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Original Article
Özgün Araştırma

Diagnosis of *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica*, *Klebsiella* spp., by real-time PCR and pathological methods in dogs with bronchopneumonia

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Abstract: There are many bacterial agents that cause respiratory diseases in dogs. Bacterial bronchopneumonia is a lung disease caused by bacteria in the lower respiratory tract and lung parenchyma. In this study, it was aimed to determine *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica*, *Klebsiella* spp., in dogs with bronchopneumonia by Real-time PCR and to compare the histopathological findings determined according to the agents. The material of the study was paraffin blocks of lung tissue taken from 37 dogs that died for different reasons and were diagnosed with bronchopneumonia during microscopic examination. Microscopically, edema in the alveolar lumens, shedding of the alveolar epithelium, shedding of the bronch and bronchial epithelium and bronchiectasis, mononuclear cell infiltration (MCI) and Polymorphonuclear leukocytes (PMNL) infiltration in the bronchial lumens, peribronchiolar MCI infiltration, Bacterial clusters localized to the bronch epithelium, PMNL infiltration in the interstitium, multifocal necrosis areas, bleeding and pleuritis were observed. Real-time PCR analysis revealed *Bordetella bronchiseptica* in 18 (48.64%) cases, *Mycoplasma* spp. in 9 (24.32%) cases, *Streptococcus* spp. in 10 (27.02%) cases, and *Klebsiella* spp. in 2 (5.4%) cases. In conclusion, this study showed that the causative agent can be determined in bacterial bronchopneumonias of dogs with Real-time PCR even in tissues without culture opportunity. In addition, this study indicates that polymicrobial lower respiratory tract infections can also be seen in dogs and reveals that more than one bacterial species should be investigated for diagnosis.

Keywords: Bronchopneumonia, Dog, Pathology, Real-time PCR

Bronkopnömonili köpeklerde *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica*, *Klebsiella* spp. 'nin Real-time PCR ve patolojik yöntemler ile teşhisi

Özet: Köpeklerde solunum yolu hastalıklarına sebep olan çok sayıda bakteriyel etken vardır. Bakteriyel bronkopnömoni, alt solunum yollarında ve akciğer parankiminde bakterilerin neden olduğu akciğer hastalığıdır. Bu çalışmada bronkopnömonili köpeklerde *Mycoplasma spp*., *Streptococcus spp*., *Bordetella bronchiseptica, Klebsiella spp.*'nin Real-time PCR ile belirlenmesi ve etkenlere göre belirlenen histopatolojik bulguların karşılaştırılması amaçlanmıştır. Çalışmanın materyalini farklı sebeplerden dolayı ölen ve mikroskobik muayenede bronkopnömoni belirlenen 37 adet köpekten alınan akciğer dokusuna ait parafin bloklar oluşturdu. Mikroskobik olarak alveol lümenlerinde ödem, alveol epitellerinde dökülme, bronş ve bronşiol epitellerinde dökülme ve bronşektazi, bronşiol lümenlerinde mononükleer hücre (MNH) ve Polimorfonükleer lökosit (PMNL) infiltrasyonu, peribronşioler MNH infiltrasyonu, bronş epitellerine lokalize olmuş bakteri kümeleri, intersitisyumda PMNL infiltrasyonu, multifokal nekroz alanları, kanama ve plöritis rastlandı. Real-time PCR incelemesinde 18 (%48.64) olguda *Bordetella bronchiseptica,* 9 (%24.32) olguda *Mycoplasma spp*., 10 (%27.02) olguda *Streptococcus spp.,* 2 (%5.4) olguda *Klebsiella spp.* saptandı. Sonuç olarak bu çalışma, Real-time PCR ile köpeklerin bakteriyel bronkopnömonilerinde kültür imkânı olmayan dokularda bile etkenin belirlenebileceğini göstermiştir. Ayrıca köpeklerde polimikrobiyal alt solunum yolu enfeksiyonlarının da görülebileceğine işaret ederek teşhis için birden fazla bakteri türünün araştırılması gerektiğini ortaya koymaktadır.

Anahtar kelimeler: Bronkopnömoni, Köpek, Patoloji, Real-time PCR

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Introduction

There are many bacterial agents that cause respiratory diseases in dogs. Bacterial bronchopneumonia is a lung disease caused by bacteria in the lower respiratory tract and lung parenchyma (Proulx et al., 2014). Bacterial bronchopneumonia is among the most commonly diagnosed diseases in dogs with acute or chronic respiratory disease. Young animals are particularly susceptible to the development of bacterial pneumonia due to their sensitive immune systems. Chemotherapy and immunosuppressive drugs also increase the risk of bacterial pneumonia (Dear, 2020).

