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Original Article
Özgün Araştırma

Real-Time PCR detection of *Neospora caninum* and *Toxoplasma gondii* in ruminant abortions in Marmara

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Abstract: Abortions associated with protozoan infections remain a significant cause of reproductive losses in ruminant production systems worldwide. Among these pathogens, *Neospora caninum* and *Toxoplasma gondii* are recognized as major etiological agents of abortion in cattle and sheep, respectively. This study aimed to simultaneously detect *N. caninum* and *T. gondii* DNA in aborted ruminant fetuses collected from the Marmara Region of Türkiye using a Real-Time PCR approach. A total of 306 aborted fetuses (84 bovine and 222 ovine) obtained from different provinces of the Marmara Region were included in the study. Tissue samples were analyzed by species-specific Real-Time PCR assays targeting *N. caninum* and *T. gondii*. DNA was detected in 22 (26.19%) bovine fetuses, whereas *T. gondii* DNA was identified in 4 (1.8%) ovine fetuses. These findings demonstrate the continued epidemiological importance of protozoan agents in ruminant abortions in the Marmara Region. To the best of our knowledge, this study provides the first simultaneous molecular evidence of *N. caninum* and *T. gondii* in aborted ruminant fetuses from this region. Further large-scale investigations, including studies on definitive hosts and transmission patterns, are warranted to better elucidate the regional epidemiology of these pathogens.

Keywords: Marmara Region, *Neospora caninum*, Real-Time PCR, ruminant abortion, *Toxoplasma gondii*

Marmara Bölgesi'nde ruminant abortlarında *Neospora caninum* ve *Toxoplasma gondii*'nin Real-Time PCR ile tespiti

Özet: Protozoon enfeksiyonlarına bağlı abortlar, dünya genelinde ruminant yetiştiriciliğinde önemli üreme kayıplarına neden olmaya devam etmektedir. Bu etkenler arasında *Neospora caninum* ve *Toxoplasma gondii*, sırasıyla sığır ve koyun abortlarının başlıca etiyolojik ajanları olarak kabul edilmektedir. Bu çalışmada, Türkiye'nin Marmara Bölgesi'nden toplanan abortlu ruminant fetüslerinde *N. caninum* ve *T. gondii* DNA'sının Gerçek Zamanlı Polimeraz Zincir Reaksiyonu (Real-Time PCR) yöntemi ile eş zamanlı olarak saptanması amaçlanmıştır. Araştırmaya, Marmara Bölgesi'nin farklı illerinden temin edilen toplam 306 abortlu fetüs (84 sığır ve 222 koyun) dâhil edilmiştir. Doku örnekleri, *N. caninum* ve *T. gondii*'ye özgü Real-Time PCR testleri kullanılarak analiz edilmiştir. *N. caninum* DNA'sı 22 (%26,19) sığır fetüsünde, *T. gondii* DNA'sı ise 4 (%1,8) koyun fetüsünde tespit edilmiştir. Elde edilen bulgular, protozoon etkenlerin Marmara Bölgesi'ndeki ruminant abortlarında epidemiyolojik önemini sürdürdüğünü göstermektedir. Bu çalışma, bilgimiz dâhilinde, söz konusu bölgede abortlu ruminant fetüslerinde *N. caninum* ve *T. gondii*'nin eş zamanlı moleküler varlığını ortaya koyan ilk araştırmadır. Bölgesel epidemiyolojinin daha iyi anlaşılabilmesi amacıyla, son konakları ve bulaşma dinamiklerini de kapsayan daha geniş ölçekli araştırmalara ihtiyaç bulunmaktadır.

Anahtar kelimeler: Marmara Bölgesi, *Neospora caninum*, Real-Time PCR, ruminant abortu, *Toxoplasma gondii*.

Introduction

Ruminant livestock production, particularly cattle and sheep farming, constitutes a major component of global agricultural systems and plays a critical role in food security and rural economies. In Türkiye, ruminant husbandry remains one of the most important agricultural activities, especially in regions where animal production represents the primary source of income for rural populations. Despite substantial contributions of cattle and sheep to national meat and milk supply chains, reproductive efficiency remains a key determinant of herd profitability and sustainability (Schares et al., 1999; Dubey, 2003; Dubey et al., 2006).

