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Comparative Analysis of *Neospora caninum* Isolation Success from Various Fetal Tissues: The Importance of Brain Samples. Technical note

Análisis comparativo del éxito del aislamiento de *Neospora caninum* a partir de diversos tejidos fetales: la importancia de las muestras cerebrales. Nota técnica

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ABSTRACT

Isolating *Neospora caninum* from naturally infected hosts is crucial for understanding the epidemiology of neosporosis. Although the parasite was reported to be isolated from various fetal tissues, including the brain, the relative successful isolation rates from different sources have not been systematically explored. This study examines the rates of isolating *N. caninum* from different fetal tissues, focusing on the importance of brain samples. Fetal tissues from 90 naturally infected sheep were analyzed for the presence of *N. caninum*. Samples were processed for molecular and cell culture isolation techniques. Tachyzoite yields and cytopathogenic effects (CPE) were compared across tissues following isolation in Vero cells. Of the 90 samples, 19 (21.1%) were positive for *N. caninum* by PCR, and 8 (42.1%) of these yielded viable isolates. Sheep brain tissue exhibited significantly higher tachyzoite yields (1.9 x 107) compared to other tissues (abomasal contents, kidney, placenta, liver and pericardial fluid). The greatest CPE effect at 80% was observed in the eighth passage. Brain samples appear to be the most reliable source for *in vitro* isolation of *N. caninum* from naturally infected fetuses. This finding has important implications for the diagnosis and study of sheep neosporosis.

Key words: Abortion; brain, *Neospora caninum*, sheep, tissue culture isolation

RESUMEN

El aislamiento de *Neospora caninum* a partir de huéspedes infectados naturalmente es crucial para comprender la epidemiología de la neosporosis. Aunque el aislamiento del parásito de varios tejidos fetales, incluido el cerebro, ha sido mencionado, las tasas relativas de éxito del aislamiento a partir de diferentes fuentes no han sido exploradas sistemáticamente. Este estudio examina las tasas de aislamiento de *N. caninum* a partir de diferentes tejidos fetales, centrándose en la importancia de las muestras cerebrales. Se analizaron los tejidos fetales de 90 bovinos y ovinos infectados naturalmente para detectar la presencia de *N. caninum*. Las muestras se procesaron con técnicas de aislamiento molecular y de cultivo celular. Se compararon la producción de taquizoítos y los efectos citopatógenos (ECP) en los tejidos después del aislamiento en células Vero. De las 90 muestras, 19 (21,1 %) dieron positivo para *N. caninum* por PCR y, de estas, 9 (47,36 %) dieron lugar a aislamientos viables. El tejido cerebral de ovejas exhibió una producción de taquizoítos significativamente más alta (1,9 x 107) en comparación con otros tejidos (contenido abomasal, riñón, placenta, hígado y líquido pericárdico). El mayor ECP se observó en el octavo pasaje, al 80%. Las muestras de cerebro parecen ser la fuente más confiable para el aislamiento in vitro de *N. caninum* a partir de fetos infectados naturalmente. Este hallazgo tiene implicaciones importantes para el diagnóstico y el estudio de la neosporosis ovina.

Palabras clave: Aborto; aislamiento de cultivo celular, cerebro, *Neospora caninum*, ovejas

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INTRODUCTION

The protozoan parasite *Neospora caninum* belongs to the phylum Apicomplexa and is an intracellular, obligate coccidian [1]. Dogs (*Canis lupus familiaris*), wolves (*Canis lupus*), dingoes (*Canis lupus dingo*) and jackals (*Canis aureus*) are the final hosts of *N. caninum*, while cattle (*Bos taurus*), sheep (*Ovis aries*) and goats (*Capra hircus*) are intermediate hosts [2]. The horizontal route (contaminated food and water) of *N. caninum* infection in pregnant cattle progresses as spore-like oocysts shed in the faeces of infected dogs develop into tachyzoites in the intestinal wall and the fetus becomes congenitally infected [1, 3].

Tachyzoites, tissue cysts, and oocysts are the three stages of the parasite's life cycle, and it primarily affects ruminants as intermediate hosts and dogs as definitive hosts [1]. *N. caninum* causes abortion in sheep, goat, and cattles [4], resulting in important economic losses to farmers and the livestock industry [2]. Although the economic loss for most farms is often estimated at 2-5% per year, in some cases, it can be as high as 20% [5]. This pathogen, which spreads both horizontally and vertically in herds, can cause stillbirth, abortion or the birth of an asymptomatic animal infected through placental infection [1].

