Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http://ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Original Article

Molecular Identification of *Neospora caninum* Infection in Aborted Fetuses of Sheep, Cattle, and Goats in Mazandaran Province, Northern Iran

Behnaz Salehi [1], Afsaneh Amouei [1,2], Samira Dodangeh [3], Ahmad Daryani [4], *Shahabeddin Sarvi [4], Mohammad Reza Safari-Kharyeki [5], Saeid Salehi [6], Seyed Abdollah Hosseini [1], Zahra Hosseininejad [1]

1. *Department of Medical Parasitology and Mycology, Mazandaran University of Medical Sciences, Sari, Iran*
2. *Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran*
3. *Medical Microbiology Research Center, Qazvin University of Medical Sciences, Qazvin, Iran*
4. *Toxoplasmosis Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran*
5. *Mahdasht Dairy and Meat Company, Panbe Choleh, Sari, Iran*
6. *Mazandaran Provincial Veterinary Department of Medical Sciences, Sari, Iran*

Received 18 Oct 2020
Accepted 03 Dec 2020

Keywords:
Neospora caninum;
Ruminants' aborted fetuses;
Iran

***Correspondence**
Email:
shahabesarvi@yahoo.com

Abstract

Background: We aimed to identify *Neospora caninum* DNA in the brain samples of aborted fetuses of cattle, goats, and sheep in Mazandaran, northern Iran, using PCR.

Methods: In total, 133 aborted fetuses (51 sheep, 78 cattle, and 4 goats) were randomly collected from different stages of gestation in various regions of Mazandaran, Iran, from Mar 2016 to May 2017. The DNA was extracted from all the brain samples using phenol chloroform isoamyl alcohol instructions. The *Nc-5* gene was used for the detection of *N. caninum* DNA by nested-PCR assay.

Results: The detection of *N. caninum* DNA was confirmed by the observation of a 227 bp band in 24 samples of 133 aborted fetuses (18.1%). The highest prevalence rate of *N. caninum* was detected in the cattle (20.5%) followed by the sheep (15.6%); however, no positive cases were reported in the goats. The highest and lowest prevalence rates of the infection were reported as 23.8% and 8.6% in Qaemshahr, and Behshahr, respectively. The prevalence rate of infection (32%) in the early gestational period was higher than those in the middle (15%) and late (3.8%) gestational periods.

Conclusion: The obtained data of the present study indicated that *N. caninum* infection may partly be responsible for abortion and economic loss in livestock farming in Mazandaran Province.

Introduction

Neospora caninum , an apicomplexan unicellular parasite, is a major and important cause of bovine abortion with worldwide distribution. Dogs and coyotes are considered definitive hosts; however, a wide range of warm-blooded animals, such as cattle, sheep, buffalos, horses, goats, rodents, and rhinoceros, are the intermediated hosts to *N. caninum* (1, 2). This intracellular parasite can be a cause of abortion at any stages of pregnancy in cattle; however, it usually occurs at 5-6 months of gestation and may happen more than once in the reproductive seasons (3).

Neosporosis can be horizontally and vertically transmitted in the herds. Congenital transmission is the main route (50-95%) of abortion due to *N. caninum* and plays an important role in the maintenance of the parasite in farms and herds (4). Nonetheless, due to the relatively limited number of studies, the clinical, epidemiological, and economic importance of neosporosis in sheep, especially in goats is still not clear (5).

There are several methods for the detection of *N. caninum* in aborted fetuses, including histopathology (6), immunohistochemistry (7), and PCR (8). Brain tissue, as well as the heart and liver, are the best sample for the diagnosis of neosporosis in fetuses. Among the PCR methods for the detection of *N. caninum* DNA, nested PCR has higher sensitivity and specificity than the other methods because it is able to amplify the small amounts of *Neospora* DNA in infected tissue (9).

The results of numerous studies performed on the seroprevalence of *N. caninum* in aborted dairy cattle in Iran indicated neosporosis should be considered a cause of economic and health problems in Iran (10-12). There is no information regarding the molecular detection of *N. caninum* in aborted fetuses in northern Iran.