Reproductive disorders, particularly abortion, represent major constraints to ruminant productivity, causing fetal losses, prolonged calving intervals, reduced milk yield, increased culling rates, and additional veterinary costs. The etiology of abortion is multifactorial, involving both non-infectious and infectious causes. Nutritional deficiencies, metabolic disorders, hormonal imbalances, environmental

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stress, and management factors have all been implicated (Speer et al., 1999; Öncel and Bıyıkoğlu, 2003; Dubey et al., 2007; Tramuta et al., 2011). However, infectious agents are increasingly recognized as predominant contributors to abortion outbreaks, particularly under endemic conditions.

Among infectious causes, protozoan parasites have received considerable attention because of their persistence within herds, potential for vertical transmission, and capacity to cause both sporadic and epidemic abortion events. In this context, *N. caninum* and *T. gondii* are widely regarded as the principal protozoan pathogens associated with reproductive failure in ruminants, sharing heteroxenous life cycles, chronic infection potential, and complex epidemiological patterns that complicate control strategies (Dubey et al., 2006; Robert-Gangneux and Dardé, 2012).

N. caninum is currently considered one of the leading infectious causes of bovine abortion worldwide, with both endogenous transplacental transmission and horizontal transmission through oocyst ingestion contributing to herd infection dynamics (Öcal et al., 2014; Salehi et al., 2021). Similarly, *T. gondii* is recognized as an important abortifacient in small ruminants, particularly sheep, where infection may result in abortion, stillbirth, or neonatal mortality depending on gestational stage (Unzaga et al., 2014; Satbige et al., 2016).

Accurate diagnosis of protozoan-induced abortion remains challenging. Conventional methods such as serology, histopathology, and immunohistochemistry may have limited etiological value due to prior exposure without active infection, tissue autolysis, or uneven parasite distribution (Sebastiani et al., 2018; Meixner et al., 2020). In contrast, PCR-based molecular methods, particularly Real-Time PCR, have emerged as highly sensitive and specific tools for detecting protozoan DNA in fetal tissues, even in samples with low parasite burden or advanced autolysis (Sebastiani et al., 2018; Meixner et al., 2020). Recent studies emphasize that simultaneous molecular investigation of *N. caninum* and *T. gondii* is important for accurately defining their relative contribution to reproductive losses, although many investigations still focus on a single pathogen or host species.

In Türkiye, the presence of both parasites has been documented through serological, histopathological, and molecular studies (Öncel and Bıyıkoğlu, 2003; Ozkaraca et al., 2017), yet data on protozoan-associated abortions remain fragmented and regional epidemiological patterns are incompletely understood. The Marmara Region, one of the country's major livestock-producing areas, remains relatively underrepresented in molecular studies targeting abortifacient protozoa.

Therefore, the aim of the present study was to molecularly detect *N. caninum* and *T. gondii* in aborted bovine and ovine fetuses from the Marmara Region of Türkiye using Real-Time PCR. By providing region-specific molecular evidence, this study seeks to improve understanding of protozoan-associated reproductive losses and support future control strategies.

Materials and Methods

Study Area and Sample Collection

The Marmara Region, located in northwestern Türkiye, represents one of the country's major livestock production areas due to its favorable climatic conditions, intensive farming systems, and high animal population density. The region includes the provinces of Istanbul, Edirne, Kırklareli, Tekirdağ, Çanakkale, Kocaeli, Yalova, Sakarya, Bilecik, Düzce, Bursa, and Balıkesir and is considered among the most intensively utilized agricultural zones in Türkiye.

Between January 2019 and May 2021, a total of 306 aborted ruminant fetuses (84 bovine and 222 ovine) were collected from different provinces of the Marmara Region. Only naturally occurring abortion cases submitted for routine diagnostic investigation were included in the study, and no experimental infections were performed. From each fetus, tissue samples including brain, heart, lung, liver, and kidney were aseptically collected. Brain tissue was prioritized, and pooled tissue homogenates were prepared from the available organs for molecular analysis. All samples were stored at −20 °C until DNA extraction.

