninum* infection and transplacental transmission in naturally infected dairy farms in Hamedan, West of Iran using serological and molecular techniques.

Materials and Methods

Area of study

Hamedan province is located in the vicinity of the Alvand Mountain, West of Iran, with dry summer, cold semi-arid climate and snowy winters. The mean temperature is 11.3°C; interestingly this rate may be reach to under −30°C in winter. It is exchange focal point of a ripe ranch district where grain is developed; therefore, horticulture and animal farming have a huge job in the region's economy.

Research design and sampling

Over six months (June–November 2017), 5 ml of blood samples were randomly taken from coccygeal vein of 476 pregnant dairy cattle (10% of examined cattle in each herd) [13,14]. Number of female new born calves (59.7%) were higher than male (40.3%). According to serology and molecular findings, infected cattle to *N. caninum* were follow-up during the pregnancy until abortion and/or full term delivery occurred and stillbirth, dystocia and gender of new born calves were recorded. Blood, brain tissue, and the fluids of abdominal cavity in all of aborted fetuses were collected for analyzing the *N. caninum* infection using molecular procedures.

Dairy farms

According to Regional Veterinary Services reports (Iranian Veterinary Organization (IVO), Hamedan), a total number of 10 dairy farms (A–J) with the highest yearly abortion (>1%–5%) were selected. There were 4760 animals in the farms (A=250, B=220, C=500, D=400, E=190, F=260, G=250, H=540, I=2000, and J=150). Holstein cattle (72.7%, 346/476) was predominant breed in examined dairy farms. According to IVO (2017), all examined animals had history of artificial insemination (A.I. and free of tuberculosis, paratuberculosis, and brucellosis). They were classified into three age groups (<2 (n=50, 10.5%), 2–4 (n=139, 29.2%), and >4 (n=287, 60.3%) years-old). Dogs were also present in all examined farms.

Serology

The sera were prepared after centrifuging at 1,000×g for 10 min and evaluated for the presence of antibodies to *N. caninum* using a commercial ELISA kit (ID-Vet Company, France) based on manufacturer instruction.

Molecular assay

DNA extraction

Genomic DNA of *N. caninum* was extracted from brain and whole blood samples by using Dyna-Bio™ Blood & Tissue Kit (Takapouzist Company, Iran).

Polymerase chain reaction (PCR)

A pair of Np21 plus and Np6 plus primers (sense: 5'-CCCAGTGCGTCCAATCCTGTAAC-3', anti-sense: 5'-CTCGCCAGTCAACCTACGTC TTCT-3') were used to amplify a 330bp-fragment-length of NC5 gene of *N. caninum* [15]. PCR reaction was carried out in 25µl of reaction mixture containing 5µl of (100ng) of genomic DNA, 0.5U of *Taq*-DNA polymerase (Qiagen, Germany), 100 mM of each dNTPs, 60mM of Tris-HCl (pH 9.0), 15 mM of $(NH_4)_2SO_4$, 1.5 mM of $MgCl_2$, and 1.0 mM of each primer with positive (DNA from tachyzoites of NC-1 strain of *N. caninum*) and negative (commercially prepared water without *N. caninum*) controls. The reaction was performed in thermal cycler (Primus gradient, MWG Biotech, Germany). The samples were subjected for a primary denaturation step at 94°C for 10min, followed by 38 cycles in 45s at 94°C, 45s at 55°C, 45s 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 85V. The gels were visualized by staining with ethidium bromide (1µg/ml).

Nested-PCR

For nested-PCR, a pairs of primers (sense: 5'-GTGTTGCTCTGCTGACGTGT-3', anti-sense: 5'-TACCAACTCCCTCGGTTCAC-3') were used to amplify a 100-bp-fragment-length of the region of the NC5 gene from *N. caninum* [16]. The first

Table 1. The risk factors associated to *Neospora caninum* abortions (n=16) in naturally infected pregnant dairy cattle (n=118) in Hamedan municipality, West of Iran