Mycoplasmas, belonging to the class *Mollicutes*, are the smallest free-living microorganisms capable of self-replication. In addition, they are difficult to grow in microbiological culture media and grow slowly. Most mycoplasma species are important pathogens for animals and cause diseases such as pneumonia, conjunctivitis, arthritis and mastitis (McAuliffe et al., 2003). Mycoplasmas are thought to be part of the normal bacterial flora found in the upper respiratory tract of dogs (Rosendal, 1982). From dogs with respiratory tract disease, *Mycoplasma bovigenitalium*, *M. canis*, *M. cynos*, *M. edwardii*, *M. feliminutum*, *M. gateae* and *M. spumans* were isolated (Armstrong et al., 1972; Rosendal, 1978).

Streptococcus species are gram-positive cocci that often appear in pairs or chains on cytological preparations and histological sections (Facklam, 2002). In general, α-hemolytic and γ-hemolytic streptococci are normally found in the upper respiratory tract, intestine, lower urinary tract, and genital tract. Typically, β-hemolytic Streptococcus species are pathogenic (Quinn et al., 1999; Lamm et al., 2010). Streptococcus infection in dogs has been associated with abortion, pneumonia, septicemia, endocarditis, lower urinary tract infections, arthritis and meningoencephalitis (Vaissaire et al., 1991; Radaelli and Platt 2002; Seguin et al., 2003; O'neill et al., 2006; Sykes et al., 2006; Pesavento et al., 2008). Pulmonary Streptococcus infection presents as bronchopneumonia or hemorrhagic form (Chalker et al., 2003; Buonavoglia and Martella 2007, Kim et al., 2007; Pesavento et al., 2008).

Bordetella bronchiseptica is a gram negative, non-spore, pleomorphic and aerobic coccobacillus that causes infectious respiratory disease syndrome or kennel cough disease in dogs (Ford, 2006). Canine infectious tracheobronchitis, caused by *Bordetella bronchiseptica*, is a highly contagious respiratory disease that affects dogs of all ages. *Bordetella bronchiseptica* can cause tracheobronchitis alone as well as with canine parainfluenza virus or canine adenovirus. In puppies, death occurs due to severe disease (Ellis et al., 2001; Taha-Abdelaziz et al., 2016). In *Bordetella bronchiseptica* infections, macroscopically, the lesions are usually in the cranial and middle lung lobes. There may be leakage of foamy, mucoid or purulent contents in the trachea and bronchi. Histologically, cilia-associated *Bordetella bronchiseptica* localizes to the bronchi and is rarely found in medium or large bronchioles. Cilia-associated bacteria appear on the apical surface of the bronchial epithelium as short bacilli that mingle with or obscure the cilia. Basophilic staining of agent creates a contrasting appearance with eosinophilic cilia (Taha-Abdelaziz et al., 2016).

Klebsiella pneumoniae, belonging to the Enterobacteriaceae family, is a bacterium commonly found in the gastrointestinal tract of healthy humans and animals (Ulstad et al., 2016; Marques et al., 2019). *Klebsiella pneumoniae* colonizes the mucosal surfaces of mammals and can also be found in the environment in shallow water, food, and soil (Jouini et al., 2015; Ulstad et al., 2016; Navon-Venezia et al., 2017). In pets, *Klebsiella* spp. can cause serious infections such as respiratory and urinary tract infections. *Klebsiella pneumoniae* has been isolated from many canine infections and causes pyometra, cystitis, prostatitis, pneumonia, meningoencephalitis, enteritis, mastitis, neonatal septicemia, hepatic abscesses, and otitis externa (Songer and Post 2004).

Today, the incidence of bacterial bronchopneumonia in pet animals is increasing day by day and regional studies are carried out to determine the etiological agent. In this study, it was aimed to determine *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica*, *Klebsiella spp*. in dogs with bronchopneumonia by Real-time PCR and to compare the histopathological findings determined according to the agents.

Materials and Methods

Animal material and microscopic examination

The material of the study consisted of paraffin blocks of the lungs, which were taken from 37 dogs that died due to different reasons and were brought to Selçuk University Faculty of Veterinary Medicine Department of Pathology for pathological diagnosis, and bronchopneumonia was detected in microscopic examination. From the lung samples taken, 17 of the dogs with bronchopneumonia determined microscopically belonged to male and 20 of them

belonged to female dogs. It was determined that 19 of these lungs belonged to dogs less than one year old, eight of them were between one and five years old, and ten of them belonged to dogs older than five years. Of the dogs examined in the study, 22 were shelter dogs, 10 were stray dogs brought by municipal authorities or animal lovers, and five were owned dogs.