The distribution of abortion cases by province and host species is presented in Table 1. Sample numbers varied among provinces and reflected passive field submissions rather than structured sampling, which is commonly encountered in regional abortion investigations (Meixner et al., 2020). The study protocol was approved by the Local Ethics Committee of the Pendik Veterinary Control Institute (Approval date: 14.12.2018; Decision no: 196).

Table 1. Distribution of aborted ruminant fetuses collected from the Marmara Region of Türkiye according to host species and province

Province	Sheep (n)	Cattle (n)	Total (n)
Istanbul	3	3	6
Balıkesir	18	9	27
Çanakkale	47	14	61
Bursa	28	4	32
Tekirdağ	22	3	25
Kırklareli	15	13	28
Yalova	3	3	6
Kocaeli	12	6	18
Düzce	10	10	20
Sakarya	6	6	12
Edirne	47	13	60
Bilecik	11	0	11
Total	**222**	**84**	**306**

Methods

DNA Extraction

DNA was extracted from pooled homogenized fetal tissue samples using the High Pure PCR Template DNA Extraction Kit (Roche, Germany; Cat. No: 11796828001) according to the manufacturer's instructions. Brain tissue was prioritized for DNA extraction based on previous reports indicating higher parasite tropism and burden in protozoan-induced abortions, whereas other organs were included when brain tissue was unavailable or insufficient (Sebastiani et al., 2018, Meixner et al., 2020).

The concentration and purity of the extracted DNA were measured spectrophotometrically using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Samples with A260/A280 ratios between 1.8 and 2.0 were considered acceptable for downstream PCR analysis. DNA concentrations were adjusted to a final range of 20–40 ng/µL and stored at −20 °C until further use.

Real-Time PCR Assay

Detection of *N. caninum* and *T. gondii* DNA was performed using singleplex TaqMan-based Real-Time PCR assays adapted from Sebastiani et al. (2018) with minor optimization of reaction conditions. The Nc5 gene of *N. caninum* and the B1 gene of *T. gondii* were selected as molecular targets because of their high copy number, analytical specificity, and extensive validation in abortion diagnostics (Düzlü et al., 2014, Satbige et al., 2016, Salehi et al., 2021).

Each 20 µL reaction mixture contained 4 µL LightCycler® TaqMan Master Mix (2×) (Roche, Germany), 0.5 µL of each primer (10 µM), 1 µL of TaqMan probe (4 µM), 9 µL nuclease-free water, and 5 µL template DNA. Amplifications for *N. caninum* and *T. gondii* were performed in separate wells under identical thermal conditions. All samples were analyzed in duplicate. Each run included a no-template control (NTC) and commercially obtained plasmid DNA controls specific for *T. gondii* and *N. caninum*.

Thermal cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and combined annealing/extension at 60 °C for 30 s. A final cooling step was performed at 40 °C for 40 s. Analytical sensitivity and the cycle threshold (Ct) cut-off value were determined using 10-fold serial dilutions of plasmid DNA (10^{-1}–10^{-6}). Detectable amplification was observed up to the 10^{-5} dilution, yielding Ct values of 14, 20, 26, 33, and 36 across the dilution series, whereas no amplification was detected at 10^{-6}. Based on these results, Ct values ≤35 were considered positive, while Ct values >35 were interpreted as negative. The use of 45 amplification cycles was selected to maximize sensitivity in samples with low parasite burden, which is commonly encountered in naturally occurring abortion cases (Meixner et al., 2020).

Statistical Analysis

Statistical analyses were performed to determine the detection rates of *T. gondii* and *N. caninum* in aborted ruminant fetuses. Detection rates were calculated as the proportion of PCR-positive samples among the total number of examined samples. The 95% confidence intervals (95% CI) were calculated using the Wilson score method.