According to Dubey *et al*. [6], the incidence of Neospora-associated abortion may follow a sporadic, endemic or epidemic trend. Although animals that have never been exposed to the pathogen are protected from epidemics by ingesting it from a single source of contamination, the pathogen can still spread by transplacental contamination in the form of chronic patterns [3].

The virulence of the pathogen, the stage of gestation or the immunity of the animal can all influence the development of infection [7]. Congenital transmission rates range from 50 to 95% and are a key factor in maintaining the parasite within herds. Therefore, it indicates the nature and duration of its presence in the herd [8]. There are practical methods of controlling *N. caninum* infection on farms, including the test-and-cull strategy and immunisation [9, 10]. There is currently no viable commercial vaccine for neosporosis, although vaccination is the greatest alternative control option [5].

There have been reports [11, 12] of *N. caninum* being recovered from cell cultures exposed to fetal brain, spinal cord and kidney homogenates. No reports of *N. caninum* being isolated from abomasal material or placenta exist.

According to Garcia-Sánchez *et al*. [13], differences in the pathogenesis of isolates significantly impact the ability of the host to mount a defence against infection. The high-virulence strains (Nc-Spain7) are more likely to invade, survive and replicate in bovine macrophages than the low-virulence strains (Nc-Spain1H) [13, 14]. *N. caninum* is difficult to isolate and attempts to do so often fail [12]. In addition, prolonged passage can alter pathogenicity and other aspects of the parasite in vivo [15]. Although culture passages greater than 15 can alter tachyzoite virulence [13], there is no information on the overall changes in the number of acquired tachyzoites associated with serial passage of cell culture. For the production of vaccines, the highest yield and ideal passage number are crucial. Therefore, it is important to establish a stock of the *N. caninum* master seed strain by monitoring and reporting passage numbers in trials [16].

The objectives of this study were to investigate *N. caninum* in different aborted lamb tissue samples, to compare tachyzoite yields obtained from cell cultures of positive tissues, to track cytopathogenic changes in cells as a function of passage number, to investigate tachyzoite yields as a function of passage number, and to determine the sample type to be used in isolation studies.

MATERIALS AND METHODS

Sampling

In this study, 90 tissue samples (abomasum content, kidney, placenta, brain, liver, pericardial fluid) from 90 aborted lambs were used (TABLE 1). These samples were sent for routine diagnosis at different times from various farms in five regions of Turkey. All aborted fetuses were in the late pregnancy. These animals did not show any clinical signs other than abortion, and the foetal samples did not show any decomposition. The aborted foetal samples were delivered to the laboratory in a cold container.

PCR protocol

Genomic DNA (gDNA) was isolated from each individual aborted lamb tissue sample using the commercial purification kit (Promega, USA). The gDNA samples were stored at -20°C (KD42VX00NE, Siemens, Germany) until PCR analysis (T100 termal cycler, Biorad, USA). The PCR reaction mixture was 25 µL in total (0.6 µL of each primer (NcgeneF 5′-CCCAGTGCGTCCAATCCTGTAAC-3′ and NcgeneR 5′-CTCGCCAGTCAACCTACGTCTTCT-3′) [17], 50 ng/µL of DNA template, 5× FIREPol® Master Mix (SolisBiodyne, Estonia), and pure water enough to complete the reaction.The PCR primers and conditions were those previously described by Tramuta et al. [17]. The NC-1 isolate of N. caninum was used as a positive control. This strain was kindly provided by the Razi Vaccine & Serum Research Institute, Agricultural Research, Education & Extension Organization (AREEO), Iran. PCR products were separated by (EC120 Mini Vertikal Jel Sistem, Thermo Scientific, USA) on 1.5% agarose gels at 60 mA for 1 h, stained with ethidium bromide and visualised under UV (UVP Ultraviolet Transilluminator, Thermo Scientific, USA). Molecular weight sizes were determined by comparison with a 50 bp DNA ladder plus (Thermo Scientific, SM0373).

