Biochemistry and Molecular Biology Journal
ISSN: 2471-8084

Open Access Research Article

Molecular Identification of *Neospora caninum* in Aborted Sheep in Garmian Region/Kurdistan of Iraq

Hiwa Essa*

Department of Veterinary, *Pasteur Institute of Iran, Tehran, Iran*

ABSTRACT

Neospora caninum is an intracellular protozoan parasite associated with bovine and ovine abortion. Little is known about the extent of *Neospora* infection in infected populations of animals. This parasite is a worldwide apicomplexan protozoan with a variety of animal hosts. The earlier report of the parasite was described in 1984 in puppies with signs of encephalomyelitis and myositis. Neosporosis is considered a major cause of abortion in many cattle-producing countries. In sheep, the first infection with *N. caninum* was diagnosed in a congenitally infected lamb in England. This study aims to estimate the existence of *Neospora caninum* in aborted sheep in the Garmian region in Kurdistan/ Iraq. There is little information about the prevalence and existence of *N. caninum* in aborted animals in Kurdistan and Iraq by PCR and diagnosis is only done by serological methods. The Polymerase Chain Reaction or PCR was used for the detection of *Neospora caninum* in the placenta tissues of aborted sheep from (89) aborted sheep in mating season 2021. The results of the study showed that 6 (6.7%) out of (89) fetal samples of ovine aborted fetuses by using PCR were positives. Our results suggest that the infection with *N. caninum* is existed and was detected in aborted fetuses. Furthermore, the study is the first one to identify *N. caninum* in this region and amended that this protozoan is one of the main causative agents of abortion *via* PCR technique.

Keywords: Abortion; Fetuses; Intracellular; Neospora; N5 gene

INTRODUCTION

Prostate *Neospora caninum* are intracellular protozoan parasites associated with bovine and ovine abortion. Little is known about the extent of *Neospora* infection in infected populations of animals. *Neospora caninum* is a worldwide apicomplexan protozoan with a variety of animal hosts. The earlier report of the parasite was described in 1984 in puppies with signs of encephalomyelitis and myositis. Neosporosis is considered a major cause of abortion in many cattle-producing countries. In sheep, the first infection with *N. caninum* was diagnosed in a congenitally infected lamb in England. *Neospora*, *Toxoplasma* and *Sarcosystosis* are genera of sarcocystidae family. The *N. caninum* life cycle is divided into three infectious stages which are tachyzoites, tissue cysts, and oocysts. The first two stages are found intracellularly in the intermediate hosts, whereas oocysts which are approximately measure 12 µm in diameter are excreted unpopulated in feces and sporulation occurs outside the host.

The final host of *Neospora* is a dog which is also stated as an intermediate host. Some studies indicate that coyotes (*Canis latrans*), dingoes (*Canis lupus dingo*), red foxes (*Vulpes*

Received:	04-April-2023	**Manuscript No:**	IPBMBJ-23-16066
Editor assigned:	06-April-2023	**PreQC No:**	IPBMBJ-23-16066(PQ)
Reviewed:	20-April-2022	**QC No:**	IPBMBJ-23-16066
Revised:	05-June-2023	**Manuscript No:**	IPBMBJ-23-16066(R)
Published:	12-June-2023	**DOI:**	10.36648/2471-8084.9.5.41

Corresponding author: Hiwa Essa, Department of Veterinary, Pasteur Institute of Iran, Tehran, Iran; E-mail: d.hiwa1980@yahoo.com

Citation: Essa H (2023) Molecular Identification of Neospora Caninum in Aborted Sheep in Garmian Region/Kurdistan of Iraq. Biochem Mol Biol J. 9:41.

vulpes), and grey wolves (*Canis lupus*), are also measured as definitive hosts of this protozoan. Dogs, the definitive hosts, infect *via* ingestion of infected tissues with tachyzoites or tissue cysts from several intermediate hosts. The natural intermediate hosts of neosporosis are cattle, sheep, goats, camels and water buffalos, some birds and cats, mice, gerbils and monkeys are experimental intermediate hosts. It occurs occasionally in horses and deer.

