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Paper

Neospora caninum in Cattle Herds: Risk Factors, Prevalence and Molecular Characterization in Western Türkiye

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Abstract

Neosporosis is a major cause of bovine abortion worldwide, leading to substantial economic losses. In western Türkiye, an area characterized by intensive cattle breeding, data on this disease remain limited, and no prior studies have investigated its risk factors. This study aimed to determine the prevalence, associated risk factors, and phylogenetic profile of *Neospora caninum* in the region. Visceral tissues from 88 aborted cattle fetuses were analyzed through genomic DNA isolation, real-time PCR (qPCR), and sequencing. In parallel, the presence of *N. caninum* antibodies was assessed in 973 serum samples using ELISA. *N. caninum* DNA was detected in 8 (9.09%) fetal samples by qPCR, with three of these further confirmed via conventional PCR and sequencing. Sequence analysis validated the presence of *N. caninum* in these three samples. Serologically, 122 of 973 serum samples (12.53%) tested positive for *N. caninum* antibodies. Statistical analysis of potential risk factors—including province, gender, age group, origin, and farm type—identified farm type as the most significant determinant of seropositivity. Notably, family-operated farms exhibited a higher prevalence of positive cases. This study represents the first large-scale investigation of *N. caninum*-associated reproductive losses in western Türkiye. The findings offer valuable insights for the development of targeted control and prevention strategies in affected cattle populations.

Keywords

Cattle abortion, Neospora caninum, Real time PCR., Risk factors, Seroprevalence

Introduction

Neospora caninum, a leading cause of reproductive disorders in domestic ruminants—particularly cattle—follows a two-host life cycle involving definitive and intermediate hosts (Kaltungo and Musa, 2013). This apicomplexan parasite is globally distributed and imposes a substantial economic burden on livestock producers due to its impact on cattle fertility. Transmission occurs when unsporulated oocysts shed in dog feces contaminate the environment and subsequently sporulate in feed or water. Ingestion of these sporulated oocysts by intermediate hosts—including cattle, sheep, goats, horses, or even dogs themselves—facilitates infection and perpetuates the parasite's life cycle (Dubey et al., 2017; Acici et al., 2019).

Abortion rates in cattle herds vary depending on production system, farm management practices, and regional husbandry conditions. While an abortion rate of 3–5% is considered within normal limits, higher rates (>5%) are regarded as indicative of pathological processes requiring investigation (Hoving, 2009; Holler, 2012). *N. caninum* is among the most important parasitic agents responsible for bovine abortion globally and has also been frequently reported in Türkiye. Seroprevalence studies conducted in various Turkish regions have reported positivity rates ranging from 2% to 37.7% (Yıldız et al., 2009; Kul, 2012; Eski & Utuk, 2018; Erol et al., 2019; Demir et al., 2020; Kasap et al., 2020; Kose et al., 2021; Kula & Gokpınar, 2021). Despite this relatively high prevalence, limited research has been conducted in the Aegean region of western Türkiye, where cattle breeding is highly concentrated.

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Horizontal transmission of *N. caninum* is sustained postnatally through the ingestion of infected placental tissues, aborted fetuses, uterine secretions, or raw viscera by dogs, thereby maintaining the biological cycle (Serrano-Martínez et al., 2019; Salehi et al., 2021). In Türkiye, small-scale, family-run livestock operations—often characterized by traditional or extensive management systems—commonly lack biosecurity measures and allow dogs unrestricted access to cattle housing and grazing areas. Additionally, communal grazing practices and the presence of stray dogs increase the likelihood of oocyst contamination in pastures (Cerqueira-Cézar et al., 2017; Tulu et al., 2018).

This study presents a comprehensive epidemiological investigation of *N. caninum* in cattle herds exhibiting reproductive failure across western Türkiye. It focuses on prevalence, key risk factors for abortion, and broader implications for disease control. Given the economic consequences of bovine abortion and the limited data available from the Aegean region, this study fills a critical knowledge gap by providing updated serological and molecular data (Şentürk et al., 2020). Moreover, it explores the phylogenetic relationships between *N. caninum* isolates detected in Türkiye and those reported in neighboring countries, contributing to the understanding of regional genetic diversity and transmission dynamics (Salehi et al., 2021; Gharekhani et al., 2022). By integrating seroepidemiological, molecular, and ecological approaches, the study offers valuable insights to inform effective disease management strategies, enhance farm-level biosecurity, and support policy development aimed at minimizing economic losses due to neosporosis.