Preparation of the samples for cell culture

The NC-1 isolate of N. caninum was used as a positive control for cell culture procedure. The samples detected as positive by PCR were cut into pieces with sterile scalpels. One gram of each sample was used. Host cell debris was separated by three passes with a 25-gauge needle [18]. These lysates were mixed with 9 mL phosphate-buffered saline (PBS, pH 7.4), 8 g/L fluconazole, and 1 mg/L vancomycin and incubated at 37 °C for 16 hours, and then the tachyzoites were collected by centrifugation (Heraeus megafure 8R centrifuge, Thermo Scientific, USA) at 400 g x 10 min.

Isolation of the tachyzoites

Cell culture was performed in a precision HEPA-filtered Glove Box BSL 3 biosafety cabinet (Labconco). Tachyzoites of N. caninum isolates were grown on a 24-h-old monolayer of African green monkey (Vero) cells in Glasgow Minimum Essential Medium (GMEM) supplemented with Streptomycin/mL (100 µg) (Invitrogen, USA), Penicillin/mL (50 U) (Invitrogen, USA), and 10% fetal calf serum (FCS) (Gibco) at 37 °C with 5% CO2 (BB15, Thermo Scientific) for 15 d. This technique was performed eight times in serial passages in order to adapt the isolates and obtain larger quantities of tachyzoites. The collected tachyzoites were centrifuged three times at 600 g for 15 min in cold PBS. A minimum of 1x106 cells were counted and added to culture flasks with a final volume of 75 mL [19, 20]. On regular d, fresh medium and cell morphology controls were added using an inverted microscope with a trinocular imaging system (DMi1, Leica, Germany).

Determination of the tachyzoite amount

The tachyzoites were extracted from infected cell cultures after lysis of 80-90% of the Vero host cells (cytopathic effect, CPE) [9, 11]. An inverted microscope (DMI1, Leica, Germany) was used to measure CPE on cells. Tachyzoite viability was also validated by Giemsa, as well as trypan blue staining. The isolation capacities of different sources and the effects of cell culture passage number were investigated based on the presence of tachyzoites. Samples from the same source (abomasum content, kidney, placenta, brain, liver, pericardial fluid) were compared to determine the optimal source for isolation and four samples were taken at different passage times (1st, 3rd, 5th and 8th). Tachyzoite yields were then determined using Real-time PCR (CFX96 Touch Real-Time PCR, Bio-Rad, USA) [21] and a cell counter (TC20, Biorad, USA) [22, 23].

Briefly, the manufacturer's protocol was followed to obtain tachyzoite DNA from collected cells using the DNA Blood and Tissue Kit (Omegabiotek). The reaction mixture contains 10 µL of 50 ng/mL of DNA template, 2 SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), and 0.1 µL of each primer (NC5F and NC5R) in a total volume of 20 µL. Real-time PCR conditions were carried out according to the protocol described by Abdelbaky et al [21]. An NC-1 isolate of N. caninum was used as a positive control. Real-Time PCR analysis was made using a thermal cycler (CFX96 Touch Real-Time PCR, Bio-Rad, USA). A cycle threshold (Ct) value of 30 for N. caninum was considered positive. Once the presence of tachizoites was confirmed, the number of tachyzoites found in the supernatant at the end of the passage was counted. It was purified by centrifugation in Ficoll 400 (Sigma Aldrich) as previously described [22, 23] and then counted using a cell counter (TC20, Biorad, USA).

RESULTS AND DISCUSSION

N. caninum was found in 19 of the 90 (21.1%) aborted fetal samples by PCR (FIG. 1). Abomasal contents were the most frequently tested sample from aborted fetuses (n=41). While the lowest positivity was determined from abomasum contents with 19.51%, the highest positivity was detected in brain samples with 50%. Interestingly, none of the 15 liver samples were positive by PCR.

Eight of the 19 PCR-positive samples were successfully cultured. Among the tissues determined to be positive by PCR, the lowest number of strains adapted to cell culture was from placenta samples (33.3%), and the highest percentage was from brain samples (100%) (TABLE 1) (FIG. 1).