Sheep can be infected either transplacentally (vertically, congenitally) from an infected dam to her fetus during pregnancy or horizontally by ingestion of sprozoites containing oocysts which are shed by the definitive host. *N. caninum* can cause abortion in sheep with fetal mummification, embryo resorption, still birth, and the birth of weak or apparently healthy but congenitally infected offspring. Lesions of ovine cerebral neosporosis were acute non-suppurative meningoencephalitis and mild to moderate non-supportive myelitis.

Until recent years, little evidence that the disease become a significant field problem in sheep was recorded, however, in some places, naturally occurring ovine neosporosis has been reported in Japan, South America and Switzerland. When the fetal immune system is little developed at the early gestation periods, the infection can be fatal with fetal resorption or abortion, while the exposure to older fetuses with a better developed immune system may result in the birth of clinically normal but congenitally infected animals. Although reports of reproductive failure and abortion have been reported in many studies, information about the description and detection of nucleic acids resulting from neosporosis in sheep is still deficient. The first report about the association between *N. caninum* and cases of abortion in naturally infected sheep was first mentioned by Hassig, et al., through PCR in the brain of fetuses from aborted sheep. Many studies about the detection of abortion induced by protozoa have been based on histopathological determination for many years this technique cannot make an accurate differentiation between infections with *N. caninum* from that with *Toxoplasma gondii* because of the morphological similarity of both parasites. Thus, to overcome these methodological limitations more sensitive and specific techniques have been applied to enable the detection of the parasite DNA and genetic characterization. PCR allows fast and methodically highly sensitive identification of *N. caninum* by subsequent and amplification of the parasite specific DNA sequences in different tissues likes brain, lung, liver, kidney, semen, and placenta. The study aimed to estimate the existence of *Neospora caninum* in aborted sheep in the Garmian region in Kurdistan/Iraq by using PCR [1-4].

MATERIALS AND METHODS

The study area is located in the Southeastern part of the region of Iraqi Kurdistan, between two latitudes(33° N and 35°N) and longitudes (41°, 44°)E. As they challenged the Sulaymaniyah governorate from the North and the provinces of Salah al-Din and Diyala from the West and South, respectively, while its Eastern borders, representing the international border with the Islamic Republic of Iran, at a distance of 153 km [5-11].

Sampling and Data Collection

A total of 89 samples including sheep aborted placental tissues were collected from aborted flocks suspected to be infected with *N. caninum*. During mating season 2021, (89) flocks, with a history of reproductive disorders like abortions, stillbirth, weak birth were investigated. Samples were collected from (89) different flocks from 23 villages. The samples were from villages distributed in the Garmian region from al Sulaymaniyah province in Kurdistan of Iraq. Collected samples were preserved freezing at −20°C until the PCR and DNA extractions were implemented (**Figure 1**) [12,13].

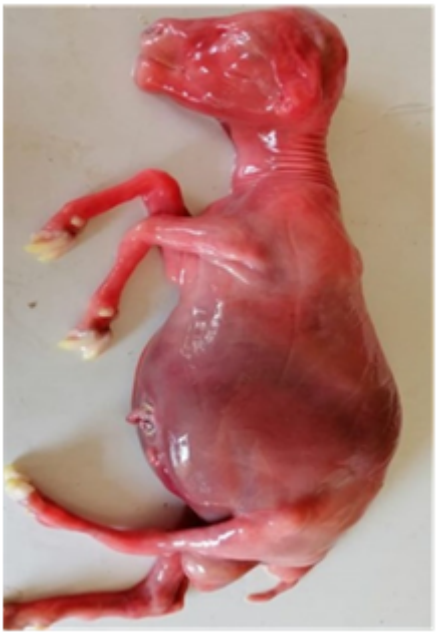

Figure 1: Fetus, lamb. Abortion occurred in about 90 day's ewe with *N. caninum* of gestation period.

DNA Extraction

Diagnostic samples were collected from the placenta of aborted fetuses for genomic DNA diagnosis about 5 g–10 g of each sample was taken selectively from aborted fetuses, and preserved in freezing -20°C for future DNA extractions.

Genomic DNA was extracted from about 20 mg–50 mg of each sample and DNA extraction was done according to the Addbio tissue extraction kit (South Korea). Finally, 100 µl volume of eluted DNA was obtained and stored at -20°C until PCR analysis.