Materials and Methods

Study Region and Samples Collection

In this study, aborted fetuses and blood serum samples were collected from cattle breeding farms located in the western provinces of Türkiye. Serological sampling was conducted during field visits between January and July 2022. During blood collection, data on each animal's gender, age group, origin, and farm type (extensive vs. intensive) were recorded through face-to-face interviews with farm owners.

For the serological investigation, the provinces of Aydın, Denizli, İzmir, Kütahya, Manisa, Muğla, and Uşak were defined as distinct epidemiological units, in accordance with livestock population data reported in the annual circular issued by the Ministry of Agriculture and Forestry (Figure 1). A total of 973 blood samples were collected—139 per province—using a simple random sampling method. The required sample size was calculated based on a 95% confidence level, an expected prevalence of 10%, and a margin of error of ±5%. To meet the sampling threshold, approximately 10% of the cattle in each farm were sampled. Around 5 mL of blood was drawn from each animal and transported under cold chain conditions to maintain sample integrity.

Figure 1. Cities in western Türkiye from which samples were collected for the study.

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For the molecular analysis, brain, heart, liver, and kidney tissues were collected from 88 aborted bovine fetuses submitted to the İzmir-Bornova Veterinary Control Institute from the same provinces between May 2021 and May 2022. Genomic DNA was extracted from these tissues following necropsy for subsequent molecular investigations.

Serological Study

Samples delivered to the laboratory were catalogued along with associated metadata. Following data entry, blood samples were centrifuged at 1500 rpm for 10 minutes to separate the serum. The resulting serum was aliquoted into 1.5 mL eppendorf tubes and stored at −20°C until serological analysis.

For the serological testing, a commercial ELISA kit (ID Screen® *Neospora caninum* Indirect, Innovative Diagnostics, France) was used in accordance with the manufacturer's instructions.

Approximately 1 g of each tissue (brain, heart, liver, and kidney) collected from aborted fetuses during necropsy—totaling 4 g per sample—was pooled, triturated with sterile sand, and homogenized in phosphate-buffered saline (PBS) at a 1:10 ratio. The resulting homogenates were centrifuged at 4 °C and 3500 rpm for 15 minutes using a refrigerated centrifuge (ThermoFisher SL 16R, Germany).

From each homogenate, 0.2 mL of the supernatant was used for genomic DNA extraction, performed with a commercial kit (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche, Germany; Product No. 03038505001), following the manufacturer's protocol.

In the preliminary phase of molecular testing, a real-time PCR (qPCR) assay targeting *Neospora caninum* was performed using a commercial kit (ID Gene™ *Neospora caninum* Duplex, France; Product No. IDNEO-100). The qPCR was conducted according to the manufacturer's instructions, with plasmid *N. caninum* DNA provided in the kit serving as the positive control. The thermal cycling protocol included an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and extension at 60 °C for 60 seconds. Samples were considered positive based on the presence of exponential fluorescence curves and recorded cycle threshold (Ct) values.

To obtain sequence data, conventional PCR was subsequently carried out on the eight qPCR-positive samples. Amplification targeted a 330 bp fragment within the Nc5 genomic region of *N. caninum*, using the following primers (Müller et al., 1996):

Forward: 3′-CCCAGTGCGTCCAATCCTGTAAC-5′

Reverse: 5′-CTCGCCAGTCAACCTACGTCTTCT-3′

The PCR reactions were prepared using a HotStart PCR kit (Grisp, Xpert Fast Hotstart Mastermix 2X, Portugal) in a final volume of 25 µL. The thermal profile included an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72 °C for 2 minutes, with a final extension step at 72 °C for 10 minutes. Amplicons were evaluated by agarose gel electrophoresis.

Bidirectional sequencing of PCR products was performed by a commercial service provider (Microsynt, Balgach, Switzerland). Raw sequences were manually corrected, aligned using DNADynamo software, and validated through BLAST (Basic Local Alignment Search Tool) analysis against NCBI database entries (Altschul et al., 1990).