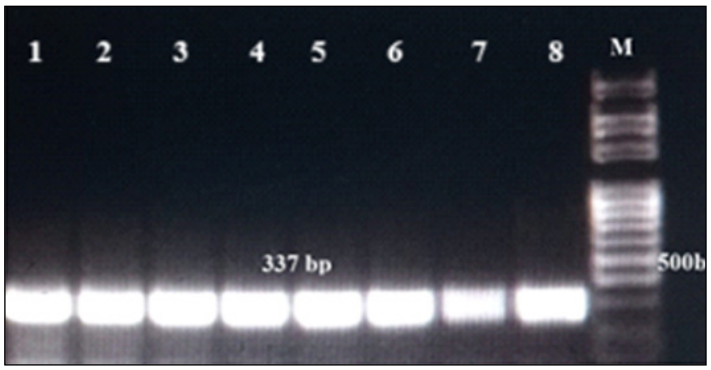

FIGURA 1. Confirmation of the presence of *Neospora caninum* in aborted fetal tissues by classical PCR. M: 100 bp marker, 1: Positive control, NC-1 isolate of Neospora caninum 2-8: Positive samples (337 bp)

TABLE 1
Detection of *Neospora caninum* from aborted foetal lambs tissue by PCR and isolation in cell culture

Isolate sources	Number of samples (n)	Positive by PCR assay (n)	Cell culture positive samples (n)
Abomasum content	41	8 (19.51%)	3/8 (37.5%)
Kidney	20	5 (25%)	2/5 (40%)
Placenta	7	3 (42.85%)	1/3 (33.3%)
Brain	4	2 (50%)	2/2 (100%)
Liver	15	-	-
Pericardial fluid	3	1 (33.3%)	-
Total	90	19 (21.1%)	8/19 (42.1%)

Comparing it with the growth curve of *N. caninum* NC-1 isolate as a positive control, the best-adapted N. caninum field strain was determined. In addition, the tachyzoite yields obtained increased according to the number of passages, and the increase in the number in the eighth passage was detected by Real time-PCR (FIG. 2).

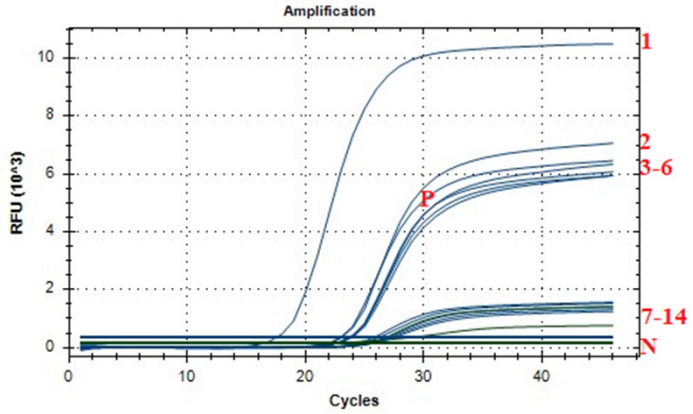

FIGURA 2. NC: Negative control, 1: Best adapted *Neospora caninum* field strain, 2: Positive control, NC-1 isolate of *Neospora caninum*, 1: Best adapted *Neospora caninum* field strains in eighth passage, 3-6: *Neospora caninum* field strain in fifth passage, 7-10: *Neospora caninum* field strain in third passage, 11-14: *Neospora caninum* field strain in first passage

The quantity and presence of tachyzoites varied according to the type of source (abomasal contents, kidney, placenta and brain), strain adaptation to cells and the number of passages. The CPE of *N. caninum* field strains was examined at the first, third, fifth and eighth passages, with the eighth passage having the greatest effect. The greatest CPE effect at 80% was observed in the eighth passage from brain isolates (FIG. 3, FIG. 4). When the number of tachyzoites obtained from different samples was compared, the highest tachyzoite yield (1.9x107) was obtained from fetal brain tissue (TABLE 2).