PCR Primers and Master-Mix

The PCR assay using the primers specific for *Neospora caninum* with an amplified product of 334 bp (**Figure 2**). Oligonucleotide primers were designed according to Muller, N et al., for amplification of the *NC5* gene of *N caninum* primers used in the PCR reaction were synthesized by Macro gene (S, Korea). The primers were received in lyophilized form and

suspended in RNASE free water to reach a final concentration of 10 Pmol/µl designed to amplify a specific segment of 334 bp. Neospora caninum specific primer pair Np21 forward (GTGCGTCCAATCCTGTAAC) and Np6 Reverse (CTCGCCAGTCAACCTACGTCTTCT) 334 bp amplicons that anneal to a repetitive region of the parasite genome and used for molecular diagnosis of the parasite.

All PCR reactions were performed in a total 20 µl volume reactions with 10 µl 2X master mix concentration containing 20 mM Tris-HCl (pH8.8), 100 mM KCl, 0.2% Triton X-100, 4 mM $MgCl_2$. Protein stabilizer, sediment, loading dye, and 0.5 mM each of dATP, dCTP, dGTP, and dTTP, primers 1,25 µl (10 pMol/ µL) from each primer, 5 µl of a genomic DNA sample, and 2.5 µl nuclease free water [14-16].

Polymerase Chain Reaction (PCR)

PCR was performed by a thermocycler (Techni, UK) with the following conditions: Initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec with a final extension of 72°C for 5 min. Amplification products were analyzed by electrophoresis through a 1.5% agarose gel, stained with safe gel stain dye (Addbio S. Korea), 10 µl of the product was loaded to the individual wells of a 1.5% agarose gel with 5 µl of a 100 bp DNA ladder And negative controls (double distilled water) were included in each PCR run and the results were seen by run VIEW real time horizontal documentation system (Cleaver Scientific, UK) and then the image was taken to check the presence of specific base pair by using run view real time horizontal documentation system (Cleaver Scientific, UK).

Sequence and Phylogenetic Analysis

About 50 µl of 334 bp PCR product of amplified *NC5* gene sequenced with forward Np_{21} and reverse primers Np_6 in macro gene (South Korea). The 306 bp nucleotide gene sequences of *N. caninum* were obtained and subjected to blast analysis by using the NCBI BLAST tool and compared with other reference *N. caninum* samples available in National Center for Biotechnology Information (NCBI). A total of 9 sequences including reference gene sequences of *N. caninum* were used for analysis. The sequences alignments and phylogeny analysis based on NC5 gene sequences belonging to *Neospora* isolates with those of the present study were aligned by the CLUSTAL W tool. GenBank accession numbers of *N. caninum* sequences used in the analysis are: Iran (MT955656.1) and (MT709296.1), USA (KF649848.1), UK (LN714476.1), Italy (KP715560.1), South Korea (FJ464412.1), Brazil (EU073599), Australia (KU253799.1), and Switzerland (X84238.1).

RESULTS

The goal of the study was to apply the PCR technique to confirm *N. caninum* induced abortion in sheep in our regions. Tissue samples from aborted fetuses were examined by PCR for the detection of *N. caninum*. *N. caninum* was detected by PCR in 6 aborted lambs, (6.74%) were positive; the primers Np21 and Np6 were used to amplify a 334 bp fragment of the repetitive region of the parasite genome (**Figure 2**).

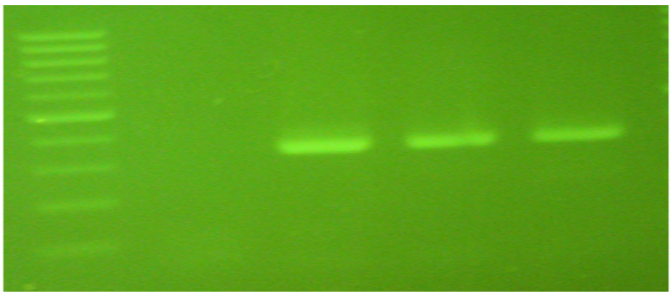

Figure 2: Agarose gel image result of PCR positive samples. Lane1 100 bp ladder lane 2 negative control; lane 3, 4, 5 positive 334 bp of NC5 gene DNA for *N. caninum*.

