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MUSTAFA AÇICI

CENK SONER BÖLÜKBAŞ

GÖKMEN ZAFER PEKMEZCİ

HANDE GÜRLER

OKTAY GENÇ

See next page for additional authors

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Authors

MUSTAFA AÇICI, CENK SONER BÖLÜKBAŞ, GÖKMEN ZAFER PEKMEZCİ, HANDE GÜRLER, OKTAY GENÇ, ALİ TÜMAY GÜRLER, SELMA KAYA, and ŞİNASİ UMUR

A diagnostic survey of *Neospora caninum* infection in aborted fetuses in the Middle Black Sea Region and Sivas Province, Turkey

Mustafa AÇICI^{1*}, Cenk Soner BÖLÜKBAŞ¹, Gökmen Zafer PEKMEZCİ², Hande GÜRLER³,
Oktay GENÇ⁴, Ali Tümay GÜRLER¹, Selma KAYA⁵, Şinasi UMUR¹

¹Department of Parasitology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey

²Department of Preclinical Sciences, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey

³Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey

⁴Department of Microbiology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey

⁵Department of Parasitology, Samsun Veterinary Control Institute, Samsun, Turkey

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Abstract: *Neospora caninum* is a major, cosmopolitan, protozoan parasite associated with abortion in cattle and water buffaloes. In this study, real-time PCR was used for the detection and quantification of the burden of *N. caninum* infections in aborted fetuses in the Middle Black Sea Region and Sivas Province of Turkey. In total, *N. caninum* DNA was detected in 44 of the 89 (49.4%) aborted fetuses of the cattle and water buffalo from 89 different farms. The authors conclude that the pervasiveness of neosporosis is a threat to cattle breeding in the Middle Black Sea Region and Sivas Province. Real-time PCR testing gave consistently reliable results, which suggests that this procedure can be used for the molecular diagnosis of abortion cases in bovine livestock.

Key words: Turkey, abortion, cow, water buffalo, neosporosis

1. Introduction

Neosporosis is recognized as a leading cause of reproductive failure in bovine herds [1]. The disease is caused by *Neospora caninum* [2], an obligate intracellular protozoan, which is widely distributed and causes substantial economic losses to producers [1,2]. Infection occurs horizontally via oocysts shed by dogs as the definitive host or vertically by transplacental infection which is important in dairy cows; up to 95% of newborn calves can be infected with *N. caninum* [1,3,4].

Since the identification of *N. caninum* [5], numerous scientific papers have been published on its effects on cattle and water buffaloes worldwide [4,6,7]. The presence of *N. caninum* in cattle and water buffaloes has frequently been confirmed in Turkey by using serological techniques [8,9,10,11]. Furthermore, the seroprevalence of neosporosis can reach 97.5% in dairy cattle herds [3] and abortions in cattle may be sporadic, endemic, or epidemic [12]. Congenitally infected cows have a 7.4-fold higher risk of abortion during the first pregnancy and a 1.7-fold higher risk of aborting the first pregnancy during their first lactation than noninfected cows [13]. Epidemic abortions are thought to be due to a primary infection of naive dams,

and abortions in 3- to 7-year-old cows occurred between 4 and 8 months of gestation [14,15].

Annual economic losses in the dairy and beef industries in New Zealand that were attributable to neosporosis were estimated to be one billion dollars [2].

There have been a few molecular studies on bovine neosporosis in parts of Turkey [11,16,17]. In Turkey, seropositivity for *N. caninum* in cattle in Kars was 8.2% with the use of ELISA [8], 7.01% with cELISA in the Eastern Anatolian region [9], 37.7% with ELISA in some parts of Turkey [11], 35.07% with IFAT in different parts of Turkey [18], 32.35% with cELISA in different parts of Turkey [19], and 7.2% with cELISA in Kars [20].

Against that background, the objective of the present study was to investigate cases of *N. caninum* infection in aborted bovine fetuses in the Middle Black Sea Region and Sivas Province of Turkey through the quantification of the burden of *N. caninum* DNA in fetal tissues by using real-time PCR.

