HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Investigation of the Role of Some Selected Virus Species in Abnormality of Calves

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Abstract: Early embryonic death, fetal resorption, fetal mummification/maceration, abortion, and abnormalities are some of the causes of losses in farm animals. In this study, it was aimed to make recommendations to clinician veterinarians by investigating the presence of bovine viral diarrhea virus (BVDV), Schmallenberg virus (SBV), Akabane virus (AKAV), and bovine herpesvirus-1 (BHV-1) viruses in calves with congenital anomalies. A total of 34 calves of different breeds and sexes, between 1 day and 2 months of age which were brought to Fırat University Animal Hospital for diagnosis and treatment and found to have congenital anomalies after a general clinical examination during visits to the neighboring villages, were included in the study. The abnormalities detected in the calves were as follows: Hydrocephalus, cerebellar hypoplasia, brahignati inferior, mandibular laterognathism, arthrogryposis, cheilognathopalatoschisis, coccygeal agenesis, atresia ani, bouleture/arqure, ocular and orbital deformities, dermoid cysts, miniature, renal and urinary system anomalies. PCR results showed pestivirus was detected in 6 of 34 (17.6%) calves with abnormalities. After sequencing, the expected PCR product (288 nucleotides) for pestivirus showed high identity (97% and above) to the BVDV-1 strain/isolates in the GenBank. In conclusion, although the primary purpose of cattle breeding is high meat and milk yield, it is also very important to continue the production process with healthy newborn calves. For this reason, even if congenital anomalies are seen at a low rate in calves, they should not be underestimated as they will cause economic losses, and we believe that clinician veterinarians should not ignore these diseases.
Keywords: *Calf, Congenital anomalies, Virus.*

Buzağı Anomalilerinde Seçilen Bazı Virüs Türlerinin Rolünün Araştırılması

Özet: Erken embriyonik ölüm, fetal rezorpsiyon, fetal mumyalama/maserasyon, abort ve anormallikler çiftlik hayvanlarında kayıpların nedenlerinden bazılarıdır. Bu çalışmada, kongenital anomalili buzağılarda sığır viral diyare virüsü (BVDV), schmallenberg virüsü (SBV), akabane virüsü (AKAV) ve sığır herpes virüsü-1 (BHV-1) virüslerinin varlığı araştırılarak klinisyen veteriner hekimlere önerilerde bulunulması amaçlanmıştır. Fırat Üniversitesi Hayvan Hastanesi'ne teşhis ve tedavi için getirilen ve civar köylere yapılan ziyaretlerde genel klinik muayene sonrasında kongenital anomalisi olduğu belirlenen değişik ırk ve cinsiyetteki, 1 günlük ila 2 aylık yaş aralığında bulunan 34 buzağı çalışmaya alınmıştır. Buzağılarda tespit edilen anormallikler: hidrosefali, serebellar hipoplazi, brahignati inferior, mandibular laterognatizm, artrogripozis, cheilognatopalatoşizis, koksigeal agenezi, atresia ani, bouleture/arqure, oküler ve orbital deformiteler, dermoid kistler, minyatür, renal ve üriner sistem anomalileri. PCR sonuçlarına göre anomalili 34 buzağının 6'sında (%17,6) pestivirüs tespit edildi. Sekanslamanın ardından pestivirüs için beklenen PCR ürünü (288 nükleotit), GenBank'ta BVDV-1 suşu/izolatlarına yüksek özdeşlik (%97 ve üzeri) gösterdi. Sonuç olarak, sığır yetiştiriciliğinde birincil amaç yüksek et ve süt verimi gibi görünse de sağlıklı yeni doğan buzağılarla üretim sürecinin devam ettirilmesi de oldukça önemlidir. Bu nedenle buzağılarda doğumsal anomaliler düşük oranda görülse bile ekonomik kayıplara neden olacağı için hafife alınmaması ve klinisyen veteriner hekimlerin bu hastalıkları göz ardı etmemeleri gerektiğine inanıyoruz.
Anahtar Kelimler: *Buzağı, Kongenital anomali, Virüs.*