DNA sequencing and BLAST nucleotides analysis from the GenBank NCBI database revealed more than 96%-98.4% identity to other Nc5 sequences samples deposited in GenBank, by using CLUSTAL w alignment of this sequence (Garmian 2020 isolate) revealed 98.4% identity with 5 nucleotides variation with nearest one which are the Iranian isolates (GenBank accession no. MT 955656.1 and MT 709296.1) strains (**Figures 3 and 4**).

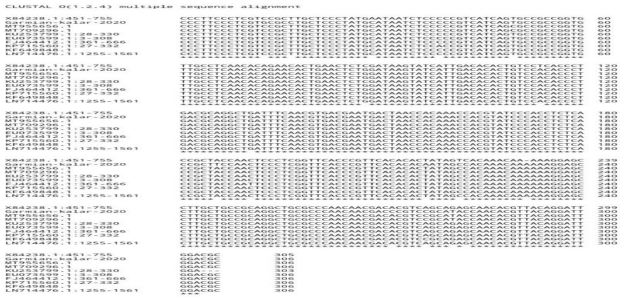

Figure 3: Sequencing of the Nc5 genomic DNA (Garmian 2020 isolate) revealed 97%–98.4% of similarity with other *N. caninum* sequences deposited in Genbank.

Figure 4: Multiple pair-wise of *Nc5* sequence between nine positive samples and (Garmian 2020 isolate) sequences. Phylogenetic tree showing 98.4% homology association of *N. caninum* isolates in our study with two Iranian *N. caninum* isolates (MT955656.1) and (MT709296.1).

DISCUSSION

The In Iraq and Kurdistan region there is little information about the diagnosis of ovine abortion associated with *N. caninum* using the PCR technique. Some molecular studies were conducted previously in Iraq like a study conducted by

Al-Shael, et al., in 2020 from Wasit province in ovine placentas, and another one conducted by Ali and Rubaie in 2020 in Al-Fallujah district in local chickens brain tissues.

The existing data from Iraq and especially our region were mostly done by serological methods. In the present study, we analyzed the *Nc5* gene of *N. caninum* that was amplified from samples in aborted fetuses. The *Nc5* gene can discriminate *N. caninum* from other related apicomplexan parasites (*T. gondii* and *Sarcocyst s* species). The *N*c5 gene has been used as a highly sensitive and specific gene for the detection of neosporosis. *Neospora caninum* can be detected in the liver, placenta, kidney, and brain of aborted fetuses. The detection of *N. caninum* DNA in the umbilical cords of calves with neurological changes might be a very useful tool to confirm disease status.

Ovine neosporosis is traditionally been associated with sporadic cases of abortion with less impact on the productivity of the flocks. But the findings of our study and other studies conclusively demonstrate an association between infection by *N. caninum* and reproductive losses in sheep.

Some serological studies were carried out for the detection of antibodies against *Neospora* in serum like a study conducted in AL-Fallujah city with a rate of (3.91%) in sheep and another study in Wasit city with about (5.6%) and (12.2%) in a study in Mosul city. Diagnosis of *N. caninum* is difficult, due to the low number of parasites in infected tissues and the absent of clear clinical signs as well as the similarity in clinical signs and pathological lesions between *N. caninum* and *Toxoplasma gondii*, and persistent of antibodies against *N. caninum* for longer time after infections.

Antibody presence in ewes does not provide conclusive diagnosis of *Neospora* in fetuses thus must be confirmed by analysis by other techniques such as PCR to detect the DNA of the parasite as well as histology to demonstrate protozoan associated lesions and/or parasite antigens.

In this study, we used the PCR technique to detect *N. caninum* in aborted ovine fetuses and prefer it on serological or other tests as Stuart, et al., and Timothy, et al., considered that molecular methods are more sensitive and specific than histopathology and immunohistochemistry tests and less affected by postmortem changes. Yamage, et al., were the first to compare the sensitivity and specificity of different primers for the diagnosis of *N. caninum*. So in our study PCR technique was used for the detection of *N. caninum* DNA in aborted fetal tissues. An investigation was done for (89) sheep flocks that suffered from abortions and neonatal deaths by molecular analyses of (89) fetuses for the possibility of the existence of *Neospora caninum* in these flocks [17-20].