2. Materials and methods

2.1. Study area and sampling

The study was conducted on aborted cattle and water buffalo fetuses selected randomly from farms in the

* Correspondence: acicim@omu.edu.tr

Middle Black Sea Region of Turkey; namely Samsun Province (Alaçam, Asarcık, Çarşamba, the central district, Bafra, Havza, Kavak, Ladik, Salıpazarı, Tekkeköy, Terme, Vezirköprü), Sinop Province (the central district, Gerze), Tokat Province (Almus, the central district, Erbaa, Reşadiye, Sulusaray, Turhal, Yeşilyurt) and Giresun Province (Şebinkarahisar), and also Sivas Province (the central district, Hafık, Zara), from March 2012 to March 2014. The numbers of cattle and water buffaloes at risk of infection on farms in the study area are shown in Table 1. In total, 89 aborted fetuses from 88 dairy cattle and a

water buffalo that originated from 89 farms with herd sizes ranging from 12 to 28 animals, were examined with real-time PCR for *N. caninum* infection (Figure).

2.2. Preparation of fetal tissues

Fresh fetal tissues, including brain, spleen, liver and lung tissue, and amniotic fluid and fetal membranes, were aseptically obtained during necropsy. An equal amount of sample of each organ of the fetus was pooled and homogenized using a Silent Crusher M (Heidolph®, Schwabach, Germany). A 25-mg portion of each mixture was poured into a sterile vial and stored at -20 °C until DNA extraction.

Table 1. Number of cattle and water buffalo farms naturally infected by *N. caninum* in the Middle Black Sea Region and Sivas Province.

| Province | County | No. of farms | No. of animals at risk | No. of aborted fetus | |
|----------|------------------|--------------|------------------------|----------------------------|----------------------------|
| | | | | <i>N. caninum</i> positive | <i>N. caninum</i> negative |
| SAMSUN | Alaçam | 1 | 22 | 1 | - |
| | Asarcık | 1 | 12 | 1 | - |
| | Bafra | 7 | 94 | 3 | 4 |
| | Central district | 1 | 12 | - | 1 |
| | Çarşamba | 2 | 53 | 1 | 1 |
| | Havza | 13 | 247 | 6 | 7 |
| | Kavak | 9 | 111 | 5 | 4 |
| | Ladik | 4 | 74 | 3 | 1 |
| | Salıpazarı | 3 | 46 | 1 | 2 |
| | Tekkeköy | 1 | 13 | 1 | - |
| | Terme | 5 | 95 | 2 | 3 |
| SİNOP | Central district | 2 | 23 | - | 2 |
| | Gerze | 1 | 12 | - | 1 |
| GİRESUN | Şebinkarahisar | 1 | 17 | 1 | - |
| TOKAT | Almus | 2 | 32 | 1 | 1 |
| | Central district | 6 | 129 | 5 | 1 |
| | Erbaa | 1 | 18 | 1 | - |
| | Reşadiye | 1 | 27 | 1 | - |
| | Sulusaray | 2 | 43 | 1 | 1 |
| | Turhal | 3 | 57 | 1 | 2 |
| | Yeşilyurt | 5 | 148 | 2 | 3 |
| SİVAS | Central district | 3 | 84 | 1 | 2 |
| | Hafık | 2 | 53 | - | 2 |
| | Zara | 5 | 137 | 1 | 4 |
| | | 1 | 10* | 1 | - |
| TOTAL | | 89 | 1667 | 44 | 45 |