Therefore, we aimed to identify *N. caninum* DNA in ruminant's aborted fetuses (sheep, goats, and cattle) using nested PCR to evaluate the association of *N. caninum* with fetus abortion in northern Iran.

Materials and Methods

Ethical approval

Ethical approval was obtained from the Ethics Committee of Mazandaran University of Medical Sciences (no. 10217).

Sample Collection

A total of 133 brain samples obtained from 51 aborted sheep, 78 aborted cattle, and 4 aborted goats were collected from several industrial farms in Mazandaran province, northern Iran, from Mar 2016 to May 2017. The brain of each aborted animal was rinsed in distilled water, packaged, and transferred to the Parasitology Laboratory of Mazandaran University of Medical Sciences for the laboratory examination.

DNA Extraction

Approximately, 5 g of brain from different segments were homogenized with 70% ethanol, and DNA was extracted using phenol-chloroform extraction method. Then, 500 µl of lysis buffer (50 mM Tris-HCL, pH (8.0); 25 mM EDTA and 400 mM NaCl), 50 µl 10% sodium dodecyl sulfate, and 35 µl proteinase K (20 µg/µl) were added to 200 µl homogenized brain samples in 1.5 ml microtubes. The suspension was incubated at 56 °C overnight. For the precipitation of debris and proteins, 200 µl of sodium chloride (6M) was added to the suspension, and it was kept at 4 °C for 30 min. After centrifugation at 14,000 rpm for 15 min, the supernatants were transferred to new microtubes for extraction with phenol chloroform isoamyl alcohol (24:24:1, v/v).

The DNA was precipitated by adding 500 µl of 100% cold ethanol and 20 µl of sodium acetate solution (3M), followed by centrifugation at 14,000 rpm for 10 min and finally kept at -20 °C for 24 h (13). The pellet was washed twice by 70% ethanol and resuspended in 60 µl of Tris-EDTA buffer. The concentration of DNA was determined by NanoDrop ND100 (Thermo Scientific). The extracted DNA was stored at -20 °C until usage.

Nested Polymerase Chain Reaction

Nested PCR was performed to detect the DNA of *N. caninum* using *Nc-5* gene with external primers, including Np21plus (5'-CCCAGTGCGTCCAATCCTGTAAC-3') and Np6plus (5' CTCGCCAGTCCAACCTACGTCTTCT-3'), as well as internal primers, including Np6 (5' CAGTCAACCTACGTCTTCT-3') and Np7 (5'- GGGTGAACCGAGGGAGTTG-3') (14, 15). The first step of PCR was carried out to amplify the fragment of 337bp in a volume of 25 µl containing 3 µl of genomic DNA, 0.75 µl of each external primer (10 pmol/µl) (BioNeer, Korea), 12.5 µl of commercial premix (Ampliqon, Denmark), and 8 µl of molecular biology grade H_2O. The PCR conditions include initial denaturation for 7 min at 94 °C, 30 cycles of denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 7 min (BioRad C1000, USA).

As the second step of PCR, a fragment of 227 bp was amplified by 1 µl primary PCR product, 0.5 µl of each internal primer (10 pmol/µl) (BioNeer, Korea), 12.5 µl of commercial premix (Ampliqon, Denmark), and 10.5 µl of molecular biology grade H_2O in a volume of 25 µl. The cycling parameters were considered the third stage, including preheating at 94 °C for 5 min, followed by 35 cycles of 20 sec at 94 °C, 20 sec at 60 °C, 20 sec at 72 °C, and final extension for 5 min at 72 °C (16). Nested PCR product was subjected to electrophoresis on a 1.5% agarose gel in a Tris-Borate-EDTA buffer at 90 V for 20 min and visualized with ultraviolet transilluminator after staining with SYBR Safe (Life Technologies Corporation, USA).

Sequence Alignments and Phylogenetic Analyses

Some positive nested PCR products were directly sequenced by targeting *Nc-5* gene marker in both directions using the aforementioned primers by Genetic Analyzer automated sequencer. The sequences of the samples were aligned and edited in consensus positions, compared to the GenBank sequences of all regional species using Sequencher Tmv.4.1.4 software. The similarity between the present sequenced isolates and sequences of other countries was determined using MegAlign software. In addition, MEGA 6 software and neighbor-joining algorithm were used in order to perform phylogenetic analysis.