Lung samples were fixed in neutral formaldehyde for 24-48 hours, and then routine tissue follow-up was performed. Afterwards, paraffin was embedded and paraffin blocks were obtained. 5 µm sections were taken from paraffin blocks to slides and stained with Hematoxylin-Eosin (H-E). In addition, sections taken from paraffin blocks on slides were stained with MacCallum Goodpasteur (MCG) staining method (Luna, 1968). Sections were viewed under a light microscope (Olympus BX51, Tokyo, Japan) and photographed (Olympus EP50, Tokyo, Japan).

Real-time PCR Analysis

For DNA isolation, 3-5 sections of 5 µm thickness were taken from paraffin blocks to slides. The sections taken were placed in a chalet with xylene added and the tissues were immersed in xylene, and they were kept in an oven at 55 °C for one hour and the paraffin was melted. Tissues were scraped from the slide and taken into Eppendorf tubes. Eppendorf tubes were vortexed by adding 96% ethanol, and then centrifuged and the supernatant was discarded. Eppendorf tubes were kept in an oven at 37°C for 10 minutes to completely remove the alcohol from the tissues. DNA isolation from paraffin blocks was performed using the commercial isolation kit (QIAamp® DNA FFPE Tissue Kit, Cat. No: 56404) according to the manufacturer's instructions. Isolated DNAs were stored at -20°C for use in Real-time PCR analysis. DNA copies of *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica* and *Klebsiella* spp. were searched with the QIAGEN Rotor Gene Q Real-time PCR device, using the primers prepared by a private company in accordance with the manufacturer's instructions. Deionized water was used as negative control. Primer sequences used in Real-time PCR analysis are given in Table 1.

Table 1. Primer sequences used in the study

SPECIES	PRIMARY SEQUENCES	
Mycoplasma spp.	F: 5'-GGTACAAAGAGACGCAATA-3' R: 5'-GCGATTACTAGCGATTCC-3'	(Hulse et al., 2018)
Streptococcus spp.	F: 5'-AGAGTTTGATCCTGGCTCAG-3' R: 5'-ACGGCTACCTTGTTACGACTT-3'	(Greisen et al., 1994)
Bordetella bronchiseptica	F: 5'-CCCCCGCACATTTCCGAACTTC-3' R: 5'-AGGCTCCCAAGAGAGAAAGGCTT-3'	(Schulz et al., 2014)
Klebsiella spp.	F: 5'-GGTGCTCTTTACATCATTGC-3' R: 5'-GCA ATG GCC ATT TGC GTT AG-3'	(Aher et al., 2012)

F: Forward, **R:** Reverse

Results

The agents amplified by Real-time PCR from the lungs with bronchopneumonia microscopically and the determination of these agents according to age and gender are given in Table 2.

Although bronchopneumonia was determined microscopically in the analyses performed with Real-time PCR, amplification could not be obtained with the primers used in five lung tissues.

The agent with the lowest Cycle Threshold (CT) value was accepted as the agent causing bronchopneumonia in dogs whose amplification was determined with primer sequences belonging to more than one agent in Real-time PCR analysis. The gram staining results of the samples prepared from paraffin blocks of the lungs in which bronchopneumonia was detected, and DNA copies of *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica* and *Klebsiella* spp. in these tissues by Real-time PCR, and CT values are given in Table 3.

The macroscopic findings in the records of the lungs examined in the study are given in Table 4.

Table 2. Determination of *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica* and *Klebsiella* spp. in lung tissues with bronchopneumonia by Real-time PCR according to age and gender

SPECIES	N (37)	MALE	FEMALE	< 1 AGE	1-5 YAŞ AGE	> 5 AGE
Mycoplasma spp.	5	2	3	–	1	4
Streptococcus spp.	5	4	1	1	2	2
Bordetella bronchiseptica	13	5	8	6	4	3
Klebsiella spp.	2	–	2	2	–	–
Mycoplasma spp. + *Streptococcus spp.*	2	1	1	2	–	–
Mycoplasma spp. + *Bordetella bronchiseptica*	2	1	1	2	–	–
Bordetella bronchiseptica + *Streptococcus spp.*	3	2	1	3	–	–
Samples for which no agents were isolated by Real-time PCR	5	2	3	3	1	1

Table 3. Gram staining results of samples prepared from paraffin blocks of lungs with bronchopneumonia, detection of DNA copies of *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica* and *Klebsiella* spp. in these tissues by Real-time PCR and CT values.