*:Water buffalo

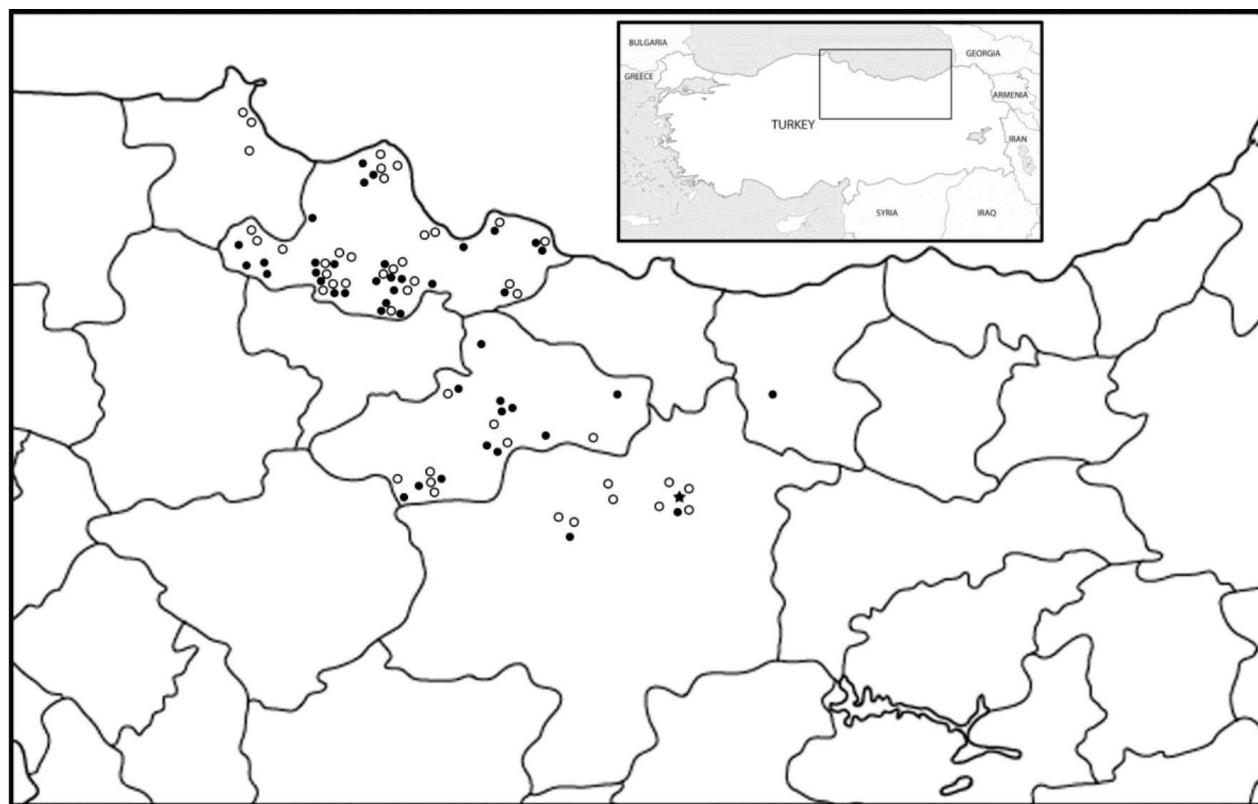


Figure. The locations of farms in Turkey from which aborted fetuses were collected for *N. caninum* examination. Black dots show the locations from which samples that were positive for *N. caninum* DNA were collected; • cattle, * water buffalo.

2.3. Genomic DNA extraction and real-time PCR analysis

The genomic DNA was extracted from 25 mg of the particular homogenized fetal tissue using a DNA purification kit (PureLink Genomic DNA Kit, Invitrogen®, Waltham, MA, USA), according to the manufacturer's instructions. The extracted DNA was then transferred into a clean, nuclease-free microcentrifuge tube and stored at -20°C for further analysis.

The genomic DNA extracted from each sample was analyzed with a quantitative, real-time PCR kit (Genesig *Neospora caninum* Nc5 marker genomic sequence kit, PrimerDesign Ltd®, Southampton, UK), according to the manufacturer's instructions. The quantification kit for *N. caninum*, which was designed to amplify a region of the parasite's genome, was used. For copy number determination and as a positive control for the PCR setup, the kit contains a positive control template and also a negative control template. Standard curve dilution series was prepared using the positive control templates (2×10^5 , 2×10^4 , 2×10^3 , 2×10^2 , 20 per μL). These series were used to generate a standard curve of *N. caninum* copy number/Cq value. All samples were run in a Rotor-Gene Q (Qiagen®, Venlo, the Netherlands) thermal cycler. During the performance of PCR, positive and negative control

samples were introduced to validate the accuracy of the PCR process. The details of the cycles for the PCR reaction were as follows: 95°C for 15 min, followed by 50 cycles of a denaturing step at 95°C for 10 s, and an annealing/extension step at 60°C for 60 s.