Results

The brain samples of 133 aborted fetuses, including 51 sheep, 78 cattle, and 4 goats, were surveyed by nested PCR for the detection of *N. caninum*; out of 133 brains samples, 24 samples (18.04%) were positive in this regard. As shown in Table 1, the prevalence rate of *N. caninum* in the aborted cattle fetuses (20.5%) was higher than that of the aborted sheep fetuses (15.6%); however, no positive case was observed in the goat fetuses. Therefore, there was no relationship between the type of livestock and prevalence of *N. caninum*. Furthermore, the results of the present study indicated that *N. caninum* caused was considerably more in the early trimester (32%) than others ($P<0.05$). The highest prevalence of this parasite was reported in Qaemshahr, Iran, followed by Sari, Babol, Amol, and Behshahr, Iran. The results of the statistical analysis did not show a significant relationship between living area and *N. caninum* caused abortion.

Table 1: Association between the types of animals, gestational period, and area with the presence of *Neospora caninum* DNA from ruminants that aborted

Variable		*Number of samples*	*Number of Positive (%)*	*Odds Ratio*	*Confidence Interval 95%*	*P value*
Type of animals	Sheep	51	8 (15.6)			
	Cattle	78	16 (20.5)	0.7	(0.2-1.9)	0.6
	Goat	4	0 (0)	-	-	-
Stage of pregnancy	Early	46	15 (32)	1		
	Middle	60	9 (15)	2.7	(1.1-7.2)	0.03
	The Late	27	1 (3.7)	12.5	(1.9-276)	0.003
Area/city	Sari	56	13 (23.2)			
	Babol	15	2 (13.3)	1.9	(0.3-20)	0.5
	Behshahr	23	2 (8.6)	3.1	(0.7-22)	0.1
	Amol	18	2 (11.1)	2.4	(0.5-17)	0.2
	Qaemshahr	21	5 (23.8)	0.9	(0.2-3.4)	0.9

The sequencing of nested PCR products obtained from three brain samples of the aborted fetuses (i.e., 1 sheep and 2 cattle) showed that the amplified sequence was *N. caninum* specific. All the data sequences of the present study were deposited in the GenBank with accession numbers of MH841974, MH795879, and MH752687. The results demonstrated our sequences shared 96% to 99% similarity with each other and 96% to 100% similarity with *N. caninum* deposited in GenBank. Phylogenetic trees showed intraspecific variations between our isolates and other *N. caninum* specimens deposited in GenBank (Fig. 1). Analysis of our sequences showed high similarity with *N. caninum* isolated from cattle in Iran (MH410658), Spain (AV494944), and China (JN634858).