Phylogenetic analyses were performed using multiple sequence alignments of the obtained *N. caninum* isolates with reference sequences from GenBank, aligned using the ClustalW algorithm in MEGA X. The optimal nucleotide substitution model was determined for each dataset. A phylogenetic tree was constructed using the maximum likelihood method with 1,000 bootstrap replicates. Pairwise nucleotide similarity was calculated, and phylogenetic relationships were further assessed using the Sequence Demarcation Tool v1.2 (SDT v1.2) (Muhire et al., 2014). All sequences generated in this study have been deposited in the NCBI GenBank database.

Statistical Analyses

Associations between test outcomes (positive vs. negative) and variables including province, gender, age group, origin, and farm type were evaluated using the chi-square (χ^2) test or Fisher's exact test, as appropriate. A p-value of <0.05 was considered statistically significant. All data were recorded and analyzed using SPSS® software (IBM®, Version 21.0; Armonk, NY, USA).

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Results

Serological and Statistical Analyses

According to the results of the serological analysis, anti-*Neospora caninum* antibodies were detected in 122 of the 973 cattle tested, corresponding to a seroprevalence of 12.53%. Seropositive animals were found in 32 of the 58 sampled cattle farms (55.17%). Statistically significant differences were observed by province and farm type; detailed results are presented in Table I.

Provinces	Number of herds	Number of animal	Number positive animals (%)	Farm type	Number of herds	Number of animal	Number positive animals (%)
Aydın	6	139	32 (23,02)	Intensive	3	72	2 (2,77)
				Extensive	3	67	30 (44,77)
Denizli	11	139	6 (4,31)	Intensive	3	46	0
				Extensive	8	93	6 (6,45)
İzmir	6	139	26 (18,7)	Intensive	2	49	5 (10,2)
				Extensive	4	90	21 (23,33)
Kütahya	6	139	12 (8,63)	Intensive	3	97	7 (7,21)
				Extensive	3	42	5 (11,9)
Manisa	11	139	18 (12,94)	Intensive	2	59	0
				Extensive	9	80	18 (22,5)
Muğla	10	139	7 (5,03)	Intensive	2	20	0
				Extensive	8	119	7 (5,88)
Uşak	8	139	21 (15,1)	Intensive	1	17	0
				Extensive	7	122	21 (17,21)
Total	**58**	**973**	**122 (12,53)**	Intensive	16	360	**14 (3,88)**
				Extensive	42	613	**108 (17,61)**
P value (interprovince)			<0,05	P value (Total interfarm type)			<0,05

Table I. Chi-square values and significance levels of N. caninum ELISA findings in the serological study.

With regard to the evaluated risk factors, no statistically significant associations were found between seropositivity and gender, age group, or origin of the animals (imported vs. locally bred) ($P > 0.05$). However, a clear difference emerged with respect to farm type: 108 of 615 animals (17.61%) raised on extensive (family-run) farms tested seropositive, compared to only 14 of 358 animals (3.88%) from intensive (integrated) farms. Statistical analysis confirmed that farm type was significantly associated with the presence of anti-*N. caninum* antibodies ($P < 0.05$).

Real-time PCR

Neospora caninum DNA was detected in 8 out of 88 pooled tissue samples (9.09%) from aborted cattle fetuses collected in the Aegean region using real-time PCR (qPCR). The detection rates by province were as follows: İzmir, 16.66% (1/6); Manisa, 15.38% (2/13); Aydın, 7.14% (1/14); Denizli, 9.09% (2/22); and Uşak, 11.11% (2/18). No positive cases were identified among the tissue pools from 9 fetuses in Muğla and 6 in Kütahya.

Sequencing and phylogenetic analyses

The phylogenetic tree was constructed by comparing the sequence data obtained in this study with reference sequences available in the NCBI GenBank database from various countries. The objective of the phylogenetic analysis was to assess the genetic relationships between the *Neospora caninum* isolates identified in this study and those previously reported from a wide range of animal hosts, including chickens, rats, dogs, deer, pigs, jackals, rabbits, wolves, sparrows, and cattle. In particular, the study aimed to evaluate the genetic relatedness of *N. caninum*

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sequences from the Aegean region of Türkiye to globally distributed isolates.