Rotor-Gene Q Series Software 2.0.2 was used for data analysis. The cycle threshold value (CT), standard curve, and threshold were determined. After reaching the threshold, the sample was considered positive.

2.4. Statistical analysis

DNA samples were tested in triplicate. For statistical analysis, the chi-square test of the SPSS v. 20.0 (IBM, Armonk, NY, USA) was employed. Differences between groups were considered statistically significant if $P < 0.05$.

3. Results

In total, *N. caninum* DNA was detected in 44 of 89 (49.4%) of the aborted fetuses tested. Forty-three of the infected fetuses (48.9%) were from cattle and one was from a water buffalo in Sivas Province (Figure). Real-time PCR analyses showed that the prevalence of *N. caninum* DNA in aborted cattle fetuses was distributed as follows: 2/10 (20%) in Sivas Province, 28/54 (51.9%) in Samsun Province, and 12/20 (60%) in Tokat Province. There were no cases of infection

recorded in Sinop Province and only 1 of 17 fetuses (5.9%) in Giresun Province was infected. Abortions occurred in animals up to the age of 7 years (Table 2). The periods of occurrence of abortion of the fetuses infected by *N. caninum* in descending order were as follows: 50% (22/44) between 6 and 7 months of gestation, 34% (15/44) between 8 and 9 months of gestation, and 16% (7/44) between 3 and 5 months of gestation (Table 3). There were significant differences between the periods of abortion for the number of fetuses infected by *N. caninum* DNA ($P < 0.05$). Separately, *N. caninum* infections were detected in different cattle breeds (Table 3) but no significant differences were observed between the breeds for the proportion of fetuses infected ($P > 0.05$). In addition, the quantity of *N. caninum* DNA in positive samples of the aborted fetuses from cattle ranged between 2 and 343,400,546 copies/mL and the water buffalo fetus had 17,548 copies/mL.

4. Discussion

The diagnosis of neosporosis is difficult in bovine abortion cases. The detection of *N. caninum* DNA in aborted fetal material is not sufficient for confirmation that it is responsible for reproductive failure because it is possible that other potential aborting agents may also be present. However, molecular analysis methods are more sensitive than serological methods in differentiating abortion materials and genital tract pathogens [1]. There have been several studies on the seroprevalence of neosporosis in cattle in Turkey [21,22,23] and also few molecular and histopathological studies specifically done on neosporosis in aborted tissues [16,17].

In the present study, 43 of 88 (48.9%) aborted fetuses from cattle and a water buffalo in the Middle Black Sea

Table 2. The number of aborted fetuses per breed and age group of the parent cows in the Middle Black Sea Region and Sivas Province.

| Breeds | Age group | | | | TOTAL |
|-----------------------|-----------|-----|-----|----|-------|
| | 2-3 | 4-5 | 6-7 | >7 | |
| Brown Swiss | 8 | 5 | 6 | 1 | 20 |
| Brown Swiss crossbred | 7 | 11 | 8 | - | 26 |
| Simmental | 6 | 3 | 2 | 1 | 12 |
| Simmental crossbred | 2 | - | 1 | - | 3 |
| Holstein | 2 | 7 | 6 | 2 | 17 |
| Holstein crossbred | 1 | 1 | 1 | - | 3 |
| Jersey | - | - | - | 2 | 2 |
| Jersey crossbred | - | - | 1 | 1 | 2 |
| Anatolian Black | 2 | 1 | - | - | 3 |
| Water buffalo | 1 | - | - | - | 1 |
| TOTAL | 29 | 28 | 25 | 7 | 89 |

