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Diagnosis of *Neospora caninum* in Bovine Fetuses by Histopathology, ELISA, Conventional and Real Time TaqMan Probe PCR

Amininajafi F[*1], Sasani F[2] and Nazemshirazi MH[2]

[1]Department of Pathology, Faculty of Veterinary Medicine, Tehran, Iran

[2]Private Veterinarian, Tehran, Iran

*** Corresponding Author**

Amininajafi F, Department of Pathology, Faculty of Veterinary Medicine, Tehran, Iran, Tel: +989123256121, E-mail: vadieamini@yahoo.com

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Abstract

Bovine neosporosis is caused by *Neospora caninum*, a protozoan parasite that causes lifelong infection. Abortion is often the only observed sign of disease. There is no effective treatment and vaccine efficacy is questionable Infection with *N. caninum* can result in early embryonic death, abortion, stillbirth or a weak, abnormal calf. *Neospora caninum* is a protozoon that was originally described as a parasite in dogs, in which it causes myositis and encephalitis. Bovine neosporosis is now recognized as a major cause of spontaneous abortion in cattle. It is highly suspected on 20% of the farms with repeated abortions and a cow that is seropositive for *Neospora caninum* has a threefold greater risk of aborting than a cow that is Neospora-negative. Neospora is responsible for 21% of spontaneous abortions occurring in an individual animal. This percentage rises to 33% for the herd as a whole. Verticle transmission is the rule (at least 80% of the calves born to seropositive cows are infected). Serotesting before the calf's first colostrum intake will reveal prenatal infection.

Keywords: Pathology

Introduction

N. caninum infections have been identified from most parts of the world indicating that 12-45% of aborted fetuses from dairy cattle are infected with the *N. caninum*. Exposure is common in United States dairy farms ranging from 16% -36% of dairy cows testing positive on serum. A classic feature of the disease is abortion at four to six months of pregnancy. Cows would be infected with this parasite in Horizontally rout by contaminated feed and water with eggs from infected dogs and other canids or vertical transmission from cow to the fetus during gestation and then this infection would be maintained as a life-long infection. Once infected, a cow can pass the organism through the placenta to her calf in every pregnancy throughout her lifetime. In some pregnancies, this fetal infection may result in abortion or weak calves. However the vast majority (95%) of calves born with the infection ("congenitally infected") from positive dams are absolutely normal but remain infected for life. A heifer calf born with the infection can transmit the infection to the next generation when she becomes pregnant, thus maintaining the infection within the herd. Vertical transmission is known to be the main rout of transmission in cattle but both horizontal and vertical transmission is necessary for parasite survival.

Abortion is thought to happen by direct fetal and placental destruction and/or the placental damage may cause discharge of maternal PGs that cause luteolysis and abortion.

Perfect identification of abortion is through finding of the *N. caninum* organism in the fetal tissues, most reliably the fetal brain. Control is based on removing positive animals, avoiding entry of infected replacements into the herd, and preventing likely methods of horizontal infection.

Materials and Methods

Clinical samples

One hundred and five samples from bovine abortions were sent to the Kowsar laboratory complex, from January 2018 to May 2019. Samples were screened for *N. caninum* in the brain, heart, kidney, liver, lung, spleen, thymus, and placenta, using Histopathology, ELISA, conventional, PCR and Real Time PCR. Samples did not include all organs from every fetus, and not all samples received were suitable for analysis.

ELISA Test

In order to determining whether neosporosis is a potential reproductive problem in cattle farms the breeding herd should be tested and daughters of seropositive dams should be excluded as replacement heifers and only negative replacement heifers should be purchased.

According to previous reports ID vet Indirect ELISA kit for the detection of anti-Neospora caninum antibodies in serum or milk has .showed excellent Se and Sp values (>95%).