Introduction

Early embryonic death, fetal resorption, fetal mummification/maceration, abortion, and abnormalities are some of the causes of losses in farm animals (Doğan and Bilge Dağalp, 2017; Gül ve İssi, 2009; Gül et al., 2013; Gürçay et al., 2013; İssi et al., 2012; İssi et al., 2013). Congenital abnormalities, also called congenital malformation, congenital defects, or teratogenicity can be defined as structural or functional disorders that appear after birth (Aksoy, 2001; Belge et al., 2000). Although congenital abnormalities are rarely seen in farm animals, it is important that they cause economic losses and be transferred on to the next generations as hereditary (Belge et al., 2000; Berber and Sözdutmaz, 2013; Yurdakul 2020). Among the factors that cause these cases, the contribution of viruses is at a level that cannot be neglected (Berber and Sözdutmaz, 2013; Doğan and Bilge Dağalp, 2017; Gürçay et al., 2013; İssi et al., 2012; İssi et al., 2013; İssi et al., 2014). Viruses, which are also teratogenic infectious agents, often cause malformations by affecting the nervous and musculoskeletal systems (Tamer and Okur Gumusova, 2017; Vandevelde et al., 2012). These malformations hydranencephaly, porencephaly, hypomyelinogenesis, demyelinogenesis, cerebellar hypoplasia, cerebellar dysplasia, and hydrocephalus in the nervous systems and are muscular atrophy and arthrogryposis in the musculoskeletal system (Maxie, 2015; Tamer and Okur Gumusova, 2017). Viral agents that cause congenital malformation as a result of fetal transplantation in cattle include bovine viral diarrhea virus (BVDV), schmallenberg virus (SBV), akabane virus (AKAV), bluetongue virus (BTV) or aino virus (AV) (Agerholm, 2015). In addition to respiratory system infections, bovine herpesvirus 1 (BHV-1) is known to cause genital tract infections and abortions (Doğan and Bilge Dağalp, 2017).

Bovine viral diarrhea virus is a major pathogen associated with gastrointestinal, respiratory, and reproductive disorders of cattle worldwide. BVDV as an enveloped, small (about 40-60 nm) positive polarity RNA virus is a member of the genus Pestivirus, family *Flaviviridae* (MacLachlan and Dubovi, 2011). The International Committee on Virus Taxonomy has defined eleven species in the pestivirus genus (A to K). Pestivirus A (BVDV-1), Pestivirus B (BVDV-2), Pestivirus D (Border disease virus), Pestivirus H (HoBi-like pestivirus), Pestivirus I (Aydin-like pestivirus) have been reported in ruminant animals (King et al., 2012; King et al., 2018). BVDV-1 subtypes range from 1a to 1q, while BVDV-2 subtypes range from 2a to 2d. BVDV-3 (pestivirus H or HoBi-like pestivirus), a member of the pestivirus genus, has been identified in cattle and buffalo (Mishra et al., 2014). BVDV consists of two biotypes (cytopathic/non-cytopathic) according to the morphological character in cell culture (Fulton et al., 2000).

Bovine herpesvirus type 1 (BHV-1) is a member of the genus Varicellovirus in the subfamily *Alphaherpesvirinae*, *Herpesviridae* family. The BHV-1 genome is approximately 135 kb in size, enveloped, double-stranded DNA virus (Muylkens et al., 2007). BHV-1 has three different subtypes, BHV-1.1, BVH-1.2a, and BHV-1.2b, according to genetic differences and varieties in clinical signs (Metzler et al., 1985). BHV-1.1 and BVH-1.2a cause respiratory and genital system disorders and abortions. BVH-1.2b is low pathogenic when compared with the other sub-types (Miller et al., 1991, Spilki et al., 2004).

AKAV and SBV which are enveloped and negative polarity RNA viruses are members of the Orthobunyavirus genus, which belongs to the *Peribunyaviridae* family. AKAV is closely associated with SBV viruses and causes reproductive disorders in cattle, sheep, and goats, as well as congenital abnormalities such as arthrogryposis-hydranencephaly (AH syndrome) (Kurogi et al., 1985; Maclachlan and Dubovi, 2016). SBV was first discovered in Germany in 2011 and has since affected nearly all of Europe (Lievaart-Peterson et al., 2015). Pestivirus and BHV-1 infections are important problems for Türkiye (Aslan et al., 2015; Berber and Sözdutmaz, 2013; İssi et al., 2012).

In this study, it was aimed to make recommendations to clinician veterinarians by investigating the presence of BVDV, SBV, AKAV and BHV-1 viruses in calves with congenital anomalies.