NO	GRAM STAIN	CT VALUES DETERMINED BY REAL-TIME PCR			
		Mycoplasma spp.	***Streptococcus spp.***	***Bordetella bronchiseptica***	***Klebsiella spp.***
1	Gram Negative			26	
2*	–				
3	–	29			
4	Gram Negative			30	
5	–		27		
6	–	31			
7	–				28
8	Gram Negative			32	
9	Gram Negative			28	
10	–		33		
11	Gram Negative			27	
12	–	30	35		
13	–			33	
14*	–				
15	–			34	
16	–			30	
17	–			32	
18	–	33			
19	Gram Negative		31	28	
20	Gram Positive		28		
21	–	28		30	
22	Gram Negative			29	

NO	GRAM STAIN	CT VALUES DETERMINED BY REAL-TIME PCR			
		Mycoplasma spp.	*Streptococcus spp.*	*Bordetella bronchiseptica*	*Klebsiella spp.*
23	–			27	
24	Gram Negative	31		27	
25	–			30	
26	–		34	29	
27	–	34			
28*	–				
29	–		32		
30	–		34		
31	–	29	34		
32*	–				
33	Gram Negative		30	28	
34	–	35			
35*	–				
36	–				26
37	Gram Negative			32	
Total	**11**	**9**	**10**	**18**	**2**

*Samples whose amplification could not be determined by the primers used in the study

Table 4. Macroscopic findings in the records of lungs with bronchopneumonia

MACROSCOPIC FINDINGS	SPECIES			
	Mycoplasma spp. *	*Streptococcus spp.* *	*Bordetella bronchiseptica* *	*Klebsiella spp.*
Red-colored, sunken, viscous areas	8/8	4/5	17/17	2/2
The cross-sectional is flooded and moist	8/8	2/5	17/17	1/2
Foamy content in the trachea and bronchi	7/8	2/5	17/17	1/2
Bleeding areas	3/8	5/5	6/17	–
Fluid in the chest cavity	2/8	4/5	10/17	1/2

* The agent with a lower CT value in polymicrobial cases was accepted as the agent causing bronchopneumonia.

Microscopically, mononuclear cell infiltration (MCI), epithelial shedding (Figure 1-A) and bronchiectasis were observed in the bronchi, bronchioles and alveolar lumens in seven of the lungs with *Mycoplasma* spp. In seven of these lungs, MCI located around the bronchi and bronchioles were noted. Pleuritis was detected in all of the lungs. Polymorphonuclear leukocyte (PMNL) infiltration, multifocal necrosis and bleeding areas were detected in one dog. Bacterial clusters were not found in the microscopic examinations of the lungs with *Mycoplasma* spp.

Microscopically, PMNL infiltration was observed in the lumen of the bronchi, bronchioles and the interstitium of the lung in all *Bordetella bronchiseptica* positive lungs. In addition, shedding was observed in the bronchi, bronchioles and alveolar epithelium. In ten of these lungs, edema in the alveoli (Figure 1-B), areas of multifocal necrosis scattered in the lung parenchyma, pleuritis and bacterial clusters localized to the bronchial and bronchiole epithelium (Figure 1-C) were noted. MCI in four dogs and bleeding areas in three dogs were determined.

Bleeding areas and hyperemia (Figure 1-D) were seen microscopically in the lungs with *Streptococcus* spp. In four of these lungs, the lumen of the bronchi and bronchioles was found to be filled with exudate mostly composed of neutrophils and erythrocytes. In addition, shedding was detected in the bronchi and bronchiole epithelium. Multifocal areas of necrosis, MCI, and pleuritis were detected in one dog, scattered throughout the lung parenchyma. In addition, coccoid bacteria clusters localized to the periphery of necrosis areas were noted in this sample.

In two dogs with *Klebsiella* spp., PMNL infiltration and shedding of bronchial, bronchiole and alveolar epithelium were observed microscopically in the bronchi and bronchiole lumens and the interstitium of the lung (Figure 1-E). In one of these dogs, areas of multifocal necrosis scattered throughout the lung parenchyma were detected.

Microscopic findings in the lungs according to the agents determined by Real-time PCR in the study are given in Table 5.