Basically, 50 ml of sera samples were incubated in the wells coated with a sonicate lysate tachyzoites specific for N. caninum. Serum sample from the exact bovine that they aborted collected serum was removed after centrifugation at 1000 g for 10 min and they were stored at-20 C until tested. They were analyzed for detection of specific N. Caninum antibodies by indirect Elisa (using commercial elisa kit (ID. VET, FRANCE)). All sera were tested for the presence of anti-Neospora IgG using ID Screen *N. caninum* Indirect ELISA (IDVet, Montpellier, France). Samples were tested according to the manufacturer's instruction.

Histopathology

Histopathologic findings are somewhat more variable. Reported microscopic lesions include hepatic necrosis and military hepatitis, granulomatous splenitis, granulomatous hepatitis, myositis, splenic lymphoid, myocarditis, gastroenteritis, and reticular cell hyperplasia, bronchopneumonia, and meningoencephalitis, with intralesional tachyzoites often evident in areas of inflammation.

The most characteristic histopathologic lesion of protozoal encephalomyelitis is a multifocal nonsuppurative variable necrotizing meningoencephalomyelitis with tachyzoites and tissue cysts occasionally identified in neurons and neuropil.

Tissues were fixed in 10% buffered formalin, embedded in paraffin, cut at 4 µm, and stained with hematoxylin and eosin (HE) for routine histology.

Polymerase chain reaction

To determine if neosporosis is affecting the herd test all cases of abortion. In this regard the genomic DNA was extracted from fresh and frozen tissues, using a commercial kit (High Pure PCR template preparation kit from Roche), following the manufacturer's instructions. Extracted DNA were stored at -20 °C until the completion of the conventional -PCR.

DNA extraction

Brain sample was taken from aborted fetus as target sample. DNA extraction was performed with the High Pure PCR template

preparation kit from Roche (Germany). About 25 to 50 mg of each brain sample, 40 µl of proteinase K and 200 µl Binding Buffer were added and incubated at 70 °C until complete lysis. Then 100 µl of Isopropanol added. This was mixed and then transferred to one High Pure Filter Tube inserted to one Collection Tube. Add 500 µl Inhibitor Removal Buffer to the upper reservoir of the Filter Tube and centrifuged 1 min at 8,000 × *g*. washed twice with 500 µl Wash Buffer and Centrifuged 1 min at 8,000 × *g*. Centrifuge the entire High Pure assembly for an additional 10 s at full speed centrifuged to ensures removal of residual Wash Buffer. The DNA was eluted in 200 µl Elution Buffer and stored at −20 °C.

Conventional PCR

Nc gene selected as the target gene for Neospora amplification and detection. PCR was performed in a 25- µl mixture containing 2.5 µl of 10x buffer (Qiagen.);0.2 mM dNTP (Qiagen); 10 pmol each of specific primers (Table 1) and 2.5 U of Hot Start Taq DNA polymerase (Qiagen); Five min incubation for denaturation of the DNA at 95°C. Amplification was done in Applied Biosystems SimpliAmp Thermal Cycler using 35 cycles with denaturation (94 °C; 1 min), annealing (63 °C; 1 min), and primer extension (74 °C; 3.5 min). After the last cycle, a primer extension was continued for 10 min at 74 °C, Amplification products were analyzed by electrophoresis through a 2% agarose gel and analysed by Gel documentation apparatus (Bio-Rad). These primers had been designed to amplify a 337-bp sequence of the Nc5 region of N. caninum.

Real time PCR

DNA was tested by real-time PCR with dual labeled probes (FAM-BHQ1) on a LightCycler 96 (Roche,).

Real Time PCR Primers and probe were selected based on S. De Craeyea, (Table 1 for primers and probes). Each PCR reaction contained 5 µl of extracted DNA, mixed with 20 µl of a PCR master mix containing 12.5 µl of PowerMix (Qiagen, Germany), 0.5 µl of each primer (Bioneer, South Korea) at a concentration of 10µM and 0.5 µl dual labeled probe at a concentration of 2 µM. Initial denaturation and activation of the Taq polymerase at 95 °C for 15 min; followed by 45 cycles including denaturation at 95 °C for 20 s and annealing &extension [60 °C, 60s]; ramp rates in all cycle steps were 4 °C/s). Fluorescence was measured at the end of each annealing phase in the "single" mode. Quantitation of PCR products was achieved by plotting the fluorescence signals versus the cycle numbers at which the signals crossed the baseline. Positive samples were identified by a fluorescence signal which accumulated to values above the baseline within 45 cycles of reaction.