In our study (6) of the (89) of the aborted fetus samples, about 6.74% were tested positive for *N. caninum* using PCR. Similar results were found by Moreno, et al., in Spain by using PCR which was 6.8% (5/74) of ovine abortions by *N. caninum* infection. Another study conducted by Al-Shael et al., in Wasit province found that (51) ovine placental samples (13.73%) tested positive for *N. caninum* DNA by using the ITS gene combined with histopathological examinations.

On the other hand, lower results were detected in Iran by Khodadi, et al., in Urmia which was 2.3% PCR positive for *N. caninum* DNA in 130 examined ovine fetuses, while in another study conducted in Iran-Mazandaran by Amoue, et al., it was found that between 70 aborted sheep, goats, and cattle fetuses samples, 4 of them were positive with a rate of 5.7% (23, 24). In addition, by using real time PCR in Germany, Meixner, et al., between 200 aborted and stillbirth fetal placental tissues found 7 samples (3.5%) were positive for *N. caninum*. Amir Abdoli, et al., found *N. caninum* in sparrows in Iran 3.68% (8/217) of sparrows by PCR by *Nc5* gene.

Higher results were detected in Iran in a study conducted by Ramzi and Nasiri that collected samples from 71 brains of aborted fetuses, *N. caninum* DNA was detected in seven (9.8%) samples. Another study was done by Asadpour, et al., in Iran by using the *Nc5* gene for aborted ovine fetuses placental tissues, the *N. caninum* DNA was detected in 8.5% out of (70) samples, while by using serological tests they detected this protozoan in only 5.8% out of 70 serums, they improved PCR superiority on serological tests. Hughes, et al., in a study from 2005 in the UK, found 18% positive records in aborted lambs by using brain tissues and nested PCR, they confirmed nested PCR is more sensitive by 5 folds and brain tissue is preferable to other tissues from aborted lamb have.

The number of infected animals depends on risk factors like direct contact between dogs, goats and sheep which are usually housed together and can contaminate the environment with *N. caninum* oocysts. This case can cause horizontal transmission of oocysts to sheep and goats through food and water contaminated with sporulated oocysts. Other risk factors are the numbers and density of dogs in an area for example urban area with a greater number of dogs can be infected by the ingestion of oocysts and may increase levels of infection, whereas in rural areas with fewer dogs, the vertical transmission may be more significant than horizontal transmission route. There are other factors rather than parasites like physical chemical and biological factors that cannot be completely ruled out. High temperature and humidity favor faster sporulation and enhanced survival of *N. caninum* oocysts in the environment. Some reports suggested that imported animals have a greater neosporosis risk than local breeds.

Multiple sequence alignment and phylogenetic tree analysis of *Nc5* sequence between nine positive samples and (Garmian-2020 isolate) sequences. Phylogenetic tree showing 98.4% association of the (Garmian-2020 isolate) with two Iranian *N. caninum* isolates (MT955656.1) and (MT709296.1), they are geno typically related to each other, which may the long border and large trade relation between Iraq and Iran, especially the import of sheep and animal products, and movement of carnivores between these two countries. To our knowledge, in our region Garmian district in Sulaimani province, this is the first molecular detection of *N. caninum* DNA in sheep that suffered from abortions with the risk of a contaminated environment where dogs could disseminate the

oocysts, or they could have been infected through the transplacental transmission of the parasite. Flocks were negative for other *Brucella, Chlamydia, Toxoplasma*, and *Campylobacters*, data was not shown.

According to our studies, *N. caninum* is considered one of the causative agents of abortions in our region and molecularly detected by conventional PCR for treatment and control of abortions we demonstrate the ability to use PCR technique to identify *N. caninum* infection in aborted fetuses, Our results showed that infection with the *N. caninum* may lead to fetal mortality, and economic loss.

CONCLUSION

This is the first report on the molecular detection of *N. caninum* in aborted sheep in the Garmian region in Iraq. Further studies are needed to confirm these molecular findings. Based on our study we advise preventing dogs and other canids from eating placentas and aborted fetuses for ovine. This work was financially supported by the Garmian veterinary directorate ministry of agriculture and water resource Iraqi Kurdistan region.

ACKNOWLEDGMENTS

Thanks to all people who help as to finish this research especially the employee at our department and our colleagues at the Garmian veterinary laboratory.

CONFLICT OF INTEREST

"The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript."