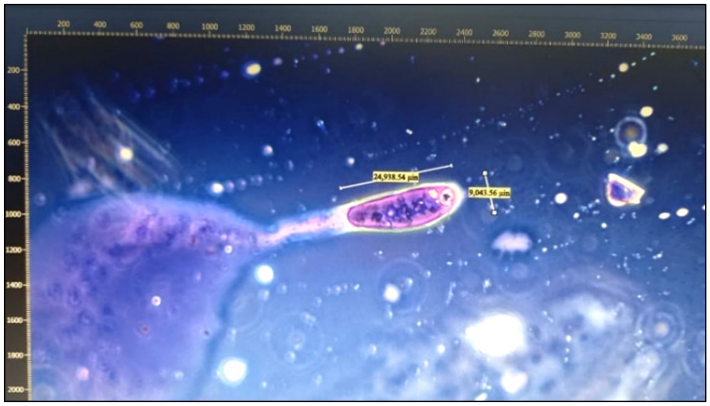

FIGURA 3. a) Tachyzoites presence shown by trypan blue in the cell culture, microscopic presentation (40x magnification)

TABLE 2
The average amount of tachyzoites (cell/ml) obtained from abomasum content, kidney, placenta, and brain samples according to the number of passages in cell culture

Passage/Sample type	1st	3rd	5th	8th
Abomasum content	1,03x10⁶	1,93x10⁶	2,15x10⁶	2,58x10⁶
Kidney	2,5x10⁶	2,92 x10⁶	2,5x10⁶	3,2x10⁶
Placenta	2,05x10⁶	2,55 x10⁶	4,05 x10⁶	5,4 x10⁶
Brain	6,05x10⁶	6,45x10⁶	9,85x10⁶	1,9x10⁷

It has been reported that *N. caninum* infection in sheep has a pathogenesis similar to that in cattle, causing recurrent abortions [24, 25, 26, 27, 28, 29]. Sheep can be used as a useful model for the study of infection. Although serological studies in sheep populations provide an idea about the contact with the agent, there is insufficient data on the prevalence of this infection in sheep [30, 31, 32]. Instead of results based on the measurement of the immune response [33], detection and quantification of the parasite and histopathological evaluation are important for neosporosis [34]. The target tissue has been identified as the central nervous system in fetuses infected with *N. caninum* in mid-gestation, in cattle [35] and sheep [36]. Therefore, histopathological examination of the fetal lamb brain appears to be the most sensitive diagnostic test for *N. caninum*-associated abortions [37]. However, brain-negative foetuses from multiple

