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Original Article
Özgün Araştırma

Determination of *Neospora caninum* in cattle fetuses from the Central Black Sea region using PCR

Rahşan Akpınar[1*], Selma Kaya[2], Coşkun Aydın[3], Şakir Önder Türlek[4], Semanur Çelik[5]

[1,2,3,4,5] *T.C. Ministry of Agriculture and Forestry, Samsun Veterinary Control Institute, Samsun, Türkiye*

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Abstract: Neosporosis is an infectious disease caused by the *Neospora caninum*, which leads to abortions in cattle. It causes significant economic losses in both global livestock farming and has recently become one of the leading causes of abortions. This study aims to determine the prevalence of *Neospora* infection in aborted fetuses from 136 cattle in enterprises where large-scale cattle farming is intensively practiced in the Central Black Sea Region (Amasya, Samsun, and Tokat provinces). For the diagnosis of *N. caninum*, DNA isolation was performed aborted fetuses, and these samples were subsequently analyzed using the PCR test method. According to the results obtained, *N. caninum* was found in 3 out of 136 aborted fetuses. In conclusion, this study conducted on cattle in the Central Black Sea Region detected the presence of *Neospora caninum* in aborted fetal samples at a rate of 2.2%. These findings indicate that *N. caninum* should not be overlooked in future studies involving aborted fetal samples. Conducting comprehensive research on the definitive hosts of the parasite will play a crucial role in controlling neosporosis and contribute to the development of effective strategies to prevent the spread of the disease.

Keywords: Cattle, Central Black Sea, Fetus, Neosporosis, PCR

Orta Karadeniz Bölgesindeki sığır fetüslerinde *Neospora caninum'un* PCR ile belirlenmesi

Özet: Neosporozis, *Neospora caninum'un* neden olduğu, sığırlarda aborta neden olan enfeksiyöz bir hastalıktır. Hastalık, büyükbaş hayvan yetiştiriciliğinde önemli ekonomik kayıplara neden olmakta ve son yıllarda abortların başlıca nedenleri arasında yer almaktadır. Bu çalışma, Orta Karadeniz Bölgesinde (Amasya, Samsun ve Tokat illeri) büyükbaş hayvancılığın yoğun olarak yapıldığı işletmelerde abort yapan 136 sığıra ait atık fetüslerde *Neospora sp. varlığını ve* prevalansını belirlemeyi hedeflemektedir. *Neospora caninum*'un teşhisi için atık fetüslerden DNA izolasyonu yapılmış ve ardından bu örnekler PCR test yöntemi ile analiz edilmiştir. Elde edilen sonuçlar doğrultusunda, 136 sığıra ait atık fetüslerden 3'ünde *N. caninum*'a rastlanmıştır. Sonuç olarak, Orta Karadeniz Bölgesindeki sığırlarda gerçekleştirilen bu çalışmada aborte fetüs numunelerinde *N. caninum*'un varlığı %2.2 oranında tespit edilmiştir. Bu bulgular, aborte fetüs numuneleri ile yapılacak gelecekteki çalışmalarda *N. caninum*'un göz ardı edilmemesi gerektiğini göstermektedir. Parazitin son konaklarıyla ilgili kapsamlı araştırmaların yürütülmesi, neosporosisin kontrolünde önemli bir rol oynayacak ve hastalığın yayılmasını engellemeye yönelik etkili stratejiler geliştirilmesine katkı sağlayacaktır.

Anahtar kelimeler: Fetüs, Neosporozis, Orta Karadeniz, PCR, Sığır

Introduction

Neosporosis is a disease caused by the protozoan *Neospora caninum*, which has a two-host heteroxenous life cycle. This disease can lead to clinical signs in various animal species, especially cattle and dogs. Neosporosis is recognized as a parasitic factor causing abortions in both wild and domestic animal species worldwide, particularly in cattle (Dubey et al. 2007; Kaltungo and Musa 2013). Transplacental infection in cattle is an important source of transmission for the parasite; however, the primary route of infection occurs through the oral intake of oocysts shed in dog feces (McAllister et al. 1998). The prevalence of *N. caninum* is attributed to the consumption of placental, aborted fetal, or uterine debris by dogs, which serves as a source of postnatal infection (Davison et al. 2001; Schares et al. 2002; Dubey 2003; Toolan 2003; Salehi et al. 2009; Goodswen et al. 2013). The presence of definitive host dogs in areas where cattle are kept and the contamination of feed and water with their feces are believed to contribute to the spread of infection (Dijkstra et al. 2002; Dubey et al. 2007).