Materials and Methods

Ethical Approval: The study was approved by the Fırat University Experimental Animals Local Ethics Committee (Decision number: 2020/07).

Clinial Specimens: This study was carried out in the province of Elazig, located in the eastern Anatolian region of Türkiye, in the second half of 2020. Brought to Fırat University Animal Hospital for diagnosis and treatment, and found to have congenital anomalies after general clinical examination during visits to surrounding villages, they were classified as different races (25 Simmental, 4 Holstein, 2 Montafon, 2 East Anatolian Red, 1 Hybrid) and (28 male, 6 females) 34 calves aged between 1 day and 2 months were included in the study. It was learned from the anamnesis information that 18 of the animals included in the study were conceived by artificial insemination and 16 by natural insemination.

The abnormalities detected in the calves were as followed: Hydrocephalus (n:1), cerebellar hypoplasia (n:1), brahignati inferior (n:5), mandibular laterognathism (n:1), arthrogryposis (n:1), cheilognathopalatoschisis (n:2), coccygeal agenesis (n:2), atresia ani (n:4), bouleture/arqure (n:4), ocular and orbital deformities (n:7), dermoid cysts (n: 2), miniature (n:2), renal and urinary system anomalies (n:2).

Blood samples taken from the jugular vein of the calves included in the study were collected into anti-coagulant tubes (with EDTA). After the blood tubes were kept at +4 ^{o}C overnight, they were centrifuged at 1500 xg for 10 minutes and plasma was obtained. The obtained plasmas were transferred to sterile eppendorf tubes and stored at -20 ^{o}C until used.

Viral Nucleic Acid Isolation: Viral nucleic acid isolation from blood plasma was performed using the QIAamp MinElute Virus Spin kit (Qiagen, Germany) and strictly

following the kit's instructions for use. Finally, viral nucleic acids were collected in 100 µL of elution buffer and quantified by measuring in a NanoDrop 2000 spectrophotometer (ThermoFisher, USA).

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR): AKAV, SBV, BVDV, and BHV-1 were detected according to the PCR conditions specified in the reference, using the primer sets in Table 1.

Table 1. Primer pairs using in PCR and RT-PCR step.

Viral agent	Primer	Region	Nucleotide sequence 5'->3'	PCR product	Reference
AKAV	AKAF1	S gene	TAACTACGCATTGCAATGGC	709	Shin et al., 2009
	AKAR1		TAAGCTTAGATCTGGATACC		
	Nested F2		GAAGGCCAAGATGGTCTTAC	230	
	Nested R2		GGCATCACAATTGTGGCAGC		
BHV-1	gB1	gB	AAGCGCAAAAACGTGTG	323	Santurde et al., 1996
	gB2		TGCAGGTACAGCTTGGC		
Pan-pestivirus	P324	5'UTR	ATGCCCWTAGTAGGACTAGCA	288	Vilcek, 1994
	P326		TCAACTCCATGTGCCATGTAC		
BVDV-3	TF-3	N^{pro}	ATGGAGTTGTTAAACTTTGAAC	494	Liu, 2009
	TR-3		GCAGCTTCCTACCCAGATGG		
SBV	SBV-S-382F	S gene	TCAGATTGTCATGCCCCTTGC		Tonbak et al., 2016
	SBV-S-469R		TCAGATTGTCATGCCCCTTGC		
	SBV-S-408FAM		FAM-TTAAGGGATGCACCTGGGCCG ATGGT-BHQ1		

PCR conditions for BHV-1 detection were performed as follows: 2 × concentrated PCR master mix solution (Hibrigen, Ist), 5 µL DNA template, 4 µL primer mix (forward and reverse primers), and PCR grade water in a 50 µL reaction. RT-PCR was performed in a 50-µL reaction volume containing reagents of the one-step RT-PCR (Qiagen, Germany) kit, 5 µL of DNA/RNA (50-100 ng/µL), probe and primer pairs (10 µM each), and water. Positive and negative controls in the Virology department of the Veterinary faculty of Fırat University were used in each PCR step.

PCR amplicons obtained in the last step of the test were electrophoresed in agarose-TAE gel (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) containing ethidium bromide at 1.5% (w/v) concentration for 40 minutes at 120 volts. Precise identification of PCR products of the expected size was performed by sequence analysis.