Risk factors	No. of infected cattle (%)	No. of aborted cattle (%)	Statistical analysis
Age groups (year)			
< 2	8	6 (75)	χ^2=9.576 *P*=0.008
2–4	14	4 (28.6)	
> 4	96 6	(6.3)	
Abortion history			
Yes	33	14 (42.4)	χ^2=32.566 *P*<0.0001 OR=30.6
No	85	2 (2.3)	
Breed			
Holstein	99	15 (15.1)	χ^2=1.329 *P*=0.248
Crossbred	19	1 (5.3)	
Stage of gestation (month)			
1–3	10	0 (0)	χ^2=0.002 *P*=0.998
3–6	62	16 (25)	
> 6	46	0 (0)	
Total	118 (25.3)	16 (13.6)	95% CI: 7.5–19.7%

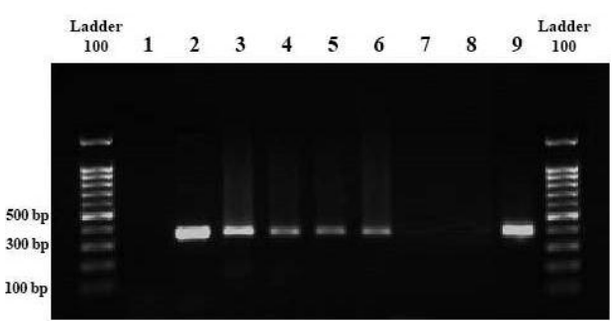

Figure 1. PCR results of *Neospora caninum* detection from samples extracted on blood cattle DNA: negative control (L1), positive control (L9), positive samples (L2-6; bands of 330 bp), negative samples (L7, 8)

amplification was: denaturation step at 95°C for 10 min, followed by 35 cycles in 95°C for 1min, 45s at 54°C, 72°C for 1 min, and 72°C for 10 min.

Data evaluation

The non-parametric Chi-square (χ^2) test was used to analyze association between positive animals and all data using SPSS 16.0 (Chicago, IL, USA). Odds ratio (OR) was calculated for all of statistical significant factors. A probability score of $P \leq 0.05$ was regarded as significant.

Ethic statement

The ethical guidelines have been consistence during the sampling, preparation of results and submitted article. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors asserted that all the procedures of this work complied with the ethical standards of the Declaration of Helsinki as revised in 2008.

Results

Prevalence and associated risk factors after delivery

Overall prevalence of *N. caninum* infection was 25.3% (CI 95%: ±3.8%) (Fig. 1 and 2). Transplacental transmission was detected in 13.6% (CI 95%: ±6.1%) of examined cattle. In infected animals, stillbirth and dystocia occurred in 2.9% (14/476) and 6.9% (33/476), respectively. Animals with stillbirth (7.1%, 1/14) (χ^2=2.409, *P*=0.120) and dystocia (12.1%, 4/33) (χ^2=3.052, *P*=0.080) were positive for *Neospora*-infection. The prevalence of *N. caninum* infection in new born calves was also higher in female (27.5%) than male (20.8%) (χ^2=2.702, *P*=0.100).

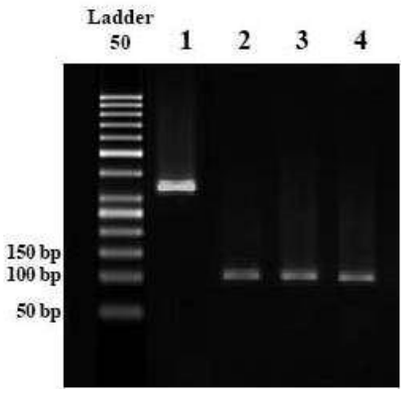

Figure 2. Nested-PCR results of *Neospora caninum* detection from samples of positive PCR: PCR product (L1 band of 330 bp) and, positive samples of Nested-PCR product (L2-4 bands of 100 bp)

Risk factors for abortion

During the follow up of positive cases, abortion occurred in 3.57% (17/476, CI 95%: ±1.6%) of pregnant animals in the second-trimester (3–6 month) stage of pregnancy (P=0.998) (Table 1). Of those, 94.1% (16/17) of aborted cattle were infected to *N. caninum* (P<0.0001). The most of abortion was occurred in pregnant cows with under 2 years-old (75%) (P=0.008). According to overall number (n=16) of abortion associated to *N. caninum,* 15.1% (15/99) and 5.3% (1/19) of those were in infected animals with Holstein and crossbred breed, respectively (P=0.248). In addition, abortion occurred in 42.4% (14/33) of infected pregnant cows with history of abortion (P<0.0001, OR=30.6) (Table 1).