Yazışma adresi / Correspondence: *Rahşan Akpınar, Yeşildere mah., Atatürk Bulvarı, Alaçam Cad., Atakum-Samsun,Türkiye*
e-mail: rahsan.akpinar@tarimorman.gov.tr

ORCID IDs of the authors: *[1]0000-0003-0075-9247 • [2]0000-0002-8934-3418 • [3]0000-0001-6244-414X • [4]0000-0001-6970-8179 • [5]0000-0001-5657-1273*

In the diagnosis of *N. caninum* infection, various serological and molecular tests, along with histopathological and immunopathological examinations using light and electron microscopy, can be employed. These methods are important tools for accurately diagnosing the infection and gaining more insight into the pathogenesis of the disease (Barber et al. 1995; Lally et al. 1996; Ortega-Mora et al. 2006; Lindsay and Dubey 2020). The determination of *N. caninum*-specific antibodies in cattle is typically preferred through serological methods (Dubey and Schares 2006). An observed increase in antibody titers during mid-pregnancy in seropositive animals is considered an indication of reactivation of latent infection (Lindsay and Dubey 2020). Neosporosis has been associated with abortion in seropositive animals (Anderson et al. 2000; Dubey 2003; Açıcı et al. 2019). However, it has been observed that approximately 95% of calves born to seropositive cows are clinically normal despite being congenitally infected (Dubey 1999a; Quintanilla et al. 2000; Dubey 2003). The most effective method for controlling the disease is the culling of infected animals once a definitive diagnosis has been made (Reichel et al. 2013). Nevertheless, the absence of clinical signs in infected cattle other than abortion complicates the diagnosis of the disease (Barber et al. 1995).

Neosporosis represents a significant parasitic threat to cattle populations, with a high prevalence reported globally. This disease is responsible for considerable economic losses (McAllister et al. 1998; Trees et al. 1999; McAllister et al. 2000). The prevalence of *N. caninum* varies by region: 3.4% to 36.2% in Africa Ayinmode et al. (2017), Abdeltif et al. (2022); 37.5% to 70% in northeast Thailand Kashiwazaki et al. (2001); 0.5% to 3.9% in the Czech Republic Václavek et al. (2007), Bártová et al. (2015); 4.1% in Germany and France Dubey et al. (2007); 2.7% to 44.4% in Australia Dubey et al. (2007), Nasir et al. (2012); 23.6% to 91.2% in Brazilian Ragozo et al. (2003), Guedes et al. (2008) and 5.2% in North Dakotato Khaitsa et al. (2006). In Türkiye, serological studies have reported the seroprevalence of neosporosis in cattle to be between 2% and 37.2% (Bıyıkoğlu et al. 2001; Aktaş et al. 2005; Pişkin and Ütük 2009; Kasap et al. 2020; Bulut et al. 2021; Köse et al. 2021; Kula and Gökpınar 2021). While some studies exist on the prevalence of the disease in Türkiye, they seem insufficient considering the cattle population in the country. Regularly conducted parasitological studies at specific intervals would particularly help in determining the prevalence of the disease. The objective of this study is to ascertain the prevalence of neosporosis in abortion cases in the provinces of Amasya, Samsun, and Tokat, where extensive cattle farming is the predominant practice.

Materials and Methods

Collection of fetal samples

The samples used in this study were obtained from the abortions of cattle sent for routine diagnosis to the Samsun Veterinary Control Institute from Amasya (n=16), Samsun (n=80), and Tokat (n=40) provinces. The organ samples taken from these calves were delivered to the Parasitology Laboratory in sterile containers, numbered and stored at -20°C until analysis. The provinces from where the fetal samples were sent are shown in Figure 1.