Table 5. Microscopic findings in the lungs according to the agents determined by Real-time PCR in the study

MICROSCOPIC FINDINGS	**SPECIES**			
	***Mycoplasma spp.* ***	***Streptococcus spp.* ***	***Bordetella bronchiseptica* ***	***Klebsiella spp.***
PMNL infiltration in bronchiole lumens	1/8	4/5	17/17	2/2
MCI in bronchiole lumens	7/8	1/5	4/17	–
Bronchial and bronchiolar epithelial shedding and bronchiectasis	7/8	4/5	17/17	2/2
Edema in alveolar lumens	3/8	3/5	10/17	–
Shedding of alveolar epithelium	7/8	4/5	17/17	2/2
PMNL infiltration in the interstitium	1/8	4/5	17/17	2/2
Peribronchiolar MCI infiltration	7/8	1/5	4/17	–
Multifocal areas of necrosis	1/8	1/5	10/17	1/2
Bleeding	1/8	5/5	3/17	–
Pleuritis	8/8	1/5	10/17	–
Clusters of bacteria localized to the bronchial epithelium	–	1/5	10/17	–

* The agent with a lower CT value in polymicrobial cases was accepted as the agent causing bronchopneumonia.

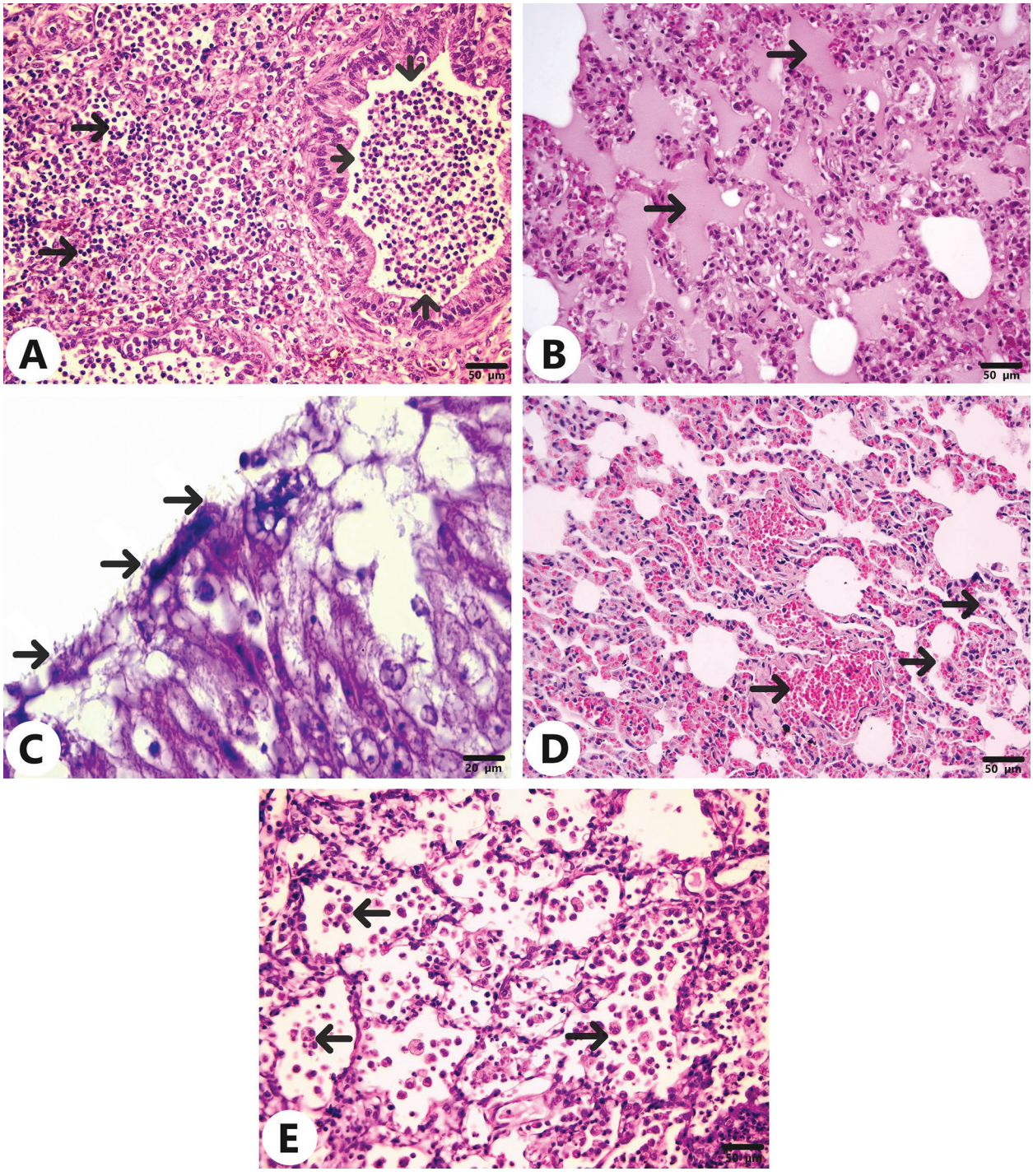

Figure 1. Histopathological findings. **A**: Epithelial shedding with mononuclear cell infiltration in the bronchiole (arrowheads) and alveolar lumens (arrows) in the lung determined as *Mycoplasma* spp., by Real- time PCR, x400, H-E, **B**: Alveolar edema (arrows) in the lung determined by *Bordetella bronchiseptica*, x400, H-E, **C**: Bacterial clusters (arrows) localized to bronchial epithelium in the lung, x1000, MCG, **D**: Bleeding and hyperemia areas (arrows) in the lung determined by *Streptococcus* spp., x400, H-E, **E**: Shedding of alveolar epithelium and alveolar macrophages (arrows) in the lung determined by *Klebsiella* spp, x400, H-E.

Discussion and Conclusion

Bronchopneumonia seen in dogs is one of the important diseases encountered in the clinic and bacterial-based infectious agents have an important place among the causes of bronchopneumonia. With this study, the role of *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica* and *Klebsiella* spp. in bacterial bronchopneumonias of dogs in our region was revealed by Real-time PCR and pathological methods.

Diagnosis of bacterial pneumonia in dogs is based on the identification of the agent by bacteriological culture. Culture for aerobic bacteria and *Mycoplasma* spp. is generally not available for diagnosis. Most of the time, contaminations formed during the collection and transport of the samples to the laboratory and the use of empirical antibiotics reduce the success of the culture method. In these cases, methods based on amplification of the genomic DNA of the bacteria are an important option to determine the appropriate antimicrobial therapy.