Primer	Sequence	Target gene
Np21	CCC AGT GCG TCC AAT CCT GTA AC	Nc gene
Np6	CTC GCC AGT CAA CCT ACG TCT TCT	Nc gene
N. caninum Forward primer	GAGAATGAGAGCGATTTCCAG	Nc gene
N. caninum Reverse primer	CTCCTGAAGTCCCAGCGA	Nc gene
N. caninum probe	FAM-CCTTCTGAGTCGGGTTGTGTTTGG-BHQ1	Nc gene

Table 1: PCR Primers and probe were selected based on S. De Craeye

Results and Discussion

In the present study convenience sampling was used, and the diagnosis of abortion by *N. caninum* was based on the presence of Histopathology, ELISA, conventional PCR and Real Time PCR.

Elisa was used as an initial screen for indication of infectious abortion and the second was histopathology and PCR, A mononuclear inflammatory infiltrate was predominant in the heart, kidney, liver, lung, brain, and placenta.

According to Dubey, Lindsay, and Speer (1998) [1], tachyzoites or cysts of *N. caninum* are rarely sufficiently numerous to be found in all histological sections from an animal, requiring the use of specific and sensitive tests to detect the parasite. For this reason, histopathology should be interpreted as complementary to tests such as Elisa, conventional -PCR and Real Time PCR which identify the causative agent.

In the Elisa test the result indicates in the table below: .

The number of animals tested	No. of positive	Seroprivalence (%)
105	36	

caninum causes mononuclear inflammatory infiltrate in various organs and focal necrosis with mononuclear infiltrate in the brain (PESCADOR et al., 2007) [2]. In the present study, these results are consistent with other studies showing that the brain is the organ of choice for the detection of the parasite, since it is the most often affected, and any part of it can be used for histological examination (DUBEY; LINDSAY, 1996; DUBEY, 2003; PESCADOR et al., 2007) [2,3]. In this case, abortion could have been due to vertical transmission of the parasite (DUBEY, 2003; DUBEY; SCHARES; ORTEGA-MORA, 2007) [3,4]. Sondgen et al. (2001) [5] believe that in some cases, the infection by *N. caninum* can lead to fetal death before lesions develop.

Non-suppurative lesions were also present in the lung, liver, kidney, and placenta, indicating that *N. caninum* infection is systemic and can reach various tissues and fetal annexes.

Due the similarity between *N. caninum* and other coccidia (*Toxoplasma gondii* and *Hammondia heydorni*), molecular characterization has been performed with sequencing of ITS1 DNA, allowing inter- and intra-species differentiation. The study by Gondim et al. (2004b) [6] showed that the sequencing of ITS1 can be applied as a complementary tool in the identification of species and strains of *Neospora*. Several studies have shown that the ITS1 region is a good marker for distinguishing members of the family Sarcocystidae (HOLMDAHL; MATTSON, 1996; PAYNE; ELLIS, 1996; MONTEIRO et al., 2007; BARRATT et al., 2008) [7,8,9,10]. The use of the ITS1 DNA region ensures the specificity of the test in the detection of *N. caninum* and BLAST analysis confirmed that the primers of the ITS1 region of the conventional -PCR JB were specific to this parasite, so no cross reaction occurred with other coccidians. The sequence of primers from the Nc5 region is not found in the genomes of *T. gondii, Sarcocystis capracanis, S. cruzi, S. miescheliana, S. moulei, S. tenella*, or *Hammondia hammondi* (YAMAGE; FLECHTNER; GOTTSTEIN, 1996; HUGHES et al., 2006) [11,12], showing that PCR PLUS has analytic specificity, too. Conventional PCR PLUS detected 22.2% (16/72) of histologically positive brain samples. This result is similar to that obtained by GOTTSTEIN et al. (1998) [13], who also used primers from the Nc5 region (genomic DNA) and detected *N. caninum* DNA in the brain of 29% of 83 aborted bovine fetuses in Sweden using conventional -PCR. Paula et al. (2004) [14] employed hemi- conventional -PCR, using primers of the Nc5 region, and found *N. caninum* in 31.3% of 32 samples of frozen brain tissue from aborted fetuses in Brazil.