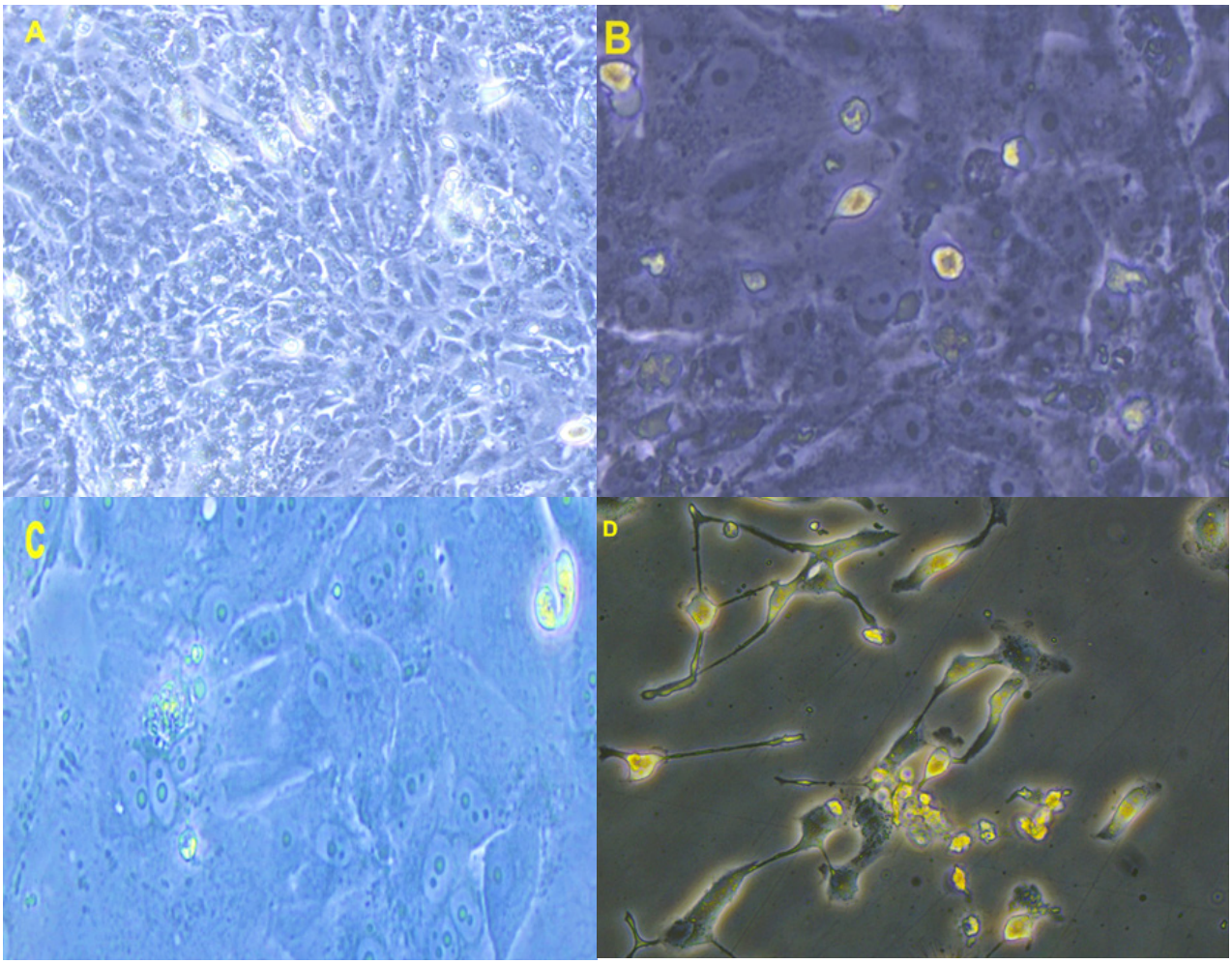

FIGURA 4. View of the cytopathogenic effect of Neospora caninum field strain related to growing in the Vero cell line at the end of the different passages from brain samples. A) First passage, B) Third passage, C) Fifth passage, D) Eighth passage

pregnancies in ewes infected mid-pregnancy have been reported [36]. In one study, foetuses and lambs with PCR-negative brains were found to be seropositive by IFAT and brain lesions were detected in their brains, suggesting a very low parasite burden [38]. In this study, 2 of 4 brain isolates were determined as positive by both PCR and culture. Brain samples have a higher yield in cell culture and could be an appropriate source to culture for further studies.

N. caninum has been isolated from dogs and some intermediate hosts such as sheep, cattle, buffalo, and white deer [6,39]. Owing to the global significance of bovine neosporosis, most of these strains have been isolated from calves and adult cattle [6] and characterized in cell culture and experimental models [12]. There are a few reports of the isolation of *N. caninum* from sheep [13, 20] and goats [40].The brain, kidney, umbilical cord, liver and heart of an aborted fetus can be used to investigate the rate of neosporosis in animals using molecular testing [41]. In a former study, it was found that kidney samples had the highest rate compared to brain samples, our findings differed from previous studies where rates ranged from 1.6% to 9.85% in the brain [32, 42]. This agent was found in the abomasal contents of aborted fetuses at a rate of 19.5% (8/41), although there is no data on N. caninum in these samples. The cell culture system has not been extensively used because it is time-consuming, difficult, expensive and somewhat insensitive [43].

Although various isolation approaches using different cell lines are accessible [11, 15], thorough investigations on different tissues are not available. According to our results, brain samples were quite useful for obtaining the highest tachyzoite yields, and this information is important for future research.

CONCLUSION

It is important to determine the best isolation protocol for *N. caninum* and to ensure adaptation with repeated passages. In addition, the type of sample used plays a critical role. This study showed that the fetal brain was suitable for obtaining the strongest cytopathogenic effect on cells, the highest tachyzoite production, and adaptation to the serial passages. Therefore, it is thought that abortion lamb brain samples will be useful in obtaining high amounts of tachyzoites.

Author contributions

All authors contributed to the design and implementation of the study. Data collection, methodology, and interpretation were performed by AB, AI, BP, FC, OE, and EET. AB and SS wrote the first draft of the manuscript, and all authors commented on it. All authors read and approved the final manuscript.

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Data availability

All relevant data and materials are included in the manuscript.

Conflict of interest

There is no personal or financial situation between the authors that could create a conflict of interest.

Ethics approval

The Faculty of Veterinary Medicine Ethics Committee at Selcuk University in Konya, Turkey, approved the study (Decision number: 2020/70).

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