In recent years, Real-time PCR has become an important diagnostic method in the field of veterinary medicine due to its rapid results, ability to detect microorganisms such as Mycoplasma spp., which are difficult to detect with conventional methods, and high sensitivity (Yoldaş et al., 2009; Tuzcu et al., 2012; Tuzcu et al., 2021; Tuzcu et al., 2022; Akcakavak et al., 2023; Tuzcu et al., 2023). In this study, agents such as *Mycoplasma* spp., *Streptococcus* spp., *Bordetella bronchiseptica* and *Klebsiella* spp., which are paraffin blocks of canine lungs with bronchopneumonia, were determined by Real-time PCR and their pathological findings were compared.

Dear (2020), based on literature data in a study in which he examined pneumonia in cats and dogs (Foster et al., 2004; Radhakrishnan et al., 2007; Johnson et al., 2013; Proulx et al., 2014) reported that 22-71% of pneumonias in dogs were associated with *Bordetella bronchiseptica*, 2-25% with *Klebsiella pneumoniae*, 30-70% with *Mycoplasma* spp., and 6-21% with Streptococcus spp. Maden et al. (2000) in a study with 31 dogs reported that 24% *Bordetella bronchiseptica*, 4% *Streptococcus* spp. and 4% *Klebsiella* spp. were isolated from the culture of bronchoalveolar lavage fluid. In a study conducted by Sayin et al. (2016) with 196 samples in the Konya region, they reported that *Bordetella bronchiseptica* was isolated at a rate of 48.7% by PCR and standard microbiological methods. In this study, the data we obtained with Real-time PCR was 48.64% for *Bordetella bronchiseptica*, 5.4% for *Klebsiella* spp., 24.32% for *Mycoplasma* spp., and 27.02% for Streptococcus spp. It has been determined that the rates found are similar to the literature data. However, in the study of Maden et al. (2000), the reason for the lower rate of *Streptococcus* spp. isolating from this study may be related to the sampling method.

In adult dogs with respiratory tract infections, polymicrobial infections were detected in seven dogs in this study, as reported in previous studies (Lamm et al., 2010; Dear, 2020). It is a remarkable result that polymicrobial infections with *Streptococcus* spp., *Mycoplasma* spp. and *Bordetella bronchiseptica* were determined in dogs younger than one year old in this study.

Jameson et al. (1995), in their study in which they examined 93 dogs with bacterial pneumonia from which transtracheal aspiration samples were taken, reported that only *Mycoplasma* spp. was isolated in seven dogs according to the *Mycoplasma* spp. culture results, while another bacterium was found together with *Mycoplasma* spp. in 58 dogs. They also reported that the majority of dogs with *Mycoplasma* spp. were older than five years and there was no gender difference in dogs with Mycoplasma spp. In this study, *Mycoplasma* spp. was determined in nine dogs and genomic DNAs of *Streptococcus* spp. and *Bordetella bronchiseptica* were amplified as well as *Mycoplasma* spp. in two of these dogs. The majority of dogs identified only with *Mycoplasma* spp. were older than five years, which is similar to the findings of Jameson et al. (1995).