Sequence Analysis: PCR products were purified from gels and sequenced by a commercial sequencing service (Macrogen Europe, The Netherlands). Sequencing was performed in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, MA, USA), using a Sanger dideoxy chain termination method and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, MA, USA). Bi-directional nucleotide sequences aligned using the Clustal-W algorithm (Thompson et al., 1994). Edited sequences verified with Basic Local Alignment Search Tool (https://pubmed.ncbi.nlm.nih.gov/) (BLASTn) were submitted to the GenBank database. A phylogenetic tree of the aligned multiple sequence data was performed using the Neigbor-joining method in 1000 replicates via the MEGA X program (Kumar et al., 2018).

Results

According to PCR results, pestivirus was detected in 6 of 34 (17.6%) calves with abnormalities. After sequencing, the expected PCR product (288 nucleotides) for pestivirus showed high identity (97% and above) to the BVDV-1 strain/isolates in the GenBank.

Table 2. Information on PCR-positive animals.

Sample No	Breed	Gender	Age (days)	Artificial insemination	Abnormality	Agent	Strain	Accession number
3	S	M	15	-	Hydrocephalus	BVDV-1l	-	
4	S	M	35	-	Brahignati inferior	BVDV-1l	TR/Elz-4-2021	MZ686434
5	S	F	7	-	Cheliognatopalotoschisis	BVDV-1r	TR/Elz-6-2021	MZ686435
14	S	M	3	+	Atresia ani	BVDV-1v	-	
23	S	M	4	+	Coccygeal agenesis	BVDV-1l	TR/Elz-23-2021	MZ686436
27	S	M	5	+	Brahignati inferior	BVDV-1v	TR/Elz-27-2021	MZ686437

S: Simmental breed; F: female; M: male.

As a result of the phylogenetic tree constructed with pestivirus nucleotide sequences from different subtypes from the same or different countries selected from Genbank, one of them was -1r, two were -1v, and three were -1l subtypes. Four different from the sequence data were submitted to the GenBank database. AKAV, SBV, and BHV-1