Discussion

The prevalence and associate risk factors to *N. caninum* infection in dairy herds are helpful for implement control program and subsequently reducing the economic losses [3]. In Iran, prevalence of *N. caninum* infection in cattle was varied of 20–23.6% in global scale [18,19]. In current study, the overall prevalence was in agreement with Razmi et al. [17].

Transplacental transmission (TT) of *N. caninum* infection in dairy cattle is up to 93.7% and known as a significant risk factor [3,4]. This way has strong association with seropositivity of animals and major impress in remaining of *Neospora*-infection through successive propagation from one generation to the next [7,17]. In the present study, TT was lower than in earlier reports from Northeastern Iran (52%) and Thailand (36.5%) [7,17]. This may be due to difference in the climate of studied regions, study design, laboratory techniques, farm management, and also associate risk factors in the farms [3].

Age and gestation stage may persist for life and influenced with levels of specific antibodies to *N. caninum* infection [13]. In some of the herds, the increment of infection occurs with age because expanding the opportunity of oocysts ingestion [1,3,4]. The most of abortion occurred in animals younger than 2 years-old. This may be due to low level of protective *Neospora*-antibodies, maternal immunity, and dominant role of horizontal transmission in these farms. Additionally, the highest abortion occurring in Holstein cattle was similar to reports from Argentina, Venezuela, and Ethiopia [3,4]. While lower risk of abortion reported from beef herds than dairy herds [3,4]. This is may be due to differences in farms management, *i.e.* the differences of access to infectious materials and food regimes [20].

In the present study the highest abortion in examined animals was in the second trimester of pregnancy. While, Marques et al. [21] noted that high infection was during the third trimesters (16.6%). The risk of TT increased by later exposure time during gestation, reactivation of chronic infection due to consumption of toxic feeds or stress and other concurrent infections, gestation number, and dose of oocysts [22–25]. Bech-Sàbat et al. [26] uncovered progesterone supplementation during mid-gestation increased abortion risk.

Abortion is the principal clinical sign associated to *N. caninum* in cattle [1]. Cows can abort repeatedly in consecutive pregnancies; because of repetition of abortion calculated 5% [27]. According to Ribeiro et al. [19] and Lefkaditis et al. [23], seropositive cattle (28.2%) with an abortion history were significantly 1.6 times more likely to abort (OR=2.6, P<0.05) than seronegative ones (2.6%). In our work, infected cattle and aborted cattle had history of abortion. Thus, the presence of seropositive cattle with history of abortion was a strong risk factor for occurring the abortion in the farms. It is therefore control of abortion in farms with high risk, TT must be blocked by selection of seronegative heifers born to seronegative dams [28].

Specific IgG1 antibody to *N. caninum* discharges via milk in infected cattle. There was significant association between level of antibodies in serum and milk [29]. Schares et al. [30] reported a high abortion rate in bulk milk positive dairy farms than

negative farms. In addition, all of the farms with high risk of abortion were positive for bulk milk test [13].

In summary, in Iran, it was the first investigation which demonstrated TT of *N. caninum* in naturally infected dairy cattle with history of abortion using molecular techniques. There was evidence of vertical transmission of *Neospora*-infection in all of the herds. Our snapshot cover the importance of *N. caninum* and abortion risk factors due to *N. caninum* infection in the region. Thus, further full investigations on hidden risk factors, design appropriate schemes for blocking *Neospora*-infection, using of infection-resistance native and crossbred cattle, culling of positive cattle with history of abortion, and reducing the economic losses are recommended.

Acknowledgements

The authors greatly appreciated staff members of Hamedan Veterinary Office for sampling, Dr. Reza Farahani-Khaltabadi for technical helping, Mrs. Sakineh Azami for supply the positive control and Mr. Mahdi Tabaghchi for preparation of map. The study was supported financially by Urmia University (a part of Ph.D thesis), Urmia, Iran.