Conventional -PCR JB detected 6.9% infected brain samples, a lower value than that published by Medina et al. (2006) [15] in Mexico, who also used conventional -PCR (primer of the ITS1 region of ribosomal DNA) and found a rate of 80% positivity in samples of infected brain tissue of aborted fetuses from dairy herds.

The rate of fetal infection with *N. caninum*, represented separately by each of the diagnostic techniques, was 8.6% with Elisa, 20.9% with Real Time PCR and 6.7% with conventional -PCR. It is important to evaluate agreement among the techniques to interpret the value of these results.

Comparing histopatology with Real Time PCR, the 0.3 kappa coefficient (confidence interval 0.079 to 0.526) was consistent with a low degree of agreement, and between Real Time and conventional -PCR, the 0.19 kappa coefficient (confidence interval - 0.105 to 0.4830) indicated no significant agreement. Sager et al. (2001) [16] also reported a weak correlation between PCR and Real Time in 47 cases shown positive by Real Time PCR. Five of these cases showed no lesions consistent with those caused by *N. caninum*, and none of these cases were positive with PCR. Molecular techniques detect DNA in small quantities of fetal samples, whatever their condition, whether mummified or in varying degrees of autolysis.

Conventional -PCR detected fetal infection at lower rates, 6.7%, when compared with Real Time PCR, 20.9%, and the kappa coefficient of 0.35 (confidence interval 0.126 to 0.57) confirmed a low agreement. As the protocol for extraction of DNA was the same in both techniques, the lower threshold of detection of nested-PCR compared to the Real Time may be related to the primers used and to the gene region amplified.

The low values of kappa coefficients among the tests show that the techniques are complementary and demonstrate the need to calculate the rate of fetal infection by taking into account at least one positive result for each of the techniques. Thus, 24.8% of samples were positive for at least one of the techniques (Real Time PCR, conventional -PCR, or Histopathology). Result for all three techniques. Pereira-Bueno et al. (2003) [17] also concluded that it is necessary to use more than one diagnostic method to increase the probability of detection of the parasite in aborted fetuses. Baszler et al. (1999) [18] suggested a protocol for the routine diagnosis of *N. caninum* in aborted bovine fetuses employing histopathology and the identification of the parasite by PCR and histopathology, as in the present work.

Another factor that may be contributing to discrepancies in

the detection rate of techniques is the tissue sampling. When fragments were trimmed out, one for histopathology and another for molecular analysis, the parasite may have been present in one portion, but not in the other.

With respect to PCR, a possible explanation for the non-detection of DNA in some fetal organs shown positive with Histopathology may be degradation of DNA by autolysis (SHIBATA, 1994) [19]. Freezing does not affect DNA, and conventional -PCR is thus a useful tool when tissues are frozen, whereas it complicates histological analysis.

The diagnosis of abortion due to *N. caninum* requires detection of the parasite through techniques that are specific, such as Histopathology, or ones such as PCR that are both specific and more sensitive, preferably associated with identification of histopathological lesions (SAGER et al., 2001) [16]. Efforts to collect organs that are as fresh as possible, including placenta and, importantly, fetal brain, should be encouraged to increase the success of the definitive diagnosis of neosporosis by tests of cnventional -PCR and Real Time PCR [20-47].

Prevent introduction of disease

- Infection is not transmitted between cattle but can be introduced by exposure to infected canine feces
- Prevent canine access to food and water sources for cattle
- Promptly dispose of all aborted tissues to prevent access by canines

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