In a study conducted by Kirchner et al. (1990), *Mycoplasma* spp. was isolated in five laboratory dogs that showed clinical signs of respiratory distress, chronic cough and respiratory tract disease and identified macroscopic pneumonia lesion during thoracic surgery. They reported that they detected bronchiectasis, purulent bronchiolitis, bronchial and bronchiolar epithelial hyperplasia and chronic nonsuppurative peribronchiolitis in these dogs histopathologically. In the present study, mononuclear cell infiltration in the lumens of bronchi and bronchioles determined microscopically, shedding and bronchiectasis in the bronchial epithelium, and mononuclear cell infiltrations around the bronchi and bronchioles were detected. Our findings are similar to those reported by Kirchner et al. (1990).

In this study, genomic DNA of *Bordetella bronchiseptica* was amplified in 18 of the lung samples analyzed by Real-time PCR. In only 10 of these cases, bacterial ciliary adhesion was detected histopathologically in the lungs, similar to the literature (Taha-

Abdelaziz et al., 2016). The fact that the genomic DNAs of *Bordetella bronchiseptica* were amplified by Real-time PCR in cases where bacteria could not be detected in the cilia microscopically in this study reveals that the diagnosis should be confirmed by PCR and immunohistochemistry.

In the tissues in which *Bordetella bronchiseptica* was detected, findings such as PMNL infiltration in the bronchi and bronchiole lumens and interstitium of the lung, shedding in the bronchi, bronchioles and alveolar epithelium, edema in the alveoli, multifocal necrosis areas scattered in the lung parenchyma, pleuritis were found to be compatible with the literature (Ellis et al., 2001; Chambers et al., 2019). However, the fact that the severity of the findings determined in each dog is different indicates that it may be related to the immunity of the dogs, their housing patterns and the genetic difference of the agent.

In a retrospective study by Lamm et al. (2010) in which they examined 393 dogs, they associated 24 of the cases with bronchopneumonia with Streptococcus infection. In these cases, histopathologically, a large number of neutrophils and bacterial colonies were detected in the lumens of the bronchi, bronchioles and alveoli. They also reported that necrotic cells and fibrin formation were observed with neutrophils in severe cases. In the microscopic examination of the lungs of two dogs with hemorrhagic form, they reported that the alveolar lumens were filled with an exudate mixed with erythrocytes, neutrophils and fibrin. The findings in our study were found to be similar to the literature.

The conclusions of Chambers et al. (2019) that *Bordetella bronchiseptica* may cause secondary infections by causing ciliostasis of respiratory epithelial cells and the finding of *Streptococcus* spp. in three dogs with Bordetella bronchiseptica in this study are parallel.

Klebsiella spp. is found in the gastrointestinal tract of healthy animals and can cause pneumonia in dogs with low immunity (Roberts et al., 2000; Ulstad et al., 2016; Marques et al., 2019; Carvalho et al., 2020). In the present study, *Klebsiella* spp. was detected in two dogs less than one year old with bronchopneumonia. This suggests that the age range of *Klebsiella* spp. identified animals may be related to insufficient breast milk intake.

In conclusion, this study showed that the causative agent can be determined in bacterial bronchopneumonias of dogs with Real-time PCR even in tissues without culture opportunity. In addition, this study indicates that polymicrobial lower respiratory tract infections can also be seen in dogs and reveals that more than one bacterial species should be investigated for diagnosis.

Ethics committee for the use of experimental animals and other ethical committee decisions and permissions: Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (SÜVDAMEK) approved the ethical compliance of the study (Approval No: 2023/077).

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Referencess

Akcakavak G, Tuzcu M, Tuzcu N, Celik Z, Tural A, Dagar O. (2023) Investigation with Real-Time PCR and Histopathology on the presence of H. felis, H. heilmannii and H. pylori in dogs, *Rev Cient*. 33, 1-7.

Aher T, Roy A, Kumar P. (2012) Molecular detection of virulence genes associated with pathogenicity of Klebsiella spp. isolated from the respiratory tract of apparently healthy as well as sick goats. *Isr J Vet Med.* 67, 249-52.

Armstrong D, Morton V, Friedman M, Steger L, Tully J. (1972) Canine pneumonia associated with mycoplasma infection. *Am J Vet Res.*, 33, 1471-1478.

Buonavoglia C, Martella V. (2007) Canine respiratory viruses. *Vet res*. 38, 355-73.