Differences in detection rates among provinces were evaluated using the Chi-square (χ^2) test. In addition, comparisons between host species were performed to assess differences in protozoan detection rates. Odds ratios (OR) with 95% confidence intervals were calculated to estimate the relative likelihood of protozoan-associated abortions between cattle and sheep. A p-value of <0.05 was considered statistically significant.

Results

Detection of *Toxoplasma gondii* in Ovine Abortions

A total of 222 ovine abortion samples were analyzed by Real-Time PCR for the presence of *T. gondii* DNA. Positive amplification was detected in 4 samples (1.8%). The provincial distribution of positive cases was as follows: Çanakkale (2/47; 4.26%), Tekirdağ (1/22; 4.55%), and Kocaeli (1/12; 8.33%). No *T. gondii* DNA was detected in samples originating from the remaining provinces. Additionally, none of the 84 bovine abortion samples yielded positive amplification for *T. gondii*. Representative amplification curves of positive samples and controls are shown in Figure 1.

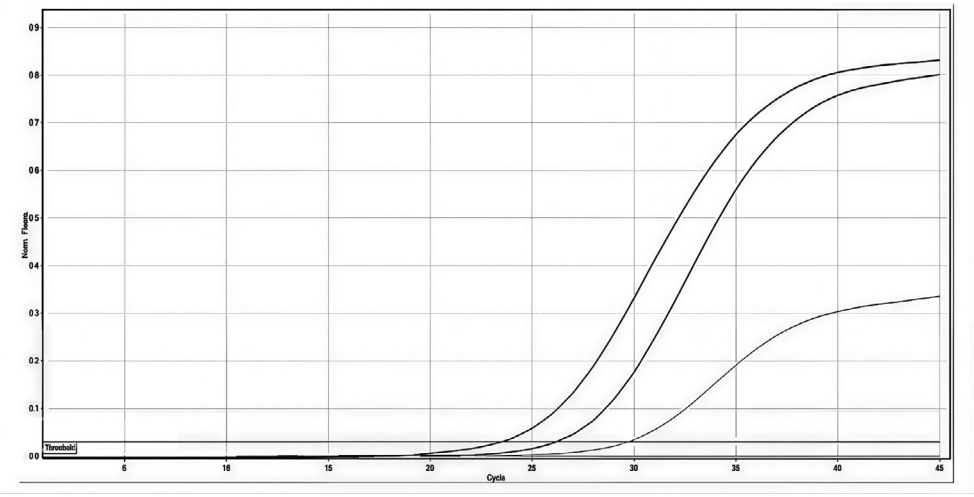

Figure 1. Real-Time PCR amplification curves for Toxoplasma gondii

Detection of *Neospora caninum* in Bovine Abortions

Among the 84 bovine abortion samples, *N. caninum* DNA was detected in 22 samples, corresponding to an overall positivity rate of 26.19%. Positive cases were identified in several provinces, with the highest detection rates observed in Kocaeli, Edirne, Istanbul, and Yalova. No *N. caninum* DNA was detected in any of the ovine abortion samples. Representative amplification curves of positive samples and controls are shown in Figure 2.