REFERENCES

1. Amir K, Seifi HA, Movassaghi AR (2014) Histopathological and molecular study of *Neospora caninum* infection in bovine aborted fetuses. Asian Pac J Trop Biomed. 4(12):990-994.
2. Fernandez EC, Zaballos A, Garcıa GA, Luis M, Mora O (2002) Quantitative detection of *Neospora caninum* in bovine aborted fetuses and experimentally infected mice by real-time PCR. J Clin Microbiol. 40(4):1194-1198.
3. Sasan F, Javanbakht J, Seifori P, Fathi S, Hassan MA (2013) *Neospora caninum* as a causative agent of ovine encephalitis in Iran. Pathol Disco. 2013:1-5.
4. Portella LP, Cadore GC, Sangioni LA, Alves MEM, Chemeris R, et al. (2016) Molecular detection of protozoa of the Sarcocystidae family in sheep from the state of Rio Grande do Sul, Brazil. Cienc Rural. 46(9):1613-1617.
5. Jitender PD, Lindsay DS (2006) Neosporosis, toxoplasmosis and sarcocystosis in ruminants. Vet Clin North Am Food Anim Pract. 22:645-671.
6. Arbabi M, Abdoli A, Dalimi A, Pirestani M. Identification of latent neosporosis in sheep in Tehran, Iran by polymerase chain reaction using primers specific for the Nc5 gene. Onderstepoort J Vet Res. 83(1):1-7.
7. Filho NADC, Oliveira PAD, Oliveira FCD, Pappen FG, Aguiar CLG, et al. PCR based identification of *Neospora caninum* in the umbilical cord of a newborn calf in Brazil. Cienc Rural. 2017;47(7):1-7.
8. Al-Jomaily AIA, AL-Rubaie HMAS. Study the prevalence of *Neospora caninum* in serum and milk of sheep in Al-Fallujah city. Al-Anbar J Vet Sci. 6(1):114-118.
9. AL-Farwachi M, AL-Badrani B, AL-Khafaji W (2012) Serodiagnosis of ovine neosporosis in Mosul city, Iraq. Eurasian j vet sci. 48(4):190-193.
10. Buxton, D (1998) Protozoan infections (*Toxoplasma gondii, Neospora caninum, and Sarcocystis* spp.) in sheep and goats: Recent advances. Vet Res. 29(3-4):289-310.
11. Asadpour R, Joozani JR, Salehi N (2013) Detection of *Neospora caninum* in ovine abortion in Iran. J Parasit Dis. 37(1):105-109.
12. Hassig M, Sager H, Reitt K, Ziegler D, Strabel D, et al. (2003) *Neospora caninum* in sheep: A herd case report. Vet Parasitol. 117:213-220.
13. Warleta MG, Hermida JAC, Cerrillo JR, Benavides J, Garcia GA, et al. (2014) *Neospora caninum* infection as a cause of reproductive failure in a sheep flock. Vet Res. 45(88):1-9.
14. Norbert M, Verena Z, Brigitte H, Bruno G (1996) Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA Hybridization Immunoassay. J Clin Microbiol. 34(11):2850-2852.
15. Al-Shaeli SJ, Ethaeb AM, Gharban AJH (2020) Molecular and histopathological identification of ovine neosporosis (*Neospora caninum)* in aborted ewes in Iraq. Vet World. 13:597-603.
16. Ali AI, Al-Rubaie HAA (2020) Molecular diagnosis of *Neospora caninum* brain tissues of local breed domesticated chickens (*Gallus gallus domesticus*) at AL-Fallujah district, IRAQ. Plant Archives. 20:7-10.
17. Ghattof HH, Faraj AA (2015) Seroprevalence of *Neospora caninum* in goats in Wasit province Iraq. Int J Curr Microbiol Appl Sci. 4(7):182-191.
18. Jung BH, Lee SH, Kwak D (2014) Evidence of *Neospora caninum* exposure among native Korean goats *(Capra hircus coreanae)*. Vet Med. 59(12):637-640.
19. Stuart P, Zittl A, Waal TD, Mulcahy G, Hawkins C, et al. (2012) Investigating the role of wild carnivores in the epidemiology of bovine neosporosis. Parasitol. 140(3): 296-302.
20. Baszler TV, Gay LJC, Long MT, Bruce A (1999) Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. J Clin Microbio. 37(12): 4059-4064.