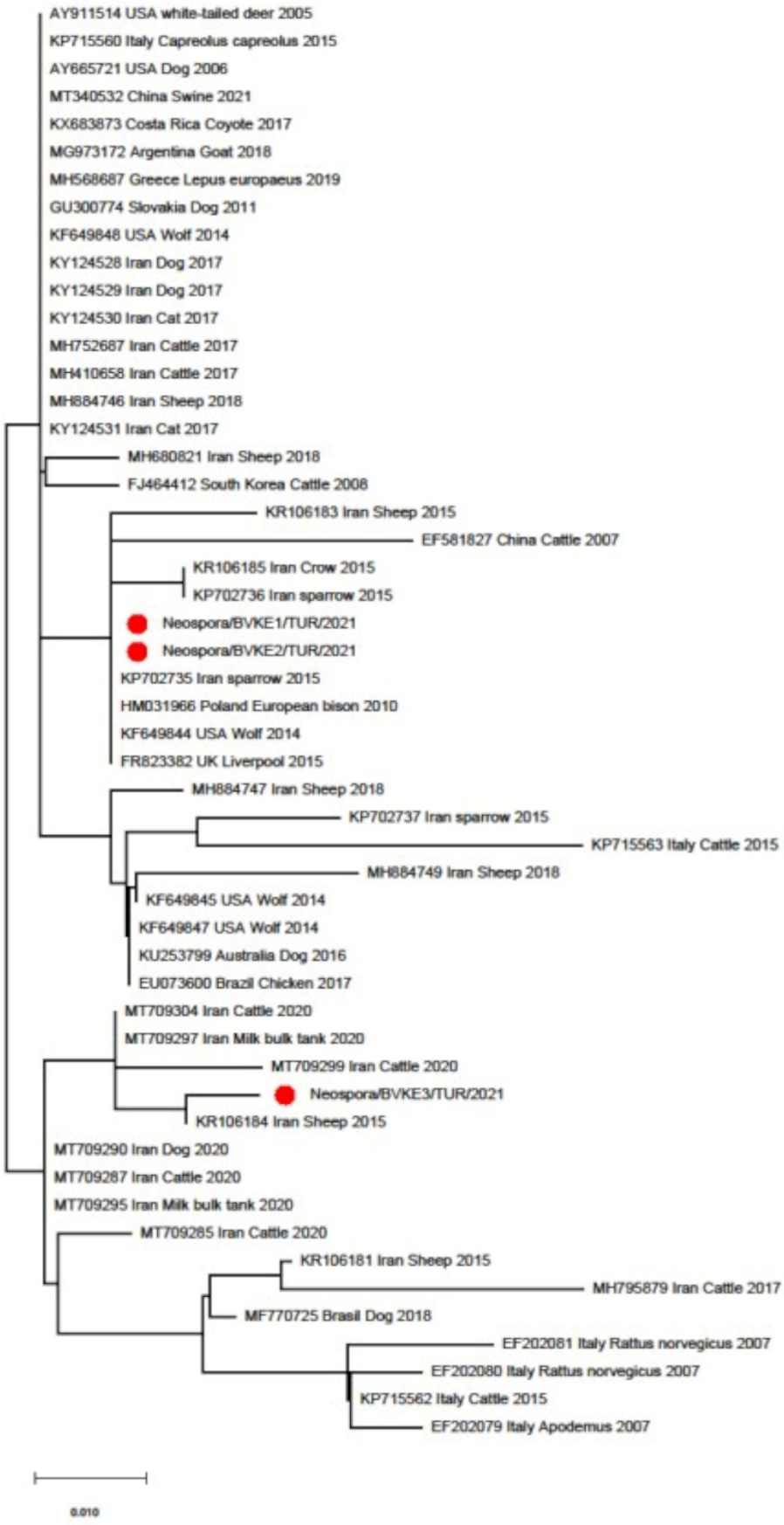

Figure 2. Phylogenetic tree of N. caninum according to the Nc5 gene region (maximum-likelihood, Tamura 3 parameter, 1000 replicates).

To confirm amplicon size, electrophoresis on a 1.5% agarose gel revealed the expected 330 bp bands in 3 out of the 8

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qPCR-positive samples. Sequence analysis was subsequently performed on one PCR product from each of the following provinces: Manisa, Denizli, and Uşak. All three sequences corresponded to a 330 bp fragment within the Nc5 region of the *N. caninum* genome. Verification using nucleotide BLAST confirmed identity with *N. caninum*, and the sequences were deposited in the NCBI GenBank under the accession numbers PP975986, PP975987, and PP975988.