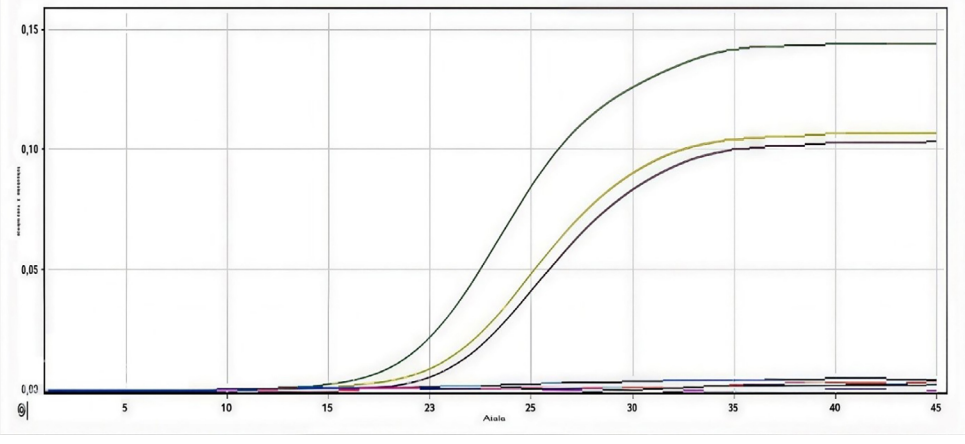

Figure 2. Real-Time PCR amplification curves for *Neospora caninum*

Statistical evaluation of protozoan detection rates

The overall detection rate of *T. gondii* in ovine abortion samples was 1.8% (4/222; 95% CI: 0.7–4.5). No statistically significant difference in detection rates among provinces was observed ($\chi^2 = 8.02$, $p = 0.71$).

In bovine abortion samples, *N. caninum* DNA was detected in 26.19% (22/84; 95% CI: 17.98–36.48) of the cases. Although detection rates varied among

provinces, the differences were not statistically significant (χ^2 = 7.04, p = 0.72).

The provincial distribution of PCR-positive abortion cases identified in the present study is summarized in Table 2. The table presents the number of examined samples together with the number of positive cases detected in each province. This distribution provides an overview of the geographical occurrence of *T. gondii* and *N. caninum* among the investigated abortion cases in the Marmara Region.

Table 2. Provincial distribution of protozoan detection in aborted ruminant fetuses

Province	Sheep samples (n)	*T. gondii* positive	Positivity rate (%)	Cattle samples (n)	*N. caninum* positive	Positivity rate (%)
Istanbul	3	0	0.00	3	1	33.33
Balıkesir	18	0	0.00	9	2	22.22
Çanakkale	47	2	4.26	14	2	14.29
Bursa	28	0	0.00	4	1	25.00
Tekirdağ	22	1	4.55	3	1	25.00
Kırklareli	15	0	0.00	13	2	15.38
Yalova	3	0	0.00	3	1	33.33
Kocaeli	12	1	8.33	6	3	50.00
Düzce	10	0	0.00	10	2	20.00
Sakarya	6	0	0.00	6	1	16.67
Edirne	47	0	0.00	13	6	46.15
Bilecik	11	0	0.00	0	0	–

Discussion

Reproductive efficiency remains a primary determinant of economic sustainability in ruminant production systems, and abortion continues to represent one of the most significant causes of productivity loss worldwide. Although the etiology of abortion is multifactorial, protozoan parasites such as *T. gondii* and *N. caninum* are widely recognized as major infectious abortifacient agents in small and large ruminants, respectively. In the present study, Real-Time PCR was employed as a highly sensitive molecular approach to investigate the presence of *T. gondii* and *N. caninum* DNA in aborted ovine and bovine fetuses from the Marmara Region of Türkiye. To the best of our knowledge, this study provides the first simultaneous Real-Time PCR-based molecular evidence targeting both *N. caninum* and *T. gondii* in ruminant abortion cases from this region.

The detection of *T. gondii* DNA in 1.8% of ovine abortion cases indicates a relatively limited molecular detection rate in the studied population. This value is lower than previously reported rates from other regions of Türkiye, such as Elazığ (8.6%) (Ozkaraca et al., 2017), and markedly below reports from South America, where PCR positivity of up to 24% has been documented (Unzaga et al., 2014). Such variability is well recognized in the epidemiology of toxoplasmosis and is commonly attributed to differences in environmental oocyst contamination, climatic conditions affecting oocyst survival, herd management practices, and methodological factors including sampling strategy and tissue selection (Robert-Gangneux and Dardé, 2012, Meixner et al., 2020).

Recent molecular reviews emphasize that PCR positivity in ovine abortion materials is frequently sporadic and generally low, even in regions with high seroprevalence, largely due to the focal distribution of parasites within fetal tissues and the transient nature of parasitemia (Sebastiani et al., 2018, Meixner et al., 2020). Therefore, the low detection rate observed in the present study should not be interpreted as absence of *T. gondii* circulation in the Marmara Region but rather as evidence that it may not represent the predominant abortifacient protozoan under the investigated field conditions.