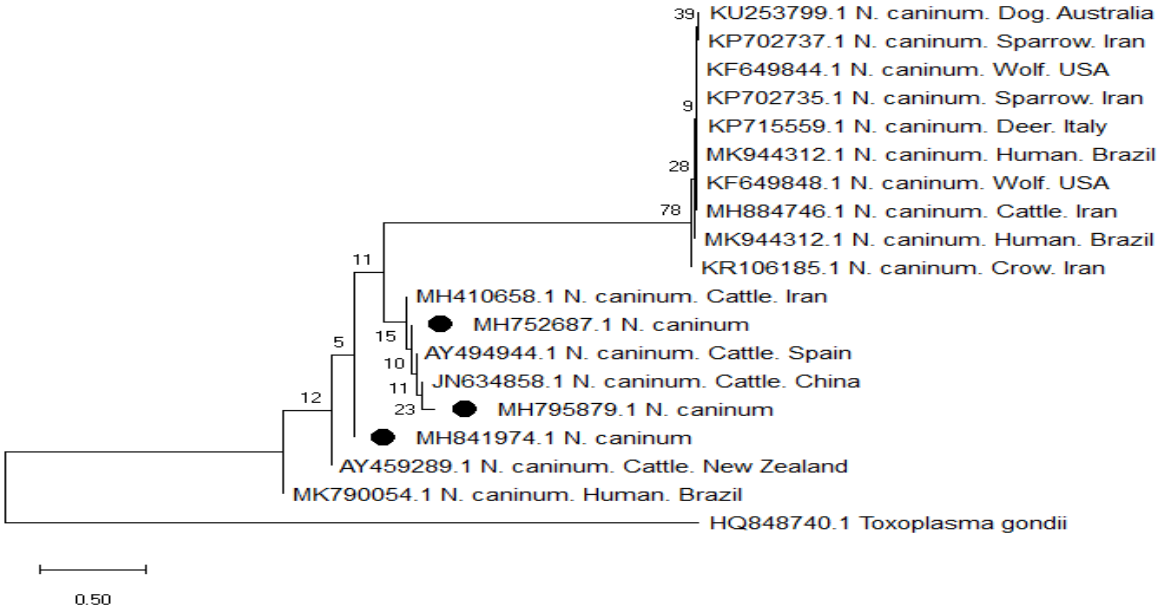

Fig. 1: Phylogenetic tree of *N. caninum* isolates from Mazandaran Province, Iran and the other isolates deposited in GenBank

Discussion

Neosporosis has emerged as one of the most common infections of abortion dairy cattle worldwide. The clinical symptoms of *N. caninum* have been observed in sheep, goats, deer, and horses. Furthermore, antibodies have been detected in the sera of camels, water buffaloes, foxes, coyotes, and felids (4).

Fetus abortion caused by *N. caninum* is the common reproductive problem that leads to major economic loss in cattle and sheep husbandry (17).

The PCR has been used for the detection of *N. caninum* since 1996, and several target genes, including *18S rDNA*, *28S rDNA*, *ITS1*, and *Nc-5*, have been used as target genes for the diagnosis of this parasite. Among these genes, *Nc-5* has been most frequently used because not found in the genome of the parasites of this protozoan family, such as *T. gondii*, *Hammondia hammondi*, and *Sarcocystiss cruzi*. The PCR, especially nested PCR has high sensitivity and specificity for the detection of parasites while using a specific gene (18). In the present study, *N. caninum* was detected in the brains of 24 (18.04%) cases out of 133 examined aborted fetuses using nested PCR.

In Iran, neosporosis has been reported from 0.9% to 8.5% in *aborted bovine fetuses* by various PCR assays (19, 20). In addition, the results of previous studies in Chahar Mahal Bakhtiari and Mashhad, Iran, showed that the prevalence rates of *N. caninum* were 11% (21) and 33% in aborted bovine fetuses, respectively (22).

In other countries, *N .caninum* was detected in the brain samples of 5%, 6.8%, 8.6%, and 18.9% of cattle fetuses in Brazil (23), aborted sheep fetuses in Spain, aborted sheep fetuses in Italy, and 18.9 aborted bovine fetuses in England (14), respectively(24, 25). The main causes of various results can be due to the frequency of definitive hosts in the studied areas (e.g., dogs), uses of various diagnostic methods, as well as climate and environmental factors (26). Therefore, farmers should further become aware of maintaining hygienic conditions and keeping livestock food out of the reach of dogs.

In this study, three positive samples were sequenced for phylogenetic analysis. The sequences results displayed high similarity with *N. caninum* isolated from cattle in Mashhad (Iran), Spain and China that clustered with them in the phylogenetic analysis. The results of phylogenetic analysis indicted that *N. caninum* from different hosts and geographical area are genetically diverse and can be classified into two main clades (Fig. 1), although the two clades proposal is inadequately supported (Bf=78%, Fig. 1). A few investigations have been performed on the phylogenetic analysis of *N. caninum* with the *Nc-5* gene. BLAST analyses of *Nc-5* gene showed greater than 94% to 97% similarities between *N. caninum* sequences deposited in GenBank. Hence, it seems the *Nc-5* gene is only a highly sensitive gene for the diagnosis of neosporosis and will be suggested to use ITS-1 and microsatellite genes for phylogenetic analyses (27).