The phylogenetic analysis included 52 *N. caninum* reference sequences retrieved from GenBank. The Tamura 3-parameter model with gamma distribution (T92+G) was identified as the most appropriate nucleotide substitution model (Tamura et al., 2013). The phylogenetic tree was constructed using the maximum likelihood method and is shown in Figure 2.

The analysis revealed that *N. caninum* isolates clustered into two primary clades with several well-defined subclades (Figure 2). The sequences obtained in this study did not exhibit any clear geographic clustering.

Instead, they shared close genetic similarity with isolates from diverse global regions. Two of the Turkish isolates (Neospora/BVKE1/TUR/2021 and Neospora/BVKE2/TUR/2021) clustered within the same branch, whereas the third (Neospora/BVKE3/TUR/2021) was located in a separate clade. Overall, phylogenetic grouping did not correlate with host species or geographic origin, as isolates from multiple species (e.g., chicken, rat, dog, deer, pig, coyote, rabbit, wolf, sparrow, and cattle) were interspersed throughout the tree.

Sequence homology analysis indicated that the Turkish isolates shared 96.26% – 99.8% nucleotide identity with the *N. caninum* Liverpool 2015 reference isolate from the UK (GenBank accession: FR823382), and 96.29%–100% identity with other global isolates. Pairwise nucleotide similarity analysis of the 52 isolates, performed using SDT v1.2 software, yielded similarity values ranging from 93% to 100%.

Discussion

The findings of this study confirm that *Neospora caninum* is a significant causative agent of bovine abortion in western Türkiye. Although various serological screenings employing different methods—including c-ELISA, i-ELISA, and IFAT—have previously been conducted to assess *N. caninum* prevalence across Türkiye (Dubey et al., 2017; Eski & Utuk, 2018; Demir et al., 2020), research in the Aegean region has been notably lacking. A meta-analysis of seroprevalence studies up to 2018 reported an average prevalence of 13.06% (1,023/7,830) (Eski & Utuk, 2018), while a subsequent review of studies through 2020 estimated an average prevalence of 14.7% (1,672/11,373) (Demir et al., 2020). The 12.53% seroprevalence reported in the current study, as determined by ELISA, aligns with these previous findings; statistical comparisons showed no significant differences in sample size ($p = 0.50$) or the proportion of positive samples ($p = 0.80$), further validating the results. Importantly, this study represents the first large-scale seroepidemiological investigation of *N. caninum* in the Aegean region, underscoring its significance in regional reproductive losses.

Several molecular studies have also investigated *N. caninum* detection in Türkiye using PCR. For example, Özkaraca et al. (2017) reported a 25.49% detection rate in aborted fetuses (26/102) from Elazığ province, possibly attributable to their focus on abortions occurring during the second trimester—when neosporosis-related losses are most frequent (Serrano-Martínez et al., 2019). In the present study, four of the eight qPCR-positive cases also occurred during the second trimester, mirroring this observation. In contrast, Irehan et al. (2022) reported a lower detection rate of 6.66% (2/30) in Eastern and Southeastern Anatolia, potentially due to smaller sample size and random sampling of abortion cases. A notably higher detection rate (49.4%) was reported in the Central Black Sea region by Acici et al. (2019), who targeted fetuses from seropositive cows in high-risk farms—an approach known to increase detection sensitivity. Supporting this, previous research indicates that seropositive cattle are 1.7 to 7.4 times more likely to abort than seronegative animals, and that transplacental transmission contributes to rising seroprevalence even in the absence of clinical abortion (Thurmond & Hietala, 1997).

Discrepancies among reported prevalence and detection rates in Türkiye likely reflect a combination of factors: geographic variation, breed and husbandry practices, sample size, gestational timing of abortion, tissue type and quantity analyzed, pathogen load, and the presence of definitive hosts such as dogs. In addition, differences in diagnostic methodologies and molecular protocols may contribute to this variability (Thurmond & Hietala, 1997; Qian et al., 2017; Gharekhani & Yakhchali, 2020; Klun et al., 2019; Cao et al., 2022).