Figure 1. The provinces from where the fetal samples were taken for analysis regarding *Neospora caninum*

This study aimed to determine the prevalence of *Neospora* infection in aborted fetal tissues (lung, heart, liver, spleen, and stomach contents) from 136 cattle that were aborted in the provinces of Amasya, Samsun, and Tokat, located in the Central Black Sea Region. Approximately 25 mg tissue pieces (lung, heart, liver, spleen, and stomach contents) were taken from each fetal sample and transferred to 7 mL cryo-tubes, followed by the addition of 3 mL of PBS. The samples were homogenized in an automatic homogenization device (Bead Ruptor Elite, Bead Mill Homogenizer, SKU 19-042E, OMNI International, USA) at 7000 rpm for 1 minute. Following homogenization, the samples were centrifuged at +4°C at 4000 rpm for 10 minutes, and 100 µL of the supernatant was taken for DNA extraction according to the manufacturer's protocol (Genomic DNA Mini Kit/Invitrogen). Specific primers for *Neospora*

caninum, Np6/Np21 primers (5′-GGG TGT GCG TCC AAT CCT GTA AC-3′, 5′-CTC GCC AGT CAA CCT ACG TCT TCT-3′) were used for PCR (Kamali et al. 2014). The PCR mixture was prepared in a total volume of 25 µL, consisting of 2.5 µL Dream Taq buffer, 0.5 µL dNTP Mix (10 mM), 0.8 µL of each primer (10 pmol), 0.4 µL Dream Taq DNA polymerase (5 U/µL), and 15 µL sterile distilled water, with 5 µL of template DNA added to reach a total volume of 20 µL. The PCR mixture was placed in a thermal cycler with the following amplification conditions: an initial denaturation at 94°C for 7 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. The process concluded with a final extension step at 72°C for 7 minutes. Subsequently, the amplified PCR products were subjected to electrophoresis in a 1% agarose gel stained with 0.05% ethidium bromide (5 mg/mL) at 90 V and 100 mA for 50 minutes, and the presence of DNA bands was visualized under UV light using a gel imaging device. A PCR product showing a band of 337 bp was considered positive.

Statistical analysis

The frequencies of aborted fetal samples from 136 cattle between 2018 and 2020 were analyzed and summarized in a frequency table 1.

Results

As a result of this study, 136 aborted fetal samples from cattle were analyzed using Polymerase Chain Reaction (PCR), and *Neospora caninum* was detected in 3 samples (2.2%). Of the 80 samples from Samsun, 2 (2.5%) were positive, and of the 40 samples from Tokat, 1 (2.5%) was positive. No *N. caninum* was found in any of the 16 samples from Amasya. The number of samples collected by province and the positivity status are presented in Table 1.

Table 1. Distribution of cattle abortion materials in this study according to provinces and results.

Provinces	Taken samples	Positive	Negative
Amasya	16	0	16
Samsun	80	2	78
Tokat	40	1	39
Total	**136**	**3**	**133**

The PCR image of the *N. caninum* abortion samples is shown in Figure 2.

Figure 2: For *Neospora caninum*, PCR analysis of the samples showed specific banding at 337 bp. M: Molecular weight marker (100 bp ladder), PC: Positive control, NC: Negative control, 1,8: Positive samples, 2-7: Negative samples

Discussion and Conclusion

Neosporosis is one of the most important parasitic causes of cattle abortions, widely observed both globally and in Türkiye, leading to significant economic losses. *N. caninum* is a parasite with a broad host spectrum and can cause infections in many domestic and wild animals, particularly cattle. This situation increases the impact of *N. caninum* on both livestock farming and natural ecosystems, resulting in serious economic losses. The diversity of hosts facilitates the spread of the parasite and complicates its control. Therefore, the prevention and management of *N. caninum* infections are crucial for animal health and productivity (McAllister et al. 1998; Dubey 1999b; Dubey et al. 2007; Şentürk et al. 2020). Congenital infections associated with *N. caninum* can lead to abortions, stillbirths, and the birth of clinically or subclinically infected calves at different stages of pregnancy. This situation poses a significant problem for cattle breeding and causes economic losses. Since the timing of these infections has a decisive impact on animal health and productivity, careful monitoring and management are required (Innes et al. 2007). One of the main reasons for abortions caused by *N. caninum* is the presence of definitive host dogs on farms. The presence of these dogs in the same environment as cattle facilitates the contamination of feed and water sources with feces from canids, thereby promoting the spread of infection (Dubey et al. 2007; Kaltungo and Musa 2013).