The prevalence rate of neosporosis was significantly higher in the cattle, compared with the sheep. Such a difference in prevalence rate between these animals could be due to the high susceptibility of cattle to neosporosis infection (28) that is in line with the findings of a study in North Africa (29). As reported clinical cases associated with *N. caninum* infection in goats are uncommon (30), no positive effect was observed in the results of the present study regarding neosporosis among the goats. The abortion of fetuses due to *N. caninum* occurs from 3 months of gestation to term (3). In this study, a higher number of abortions caused by *N. caninum* in the early trimester of gestation (32%) was similar to the results of other studies (within the range of 6-8 months) (31, 32).

The prevalence rates of aborted fetuses infected with *N. caninum* were reported. This difference could be related to the livestock

rearing system and number of dogs present in these cities. However, fetal mortality can be prevented in bovines by vaccination. Currently, there is no commercial vaccine for neosporosis (33); therefore, must be prevented from consuming placentas and fetuses by dogs and wild canids. Fetal tissues and placentas should be collected and disposed as far as possible.

Low sample size especially in goat samples and unavailability of aborted fetal samples in traditional farms are the limitations of the present study.

Conclusion

The detection of *N. caninum* DNA in the aborted fetuses of cattle and sheep showed that *N. caninum* could be the main agent of abortion in these ruminants. Awareness programs should be established for both farmers and veterinarians regarding the risks associated with this parasite. In addition, it is required to perform further investigations using molecular methods and larger sample sizes to confirm the above-mentioned hypothesis in ruminants' aborted fetuses in Iran.

Acknowledgements

The authors would like to express gratitude to the Vice Chancellor for Research of Mazandaran University of Medical Sciences for funding this research project (no.10217). In addition, the authors thank the Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran for generous support of this study.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Dubey J, Schares G. Neosporosis in animals the last five years. Vet Parasitol. 2011;180(1-2): 90-108.
2. Shaapan RM. The common zoonotic protozoal diseases causing abortion. J Parasit Dis.2016;40(4): 1116-1129.
3. Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Vet Parasitol. 1996;67(1-2): 1-59.
4. Dubey JP. Review of *Neospora caninum* and neosporosis in animals. Korean J Parasitol. 2003;41(1):1-16.
5. Anastasia D, Elias P, Nikolaos P, et al. *Toxoplasma gondii* and *Neospora caninum* seroprevalence in dairy sheep and goats mixed stock farming.Vet Parasitol. 2013;198(3-4):387-90.
6. Pescador C, Corbellini L, Oliveira E, et al. Histopathological and immunohistochemical aspects of *Neospora caninum* diagnosis in bovine aborted fetuses Vet Parasitol.2007;150(1-2): 159-63.
7. Uzêda R, Schares G, Ortega-Mora LM, et al. Combination of monoclonal antibodies improves immunohistochemical diagnosis of *Neospora caninum*. Vet Parasitol. 2013;197(3-4): 477-86.
8. Tian A-L, Elsheikha HM, Zhou D-H, et al. A novel recombinase polymerase amplification (RPA) assay for the rapid isothermal detection of *Neospora caninum* in aborted bovine fetuses. Vet Parasitol. 2018;258(15): 24-29.
9. Habibi GR, Hashemi-Fesharki R, Sadrebazzaz A, et al. Seminested PCR for Diagnosis of *Neospora caninum* Infection in Cattle. Archives of Razi Institute. 2005;59(2): 55-64.
10. Hajikolaei MRH, Goraninejad S, Hamidinejat H, et al. Occurrence of *Neospora caninum* antibodies in water buffaloes (Bubalus bubalis) from the south-western region of Iran. Bulletin- Veterinary Institute in Pulawy. 2007;51(2): 233-235.
11. Fard SRN, Khalili M, Aminzadeh AJVA. Prevalence of antibodies to *Neospora caninum* in cattle in Kerman province, South East Iran. Vet Arhiv. 2008;78(3): 253-259.
12. Nematollahi A, Jaafari R, Moghaddam Gh. Seroprevalence of *Neospora caninum* infection in