References

[1] Dubey J.P. 2003. Review of *Neospora caninum* and neosporosis in animal. *Korean Journal of Parasitology* 41: 1-16. https://dx.doi.org/10.3347/2Fkjp.2003.41.1.1

[2] Dubey J.P., Knickman E., Greene C.E. 2005. Neonatal *Neospora caninum* infections in dogs. *Acta Parasitologica* 50: 176-179.

[3] Dubey J.P., Schares G., Ortegamora L.M. 2007. Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Review* 20: 323-369. doi:10.1128/cmr.00031-06

[4] Dubey J.P., Schares G. 2011. Neosporosis in animals - the last five years. *Veterinary Parasitology* 180: 90-108. https://doi.org/10.1016/j.vetpar.2011.05.031

[5] Moore D.P., Campero C.M., Odeón A., Bardón J., Silva-Paulo P., Paolicchi F.A., Cipolla A.L. 2003. Humoral immune response to infectious agents in aborted bovine fetuses in Argentina. *Revista Argentina Microbiologica* 35: 143-148.

[6] Guido S., Katzer F., Nanjiani I., Milne E., Innes E.A. 2016. Serology based diagnostics for the control of bovine neosporosis. *Trends in Parasitology* 32: 131-143. doi:10.1016/j.pt.2015.11.014

[7] Japa O., Nuangmek A., Prakhammin K., Flynnd R. 2019. Prevalence of transplacentally transmitted *Neospora caninum* amongst beef cattle in Phayao, Thailand. *Parasitology International* 70: 98-101. https://doi.org/10.1016/j.parint.2019.02.008

[8] Santos T., Simões R., Mateus T., Lopes A. 2016. Updates on *Neospora caninum,* Economical impact. *Experimental Pathology Health Science* 8: 49-50.

[9] Sadrebazzaz A., Haddadzadeh H., Esmailnia K. 2004. Serological prevalence of *Neospora caninum* in healthy and aborted dairy cattle in Mashhad, Iran. *Veterinary Parasitology* 124: 201-204. https://doi.org/10.1016/j.vetpar.2004.06.027

[10] Razmi G., Mohammadi G., Garrosi T., Farzaneh N., Fallah A., Maleki M. 2006. Seroepidemiology of *Neospora caninum* infection in dairy cattle herds in Mashhad area, Iran. *Veterinary Parasitology* 135: 187-189. https://doi.org/10.1016/j.vetpar.2005.09.004

[11] Gharekhani J., Tavoosidana G.R., Akbarein H. 2014. Serological study of *Neospora caninum* infection in dogs and cattle from west of Iran. *Comparative Clinical Pathology* 23: 1203-1207. https://doi.org/10.1007/s00580-013-1763-z

[12] Ansari-Lari M., Rowshan A., Jesmani H., Masoudian M., Badkoobeh, M. 2017. Association of *Neospora caninum* with reproductive performance in dairy cows: a prospective study from Iran. *Veterinary Research Forum* 8: 109-114.

[13] Gharekhani J., Yakhchali M. 2019. *Neospora caninum* infection in dairy farms with history of abortion in West of Iran. *Veterinary and Animal Science* 8: 100071. https://doi.org/10.1016/j.vas.2019.100071

[14] Thrusfield M. 2018. Veterinary Epidemiology. 4nd ed. John Wiley & Sons Ltd Press, London, England.

[15] Müller N., Zimmermann V., Hentrich B. 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. *Journal Clinical Microbiology* 34: 2850-2852.

[16] Barbosa de Macedo C.A., Barbosa de Macedo M., Cardim S., Paiva M., Taroda A., Barros L., Leme da Cunha I., Zulpo D., Garcia J. 2013. *Neospora caninum*: evaluation of transplacental transmission in slaughtered dairy cows. *Brazilian Journal Veterinary Parasitology* 22: 13-17.