In contrast, *N. caninum* DNA was detected in 26.19% of bovine abortion cases, supporting the substantial role of neosporosis in cattle reproductive losses in the Marmara Region. This finding aligns with the well-established status of *N. caninum* as one of the leading infectious causes

of bovine abortion worldwide (Dubey et al., 2006, Salehi et al., 2021). When compared with previous reports, the detection rate observed here can be considered moderate, falling below values reported from Türkiye (49.4%) (Açıcı et al., 2019) and Iran (45%) (Kamali et al., 2014), but exceeding those documented in Switzerland (16.1%) (Reitt et al., 2007) and Brazil (6.7%) (Cabral et al., 2009).

Regional variation in *N. caninum* detection is strongly influenced by herd-level transmission patterns, particularly the efficiency of vertical transmission and the degree of contact between cattle and definitive hosts (Salehi et al., 2021; Meixner et al., 2020).The Marmara Region is characterized by intensive livestock production and mixed farming systems, conditions that may facilitate environmental contamination when dogs have uncontrolled access to cattle premises and abortion materials. Such management factors could contribute to sustained horizontal exposure and help explain the molecular detection rate observed in this study.

It should be noted that the statistical comparison between provinces was limited due to the relatively small number of samples and positive cases in several provinces. Since the abortion materials were collected through passive submissions to the diagnostic laboratory rather than through a structured sampling design, the number of samples differed substantially among provinces. Therefore, the provincial distribution of positive cases was interpreted descriptively rather than as a basis for robust statistical comparison.

The application of Real-Time PCR in the present investigation likely enhanced the analytical sensitivity of protozoan detection. Real-Time PCR is widely regarded as one of the most sensitive and specific diagnostic tools for identifying protozoan DNA in abortion materials, particularly in samples affected by low parasite burden or autolysis (Hurtado et al., 2001, Espy et al., 2006). Comparative studies have consistently demonstrated superior performance of Real-Time PCR over conventional PCR and serological approaches for confirming the etiological involvement of *N. caninum* in bovine abortions (Sebastiani et al., 2018, Salehi et al., 2021).

In the present study, *N. caninum* DNA was not detected in ovine abortion samples, and *T. gondii* DNA was not identified in bovine cases, a pattern consistent with host-associated epidemiological trends reported in previous molecular investigations. Although cross-species infections are biologically possible, accumulated evidence indicates that *N. caninum* predominantly affects cattle, whereas *T. gondii* plays a more prominent role in small ruminant reproductive failure (Dubey et al., 2006, Robert-Gangneux and Dardé, 2012).

It should be emphasized that PCR-based assays detect parasite DNA and do not provide direct information on parasite viability or the precise timing of infection. Nevertheless, current consensus holds that the detection of protozoan DNA in fetal tissues constitutes strong molecular evidence supporting an etiological association with abortion, particularly when samples are collected directly from aborted fetuses (Meixner et al., 2020). Within this framework, the findings of the present study contribute meaningful region-specific molecular data on protozoan-associated abortions.

The relatively low detection of *T. gondii* in ovine abortions further suggests that other infectious agents may play a more prominent role in sheep reproductive losses in the Marmara Region. Previous studies in Türkiye have identified *Chlamydia abortus*, *Coxiella burnetii*, and *Leptospira* spp. among important contributors to ovine abortion (Arslan, 2019, Malal, 2020). Accordingly, multi-pathogen molecular screening strategies have been increasingly recommended to improve diagnostic yield and to better characterize the multifactorial nature of abortion syndromes (Sebastiani et al., 2018).

From a preventive standpoint, the present findings highlight the importance of biosecurity measures aimed at limiting environmental contamination with protozoan oocysts. Restricting access of dogs and cats to livestock facilities and abortion materials, ensuring proper disposal of fetal tissues, and implementing controlled replacement policies remain key components of integrated neosporosis and toxoplasmosis control programs. In the absence of fully effective vaccines or curative treatments, sustained molecular surveillance continues to represent a cornerstone of evidence-based control strategies (Salehi et al., 2021).