dairy cattle in Tabriz, Northwest Iran. Iran J Parasitol. 2011;6(4): 95-98.
13. Kamali A, Seifi HA, Movassaghi AR, et al. Histopathological and molecular study of *Neospora caninum* infection in bovine aborted fetuses. Asian Pacific J Trop Biomed. 2014;4(12):990-994.
14. Hughes J, Williams R, Morley E, et al . The prevalence of *Neospora caninum* and co-infection with *Toxoplasma gondii* by PCR analysis in naturally occurring mammal populations. Parasitology. 2006; 132(Pt 1):29-36.
15. Müller N, Zimmermann V, Hentrich B, et al. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. J Clin Microbiol. 1996;34(11): 2850-2852.
16. Amouei A, Sharif M, Sarvi S, et al. Aetiology of livestock fetal mortality in Mazandaran province, Iran. Peer J. 2019; 6: e5920.
17. Reichel MP, Ayanegui-Alcérreca MA, Gondim LF, et al. What is the global economic impact of *Neospora caninum* in cattle–the billion dollar question. Int J Parasitol. 2013;43(2): 133-42.
18. Yao L, Yang N, Liu Q, et al . Detection of *Neospora caninum* in aborted bovine fetuses and dam blood samples by nested PCR and ELISA and seroprevalence in Beijing and Tianjin, China. Parasitology. 2009;136(11):1251-6.
19. Asadpour R, Jafari-Joozani R, Salehi N. Detection of *Neospora caninum* in ovine abortion in Iran. J Parasit Dis. 2013;37(1): 105-9.
20. Sasani F, Javanbakht J, Seifori P, et al. *Neospora caninum* as causative agent of ovine encephalitis in Iran. Pathol Discov.2013;1(1): 5.
21. Rafati N, Jaafarian M. The determination of prevalence of *Neospora caninum* in aborted fetuses in dairy cattle of Shahrekord area, Chahar Mahal Bakhtiari province, by Nested-PCR. Journal of Veterinary Laboratory Research. 2014;6(1): 45-50.
22. Sadrebazzaz A, Habibi G, Haddadzadeh H, et al. Evaluation of bovine abortion associated with *Neospora caninum* by different diagnostic techniques in Mashhad, Iran. Parasitol Res. 2007. 100(6):1257-60.
23. Santos SL, de Souza Costa K, Gondim LQ, et al. Investigation of *Neospora caninum, Hammondia* sp., and *Toxoplasma gondii* in tissues from slaughtered beef cattle in Bahia, Brazil. Parasitol Res. 2010;106(2): 457-61.
24. Masala G, Porcu R, Daga C, et al. Detection of pathogens in ovine and caprine abortion samples from Sardinia, Italy, by PCR. J Vet Diagn Invest. 2007;19(1): 96-8.
25. Moreno B, Collantes-Fernández E, Villa A, et al. Occurrence of *Neospora caninum* and *Toxoplasma gondii* infections in ovine and caprine abortions. Vet Parasitol. 2012;187(1-2): 312-8.
26. Gharekhani J. Seroprevalence of *Neospora caninum* and *Toxoplasma gondii* infections in aborted cattle in Hamedan, Iran. J Adv Vet Anim Res. 2014;1(2): 32-35.
27. Al-Qassab SE, Reichel MP, Ellis JT. On the biological and genetic diversity in *Neospora caninum*. Diversity. 2010 Mar;2(3):411-438.
28. Pan Y, Jansen G, Duffield T, et al. Genetic susceptibility to *Neospora caninum* infection in Holstein cattle in Ontario. J Dairy Sci. 2004;87(11): 3967-75.
29. Amdouni Y, Rjeibi M, Awadi S, et al. First detection and molecular identification of *Neospora caninum* from naturally infected cattle and sheep in North Africa. Transbound Emerg Dis. 2018;65(4): 976-982.
30. Eleni C, Crotti S, Manuali E, et al . Detection of *Neospora caninum* in an aborted goat foetus. Vet Parasitol. 2004;123(3-4): 271-4.
31. De Meerschman F, Speybroeck N, Berkvens D, et al. Fetal infection with *Neospora caninum* in dairy and beef cattle in Belgium. Theriogenology. 2002;58(5): 933-45.
32. Sager H, Fischer I, Furrer K, et al. A Swiss case–control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. Vet Parasitol. 2001;102(1-2): 1-15.
33. Innes EA, Andrianarivo AG, Björkman C, et al. Immune responses to *Neospora caninum* and prospects for vaccination. Trends Parasitol. 2002;18(11): 497-504.