This study further demonstrated that cattle raised under extensive farming systems had significantly higher seropositivity rates than those in intensive production systems, in line with previous findings from global literature. One of the most critical risk factors is the presence of definitive hosts (dogs), which serve as sources of environmental

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contamination with oocysts (Dubey et al., 2007; Kaltungo & Musa, 2013). The management system and husbandry practices play a pivotal role in determining the likelihood of infection. Studies consistently report lower *N. caninum* prevalence in intensive farms—where biosecurity and dog access are tightly controlled—compared to extensive or family-run farms, where contact with free-roaming dogs is more frequent (Mor & Akca, 2012; Noori et al., 2019; Klun et al., 2019; Cao et al., 2022). In the present study, family-run farms exhibited a significantly higher rate of seropositive animals than integrated farms ($P < 0.05$), likely due to the presence of uncontrolled dog populations on such farms.

To further characterize the isolates, phylogenetic analyses based on Nc5 gene sequences were performed. The three sequences obtained in this study clustered within broader groups of *N. caninum* isolates from various animal species and geographical regions. The phylogenetic tree, constructed from 52 reference sequences, was divided into two major branches with several subclades. Consistent with previous studies, the isolates showed high sequence similarity, and no clear geographic or host-based clustering pattern was observed. This lack of differentiation supports the notion that *N. caninum* exhibits limited genetic divergence across host species and regions. Pairwise similarity values among the 52 sequences ranged from 93% to 100%, confirming the strong genetic conservation of the Nc5 region across global *N. caninum* populations.

The detection of genetically similar *N. caninum* strains in both domestic and wild animals, including in urban settings, reinforces the hypothesis of interspecies transmission facilitated by ecological overlap and anthropogenic factors. These findings suggest that *N. caninum* plays a consistent and widespread role in bovine abortions in Türkiye, similar to trends observed globally. Moreover, the data highlight the potential risk of pathogen spread through uncontrolled animal movement and dog-wildlife interactions, particularly in areas with low biosecurity.

In conclusion, this study underscores the importance of *N. caninum* as a major contributor to calf losses in the Aegean region of Türkiye. The integration of serological, molecular, and phylogenetic data offers a robust framework for understanding the epidemiology of neosporosis and provides critical insights for designing targeted prevention and control strategies (Salehi et al., 2021; Gharekhani et al., 2022).

Conclusion

This study provides the first comprehensive epidemiological assessment of *Neospora caninum* in western Türkiye, employing both direct and indirect diagnostic methods. Molecular analyses were conducted to evaluate the genetic diversity of *N. caninum* and its phylogenetic relationships with isolates from other countries. The findings revealed no statistically significant associations between seropositivity and factors such as gender, age group, or animal origin (imported vs. local). However, farm type emerged as a significant determinant, with extensive (family-run) farms exhibiting higher rates of seropositivity than intensive systems.

These results emphasize the importance of integrated and well-structured cattle farming systems, which can limit the free movement of definitive hosts—particularly dogs—that play a key role in the transmission of *N. caninum*. The findings underscore the need for effective disease prevention and control strategies in livestock operations to mitigate reproductive losses.

In conclusion, this study highlights the ongoing relevance of *N. caninum* as a cause of abortion and calf loss in the region and provides foundational data for future surveillance and control efforts. Further research is warranted to better elucidate the phylogenetic structure and transmission dynamics of *N. caninum* in Türkiye. In particular, we recommend longitudinal studies in farms where biosecurity and preventive measures are actively implemented, including monitoring for the presence of *N. caninum* in fecal samples from definitive host dogs. Such investigations will be essential for tracking changes in prevalence and reducing the economic impact of neosporosis in cattle populations.

Ethical Approval

This study was carried out with the approval of the İzmir/Bornova Veterinary Control Institute Directorate Animal Experiments Local Ethics Committee, dated 16.04.2021 and numbered E-71705440-770-1262115.

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Author contributions

Conceptualization: Dr. Ömer Faruk Gökcecik; Methodology: Dr. Ömer Faruk Gökcecik, Prof. Hasan Eren; Investigation: Dr. Ömer Faruk Gökcecik; Writing - original draft: Dr. Ömer Faruk Gökcecik; Writing - review and editing: Dr. Ömer Faruk Gökcecik, Prof. Hasan Eren; Visualization: Dr. Ömer Faruk Gökcecik; Supervision: Prof. Hasan Eren. All authors have read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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