When evaluating the infection rates detected in the Marmara Region, the role of domestic and stray cat and dog populations in the epidemiology of both parasites warrants particular consideration. As the only known definitive hosts of *Neospora caninum*, dogs, including farm dogs and free-roaming strays, can initiate environmental contamination through oocyst shedding after ingestion of infected placental or fetal tissues. This transmission route constitutes a substantial risk, particularly in open-barn production systems where dogs may

have unrestricted access to livestock and abortion materials (McAllister et al., 1998). Several studies have reported a significant association between the presence of farm dogs and *N. caninum* seropositivity in cattle, emphasizing the importance of the dog–cattle transmission cycle in maintaining infection within herds (McAllister et al., 1998; Dubey & Lindsay, 2006).

With respect to *Toxoplasma gondii*, cats, as the sole definitive hosts capable of shedding environmentally resistant oocysts, represent a major source of contamination for susceptible intermediate hosts. These oocysts may persist in soil and water for prolonged periods under favorable environmental conditions, thereby increasing the likelihood of indirect transmission to grazing ruminants (Tenter et al., 2000). Both owned and free-ranging cats contribute to environmental contamination, and the growing stray cat population in both urban and rural areas of the Marmara Region may represent an important reservoir for pasture and water source contamination (Dubey, 2010). Epidemiological studies in livestock systems have consistently demonstrated that *T. gondii* seroprevalence in small ruminants is associated with local felid density, with animals exposed to cat-contaminated grazing areas showing markedly higher infection rates (Opsteegh et al., 2011).

In addition, the presence of cats and dogs, grazing management, herd size, and farm hygiene have been identified as significant risk factors associated with seroprevalence of both *T. gondii* and *N. caninum*, a pattern also reflected in studies from neighboring countries and Türkiye, where increased stray animal density has been associated with elevated exposure rates in ruminants (Yıldız et al., 2017). Collectively, these findings suggest that prevention of *N. caninum*- and *T. gondii*-associated abortions requires not only herd-level management strategies but also a broader One Health-oriented approach integrating farm biosecurity, control of stray animal populations, and restriction of canine and feline access to livestock housing, feed storage areas, and abortion materials.

In conclusion, the Real-Time PCR-based results of this study indicate that *Neospora caninum* constitutes a significant contributor to bovine abortions, whereas *Toxoplasma gondii* appears to play a comparatively limited role in ovine abortion cases in the Marmara Region. These findings underscore the diagnostic value of molecular approaches and support the need for broader, multi-agent surveillance studies to further elucidate the epidemiology of reproductive losses in ruminant populations.

Conclusion

This study provides region-specific molecular evidence for the occurrence of *Toxoplasma gondii* and *Neospora caninum* in ruminant abortions in the Marmara Region of Türkiye based on Real-Time PCR analysis. The detection of *N. caninum* DNA in a considerable proportion of bovine aborted fetuses (26.19%) underscores its significant role as a major etiological agent of reproductive failure in cattle in the region. In contrast, the low detection rate of *T. gondii* DNA in ovine abortion cases (1.8%) suggests a comparatively limited contribution of this parasite to abortion etiology under the investigated regional conditions.

The findings further demonstrate the value of Real-Time PCR as a sensitive and specific tool for the direct detection of protozoan DNA in aborted fetal tissues, particularly in cases characterized by low parasite burden. These results highlight the importance of sustained molecular surveillance for the accurate identification of protozoan-associated abortions and for supporting evidence-based herd health management strategies.

Future investigations incorporating larger sample sizes, broader temporal coverage, and parallel assessment of definitive hosts are warranted to more comprehensively elucidate the epidemiology of these protozoan infections and to strengthen control measures aimed at reducing reproductive losses in ruminant populations.

Ethical Approval: Ethical approval for the study was obtained from the Ethics Committee of the Pendik Veterinary Control Institute (Pendik, Türkiye) on 14 December 2018 (Approval No: 196).

Author Contributions: Mesut Şenel: Writing – original draft; analysis.

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Conflict of Interest: The author declares that there are no conflicts of interest.

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