Region and Sivas Province of Turkey were infected with *N. caninum*. Although there is little known of the role of *N. caninum* in causing abortion, the water buffalo is a natural host [24]. Neosporosis does cause abortion in water buffalo [25] but few studies have reported the reasons for the abortion of buffalo fetuses [4,7]. Exposure to *N. caninum* infection appears to be at least three times higher in water buffalo than in cattle [26]. Separately, the abortion risk due to *N. caninum* infection has been shown to be higher in heifers than in subsequent gestations [3]. In the present study, the aborted fetuses infected by *N.*

Table 3. Period of abortion of the aborted fetuses infected by *N. caninum*.

| Breeds | Abortion period (days) | | | TOTAL |
|-----------------------|------------------------|----------|----------|-------|
| | 90-150≤ | 180-210≤ | 240-270≤ | |
| Brown Swiss | 2 | 4 | 5 | 11 |
| Brown Swiss crossbred | 1 | 8 | 3 | 12 |
| Simmental | 1 | 2 | 2 | 5 |
| Simmental crossbred | - | - | 2 | 2 |
| Holstein | 1 | 4 | 1 | 6 |
| Holstein crossbred | - | 2 | - | 2 |
| Jersey | - | - | 1 | 1 |
| Jersey crossbred | 1 | - | 1 | 2 |
| Anatolian Black | 1 | 1 | - | 2 |
| Water buffalo | - | 1 | - | 1 |
| TOTAL | 7 | 22 | 15 | 44 |

caninum were from dairy cows aged between 2 and 7 years and from a single water buffalo aged 3 years. *N. caninum* infection was most commonly recorded in the fetal tissues of aborted fetuses from dairy cattle aged between 2 and 5 years and in the midgestation stage. In addition, there were more aborted fetuses infected by *N. caninum* from Brown Swiss cattle and their crossbreds than the other breeds in the study area (Table 3). However, there were no significant differences between the cattle breeds with respect to the incidence of infection with *N. caninum* ($P > 0.05$). The abortion of the other 51.1% of fetuses, i.e. those not infected with *N. caninum*, may be attributable to various genetic, microbial, and metabolic factors. Nevertheless, 48.9% of the aborted bovine fetuses examined in the current study were determined to be infected with *N. caninum*. This proportion was much higher than the 15.3% reported from aborted bovine fetuses in Spain using PCR [27] but was much lower than the 80% determined in Mexico with nested PCR [28].

Serological studies on *N. caninum* infections in ruminants have been carried out in different parts of Turkey [10,21,23,29,30,31]. There have been a few molecular studies on the aborted fetuses of ruminants infected by *N. caninum* across the world [7,32,33,34].

In the current study, the real-time PCR result for the proportion of aborted bovine fetuses infected with *N. caninum* (48.9%) was considerably higher than those reported from the vicinity of Elazığ in Turkey (25.49%) with the use of duplex PCR [17] and the 35.07% and 32.35% for *N. caninum* antibodies in cattle in different parts of Turkey reported by Pişkin and Ütük [18] and Erol et al. [19], respectively. Anatolian sheepdogs kept with

sheep flocks close to cattle herds might be a potential risk factor for bovine infection in the area where the present study was conducted. Furthermore, the contamination of pastures with oocysts shed by wild carnivores may also be a risk factor.

To the best of our knowledge, the detection of *N. caninum* DNA with real-time PCR in an aborted water buffalo fetus in the present study is the first report from Turkey. Ingestion of *N. caninum* oocysts may occur through contaminated feed, water, or soil. Even though cattle and water buffalo farms were separate, the animals were grazed on the same pasture. Furthermore, the prevalence of *N. caninum* infection was considerably higher in aborted bovine fetal tissues in the Middle Black Sea Region and Sivas Province than in other parts of Turkey. For a better understanding of the level of *N. caninum* infection and hence the threat posed to the reproductive success of bovine livestock in Turkey, systematic molecular studies should be carried out on pregnant animals and their fetuses, and on dogs in areas where there is the potential for interaction with bovines.

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