Abortions related to neosporosis can occur in any season of the year (Anderson et al. 1991; Moen and Wouda 1995; Thurmond et al. 1995). Numerous studies using various serological methods have been conducted on cattle in different countries around the world. The prevalence of *N. caninum* has varied, with rates of 56.9% in Argentina Campero et al. (1998), 0.5% to 3.9% in the Czech Republic Václavek et al. (2007), Bártová et al. (2015); 12.5% in Wales and England Davison et al. (1999); 36.8% in Spain Quintanilla-Gozala et al. (1999), 15.6% in Poland Cabaj et al. (2000), and 10.7% in Sudan (Ibrahim et al. 2012). In Türkiye, a study by Eşki and Ütük covering *N. caninum* seroprevalence research up to 2018 reported an average prevalence of 13.06% (1023/7830) in cattle. Similarly, a study conducted by Demir et al. in 2020 reviewed all serological studies on cattle in Türkiye and reported an average seroprevalence of 14.7% (1672/11,373) for *N. caninum*.

Globally, PCR diagnostic studies for *Neospora caninum* in aborted cattle fetuses have been conducted. Sager et al. (2001) reported 21% positivity in 58 out of 242 samples in Sweden, while Sadrebazzaz et al. (2004) found *N. caninum* in 33% of 12 aborted fetuses in Iran. In Brazil, Cabral et al. (2009) detected *N. caninum* in 6.7% of 105 aborted fetuses, and Şuteu et al. (2012) found 38.9% positivity in 21 aborted fetal samples. Macedo et al. (2017) detected *N. caninum* DNA in 38.8% of 14 tissue samples from 35 aborted fetuses. In Türkiye, PCR studies on aborted calf fetuses have reported varying results; Özkaraca et al. (2017) found *N. caninum* in 25.49% of 102 aborted fetuses in Elazığ, while Açıcı et al. (2019) reported a 49.4% positivity rate in 44 aborted fetuses from 89 farms using Real-time PCR. In Şenel (2022) doctoral thesis, which investigated *N. caninum* in the Marmara Region, DNA from brain, heart, liver, lung, spleen, and kidney tissues of 84 aborted samples revealed *N. caninum* DNA in 26.19% of cases. Additionally, a study by İrehan et al. (2022) detected *N. caninum* in 8 out of 30 aborted fetuses using Real-time PCR, with only two of these also testing positive by conventional PCR. The lower positivity rate found in our study (2.2%) compared to the 49.4% reported by Açıcı et al. (2019) could be attributed to the lower detection rate of conventional PCR. Furthermore, the lower prevalence of *N. caninum* in the Central Black Sea Region compared to previous studies may be explained by the high level of integrated farming practices in the provinces where this study was conducted and the minimization of contact between cattle and uncontrolled dogs.

The type of cattle farming systems and management strategies are significant risk factors influencing the prevalence of *N. caninum*. Studies have shown that the seroprevalence of *N. caninum* is lower in integrated farms compared to rural family farms. It has been noted that cattle in rural family farms are more exposed to uncontrolled dogs, and those cattle that come into contact with these dogs carry a higher risk of infection (Öcal et al. 2014; Noori et al. 2019). The variability in results from PCR studies for *N. caninum* diagnosis conducted globally and in our country may stem from differences in regions, cattle breeds and rearing conditions, sample sizes, types and quantities of examined tissues, parasitic load, the presence of risk factors associated with *N. caninum*, and the different tests used. Therefore, the presence of definitive host dogs in areas where cattle are located, which can contaminate feed and water with their feces, is thought to play a significant role in the spread of infection (Dijkstra et al. 2002; Dubey et al. 2007).

In conclusion, understanding the relationship between intermediate and definitive hosts of *Neospora caninum* and implementing preventive measures is of great importance. Additionally, since calves born from *N. caninum* infections can transmit the infection from generation to generation, and due to the lack of effective treatment or vaccines, it is considered essential to conduct herd-wide screenings and remove infected animals from the herd. Informing veterinarians and farmers about infections that cause abortion in cattle is crucial for combating these infections and, consequently, for the national economy. Furthermore, the use of double or triple test combinations in diagnosing *N. caninum* has been signficantly evaluated for accurate diagnosis.

Ethical Statement: This study was conducted with permission from the Local Ethics Committee for Animal Experiments of the Samsun Veterinary Control Institute, under the letter dated 07.07.2022 with reference number 19572899/031-65 (Decision no: 2022/5).

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Forestry. Neither the the Republic of Türkiye Ministry of Agriculture and Forestry can be held responsible for". This text is included in accordance with the recommendation of our Ministry.

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