[17] Razmi G., Zarae H., Nourbakhash M.F., Naseri Z. 2013. Estimating the rate of transplacental transmission of *Neospora caninum* to aborted fetuses in seropositive dams in Mashhad area, Iran. *Iranian Journal Veterinary Medicine* 7: 253-256.

[18] Ansari-Lari M. 2020. Bovine neosporosis in Iran: A systematic review and meta-analysis. *Preventive Veterinary Medicine* 176: 104913. https://doi.org/10.1016/j.prevetmed. 2020.104913

[19] Ribeiro C.M., Soares I., Mendes R.G., Bastos P., Katagiri S., Zavilenski R.B., Abreu H.F., Afreixo V. 2019. Meta-analysis of the prevalence and risk factors associated with bovine neosporosis. *Tropical Animal*

Health Production 51: 1783-1800.

[20] Liu Y., Reichel M.P., Lo W.C. 2020. Combined control evaluation for *Neospora caninum* infection in dairy: Economic point of view coupled with population dynamics. *Veterinary Parasitology* 227: 108967. https://doi.org/10.1016/j.vetpar.2019.108967

[21] Marques F.A., Headley A.S., Figueredo-Pereira V., Taroda, A., Barros L., Cunha I., Munhoz K., Bugni F., Zulpo D., Igarashi M., Vidotto O., Guimarães Junior J., Garcia J. 2010. *Neospora caninum*: evaluation of transplacental transmission in slaughtered beef cows. *Parasitology Research* 108: 1015-1019.

[22] Gondim L.F.P., Mc Allister M.M., Anderson-Sprecher R.C., Bjorkman C., Lock T.F., Firkins L.D., Gao L., Fischer W.R. 2004. Transplacental transmission and abortion in cows administered *Neospora caninum* oocysts. *Journal of Parasitology* 90: 1394-1400. https://doi.org/10.1645/ge-359r

[23] Lefkaditis M., Mpairamoglou R., Sossidou A., Spanoudis K., Tsakiroglou M. 2020. *Neospora caninum*, a potential cause of reproductive failure in dairy cows from Northern Greece. *Veterinary Parasitology: Regional Studies and Reports* 19: 100365. https://doi.org/10.1016/j.vprsr.2019.00365

[24] Gharekhani J., Haddadzadeh H., Bahonar A. 2014. Prevalence of immunoglobulin G (IgG) antibody to *Neospora caninum* in dairy cattle of Hamedan province, West of Iran. *Veterinary Research Forum* 5: 149-152.

[25] Yaniz J.L., Lopez F., Garcia G., Serrano B., Nogareda C., Sanchez-Nadal J., Almeria S., Santoloria P. 2010. Some factors affecting the abortion rate in dairy herds with high incidence of *Neospora*–associated abortions are different in cows and heifers. *Reproductive Domestic Animal* 45: 699-705.

[26] Bech-Sàbat G., Serrano B., García-Ispierto I., Santolaria P., Yániz J.L., Almería S., López Gatius F. 2006. Effect of progesterone supplementation during early foetal period in *Neospora caninum* seropositive dairy cows. *Reproduction in Domestic Animal* 41: 104.

[27] Anderson M.L., Palmer C.W., Thurmond M.C., Picanso J.P., Blanchard P.C., Breitmeyer R.E., Layton A.W., Mc Allister M., Daft B., Kinde H., Read D.H., Dubey J.P., Conrad P.A., Barr B.C. 1995. Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. *Journal of American Veterinary Medicine Association* 207: 1206-1210.

[28] Davison H., Otter A., Treesa A., 1999. Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. *International Journal for Parasitology* 29: 1683-1689. https://doi.org/10.1016/s0020-7519(99)00129-0

[29] Sekiya M., Zintl A., Doherty M.L. 2013. Bulk milk ELISA and the diagnosis of parasite infections in dairy herds: a review. *Irish Veterinary Journal* 66: 14.

[30] Schares G., Staubach C., Wurm R., Rauser M., Conraths F.J., Schroeder C. 2004. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. *Veterinary Parasitology* 120: 55-63. https://doi.org/10.1016/j.vetpar.2003.11.016

Received 02 June 2020
Accepted 03 August 2020