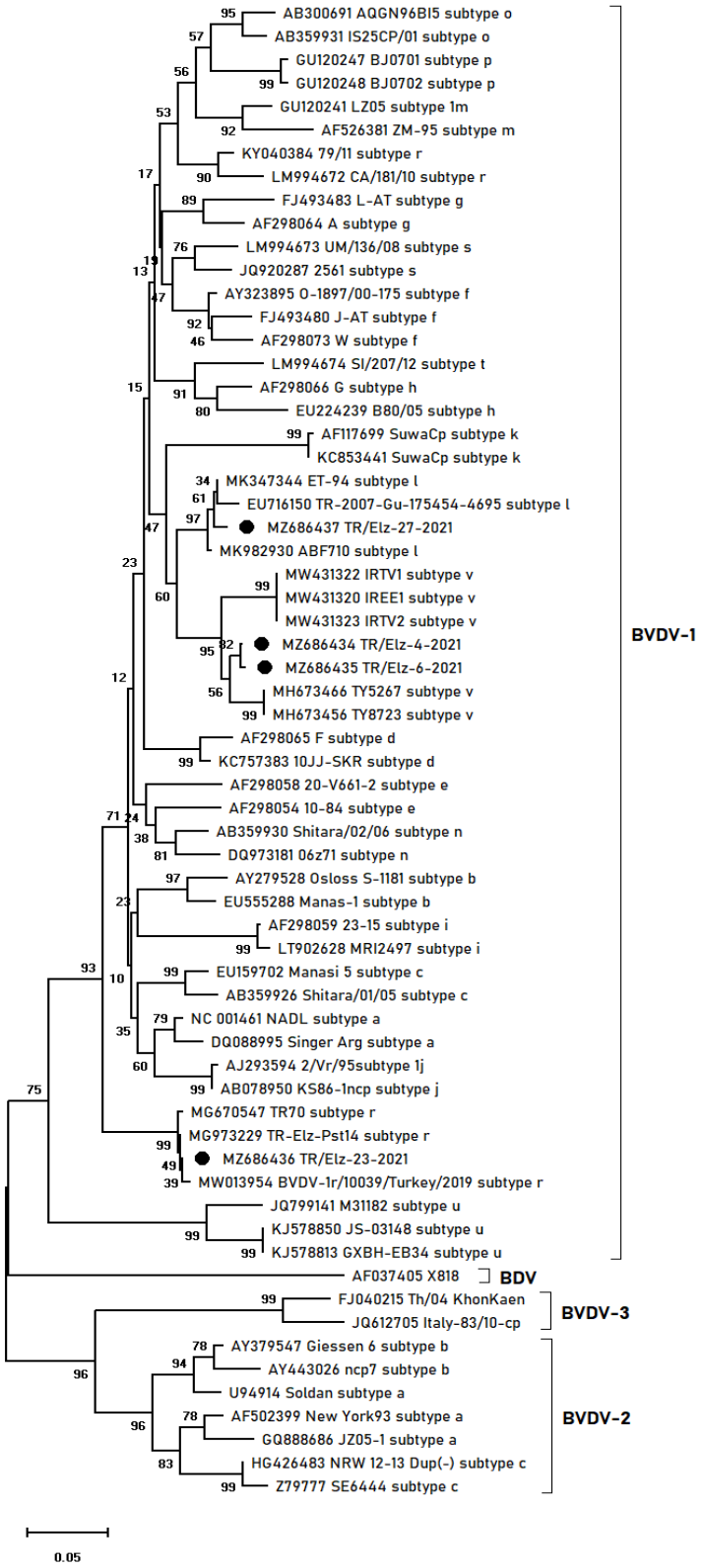

Figure 1. Phylogenetic tree based on the nucleotide sequence of the 5'UTR regions of BVDV and BDV strains/isolates and constructed by the Neigbor-joining method (1000 replicates). Black circles represent different strains of BVDV-1 from this study.

were not found in calves with abnormalities. In each reaction, the positive controls produced the expected size of the PCR product while the negative controls did not. Two of 6 BVDV-1 positive calves had brachignati inferior one had coccygeal agenesis, one had hydrocephalus, one had cheliognatopalotoschisis, and one had atresia ani. Information on PCR-positive animals is shown in Table 2. The nucleotide sequences of the BVDV-1 strains obtained in this

study were 86.00-100% identical to each other. These ratios were 75.00-99.00, 69.00-77.00%, 60.00-65.00%, and 66.00-70.00%, respectively, of some BVDV-1 BVDV-2, BVDV-3, and BDV strains/isolates selected in this study. Phylogenetic tree based on the nucleotide sequence of the 5'UTR regions of pestiviruses shown in Figure 1.

Discussion

The exact cause of congenital abnormalities is not known, but it is stated that they may occur commonly due to genetic, environmental, or genetic-environment interactions at various stages of embryogenesis or fetal development (Gumusova, 2015; Gül and İssi, 2009; Gül et al., 2013; İssi et al., 2012; İssi et al., 2013; Maxie, 2015; Tamer and Okur Yildiz et al., 2022). Hereditary transmission of congenital abnormalities to the next generation causes worldwide concern. The use of artificial insemination, embryo transfer, and international trade in germplasm has led to the proliferation of economically important traits as well as some defective recessive genes with adverse effects on fertility and animal health (Windsor and Agerholm, 2009; Windsor et al., 2011). In this study, more than half of the calves with abnormalities (18/34, 53%) were born as a result of pregnancy after artificial insemination. This result may suggest the necessity of examining the hereditary characteristics of animals used in artificial insemination in more detail. These data should be supported by further studies. In this study, most of the abnormalities examined in calves were musculoskeletal anomalies (18/34, 53%). These results are in line with previous studies (Herschler et al., 1962; Ozaydın et al., 1995). Musculoskeletal abnormalities were followed by ocular/orbital (6/34) and the abdominal wall and gastrointestinal system (4/34) deformities. In other studies, gastrointestinal system abnormalities were the most common, followed by musculoskeletal abnormalities (Aksoy et al., 2006; Belge et al., 2000). Simmental breed and male gender were dominant in the calves examined in this study, and this result was in line with a previous report (Aksoy et al., 2006).

In this study, BVDV-1 was detected in 6 calves with abnormalities (6/34) and was mostly associated with musculoskeletal disorders (4/6) and less frequently with nervous (1/6) and abdominal wall/gastrointestinal system abnomalities (1/6). Pestivirus-positive calves had one of some abnormalities such as hydrocephalus, brahignati inferior (n:2), cheliognatopalotoschisis, atresia ani, and coccygeal agenesis. In previous studies (Gül et al., 2013; Gürçay et al., 2013; İssi et al., 2006; İssi et al., 2014; Ok 2010; Radostits et al., 2008), it has been reported that BVDV causes diarrhea, acute and chronic mucosal disease, immunotolerance, persistent infection, thrombocytopenic and hemorrhagic disease, reproductive failure, and congenital defects in cattle. Congenital disorders usually occur when developing in the period of immunocompetence. In calves due to BVDV infections are common microencephalopathy, porencephaly, hydranencephaly, hydrocephalus, cerebellar hypoplasia, and hypomyelination (Bielefeldt Ohmann, 1983). However, it is stated that cataract, microphthalmia, retinal degeneration, optic neuritis, thymic hypoplasia, hypotrichosis and alopecia, curly hair coat, hyena disease, deterioration in osteogenesis, brachygnathia inferior and growth retardation may be other rare congenital defects (Radostits et al., 2008). Half of the 6 BVDV-1 strains examined in this study were of the -1l subtype and constituted the majority of the other detected subtypes (-1r, 1v), this finding is in line with previous studies (Oğuzoğlu et al., 2012; Timurkan and Aydın, 2019). To our knowledge, the -1v subtype has been previously identified in Türkiye and is reported for the first time in this study. These results indicate that the genetic diversity of BVDV-1 continues to increase. These results are important for control and eradication programs for BVDV.

AKAV causes infection in cattle, sheep and goats without any clinical signs. Intra-uterine infections in pregnant cattle cause abortion, premature and stillbirth as well as offspring with congenital abnormalities (Bowen, 2011). It affects varying degrees in the brain, spinal cord, and skeletal muscles and constitutes arthrogryposis and different vertebral malformations (torticollis, lordosis, kyphosis scoliosis). Pathological findings in the central nervous system range from encephalomyelitis, porencephaly, and hydranencephaly to microcephaly (Charles, 1994; Peperkamp et al., 2015; van den Brom et al., 2012). In cattle, AKAV infection in the first two months of pregnancy has almost no effect on the fetus (Radostits et al., 2008). AKAV causes hydranencephaly and porencephaly at days 76-104 of pregnancy, while it causes calves with and without vertebral deformities such as arthrogryposis, torticollis, scoliosis or kyphosis at days 105-170 of pregnancy. Malformations from infection in the last trimester of pregnancy are less severe. It is thought that SBV, which is in a similar serogroup with AKAV, also causes infections with similar characteristics (Charles 1994; Peperkamp et al., 2015, Tonvak et al., 2013). Although the calves examined in this study showed clinical features of arthrogryposis (n:1) and hydrocephalus (n:1) and the sampling was done during the high mosquito population, AKAV and SBV positivity could not be detected. The inclusion of calves with AH syndrome, an important feature of infections with these viruses, and a combination of both clinical features, could have increased the probability of detecting viruses.

BHV-1 is the causative agent of respiratory and genital tract infections such as infectious rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, and abortion. Due to breeding synchronization in cattle, BHV-1 may also cause abortion storms (Ackermann, 2006). Although congenital abnormalities from BHV-1 have not been reported so far, they have been rarely associated with the central nervous disorder as well as stillbirth in calves. BHV-1 was not found in calves with congenital abnormalities in this study.

Although the primary purpose in cattle breeding seems to be high meat and milk yield, it is also very important to continue the production process with healthy newborn calves. For this reason, even if congenital anomalies are seen at a low rate in calves, they should not be underestimated as they will cause economic losses and we believe that clinician veterinarians should not ignore these diseases.

Conflict of interest

The authors declared that there are no actual, potential, or perceived conflicts of interest for this article.

Ethical permission

This study was performed with the permission of the Experimental Animals Local Ethics Committee in Fırat University with 2020/05/09 dated 391732 approval number. In addition, the authors declared that they comply with the Research and Publication Ethics.

Similarity Rate

We declare that the similarity rate of the article is 15% as stated in the report uploaded to the system.

Author Contributions

Motivation/Concept: Mİ, SB
Design: Mİ, SB, HA
Control/Supervision: MI, ŞT, SB
Data Collection and/or Processing: SB, HA, KÇT, EP
Analysis and/or Interpretation: MI, HA, SB
Source Search: Mİ, SB, HA, KÇT, EP
Writing the Article: Mİ, SB, HA
Critical Review: Mİ, ŞT, SB

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