Carvalho I, Alonso CA, Silva V, Pimenta P, Cunha R, Martins C, Igrejas G, Torres C, Poeta P. (2020) Extended-spectrum beta-lactamase-producing Klebsiella pneumoniae isolated from healthy and sick dogs in Portugal. *Microb Drug Resist.* 26, 709-715.

Chalker VJ, Brooks HW, Brownlie J. (2003) The association of Streptococcus equi subsp. zooepidemicus with canine infectious respiratory disease. *Vet Microbiol.* 95, 149-156.

Chambers J, Matsumoto I, Shibahara T, Haritani M, Nakayama H, Uchida K. (2019) An outbreak of fatal Bordetella bronchiseptica bronchopneumonia in puppies. *J Comp Pathol.* 167, 41-45.

Chandler JC, Lappin MR. (2002) Mycoplasmal respiratory infections in small animals: 17 cases (1988–1999). *JAAHA* 38, 111-119.

Dear JD. (2020) Bacterial pneumonia in dogs and cats: An update. Veterinary Clinics: *J Small Anim Pract.* 50, 447-465.

Ellis JA, Haines DM, West KH, Burr JH, Dayton A, Townsend HG, Kanara EW, Konoby C, Crichlow A, Martin K. (2001) Effect of vaccination on experimental infection with Bordetella bronchiseptica in dogs. *J Am Vet Med Assoc*. 218, 367-375.

Facklam R. (2002) What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin Microbiol Rev.*, 15, 613-630.

Ford RB. (2006) Canine infectious tracheobronchitis. *Infectious diseases of the dog and cat*.

Foster S, Martin P, Braddock J, Malik R. (2004) A retrospective analysis of feline bronchoalveolar lavage cytology and microbiology (1995–2000). *J Feline Med Surg*, 6, 189-198.

Greisen K, Loeffelholz M, Purohit A, Leong D. (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J Clin. Microbiol*. 32,335-351.

Hulse LS, Hickey D, Mitchell JM, Beagley KW, Ellis W, Johnston SD. (2018) Development and application of two multiplex real-time PCR assays for detection and speciation of bacterial pathogens in the koala. *J Vet Diagn Invest.* 30, 523-529.

Jameson P, King L, Lappin M, Jones R. (1995) Comparison of clinical signs, diagnostic findings, organisms isolated, and clinical outcome in dogs with bacterial pneumonia: 93 cases (1986-1991). *J Am Vet Med Assoc*, 206, 206-209.

Johnson L, Queen E, Vernau W, Sykes J, Byrne B. (2013) Microbiologic and cytologic assessment of bronchoalveolar lavage fluid from dogs with lower respiratory tract infection: 105 cases (2001–2011). *J Vet Intern Med.*, 27, 259-267.

Jouini A, Klibi N, Dziri R, Alonso CA, Boudabous A, Slama B, Torres C. (2015) Detection of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in vegetables, soil and water of the farm environment in Tunisia. *Int J Food Microbiol.* 203, 86-92.

Kim M, Jee H, Shin S, Lee B, Pakhrin B, Yoo H, Yoon J, Kim D. (2007) Outbreak and control of haemorrhagic pneumonia due to Streptococcus equi subspecies zooepidemicus in dogs. *Vet Rec.* 161, 528-530.

Kirchner B, Port CD, Magoc T, Sidor M, Ruben Z. (1990) Spontaneous bronchopneumonia in laboratory dogs infected with untyped Mycoplasma spp. *Lab Anim Sci.* 40, 625-628.

Lamm C, Ferguson A, Lehenbauer T, Love B. (2010) Streptococcal infection in dogs: a retrospective study of 393 cases. *Vet Pathol.*, 47, 387-395.

Luna L. (1968) Routine staining procedures. Manual of histologic staining methods of the Armed Forces Institute of Pathology. Blakiston Division McGraw-Hill, New York, 258 p.

Maden M, Birdane FM, Atkan F, Hadimli HH, Şen İ, Aslan V. (2000) Clinical, cytologic, bacteriologic and radiographic analysis of respiratory diseases in dogs. *Eurasian J Vet Sci.* 16, 43-50.

Marques C, Belas A, Aboim C, Cavaco-Silva P, Trigueiro G, Gama LT, Pomba C. (2019) Evidence of sharing of Klebsiella pneumoniae strains between healthy companion animals and cohabiting humans. *J Clin Microbiol.* 57, e01537-18.

McAuliffe L, Ellis RJ, Ayling RD, Nicholas RA. (2003) Differentiation of Mycoplasma species by 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis fingerprinting. *J Clin Microbiol.*, 41, 4844-7.

Navon-Venezia S, Kondratyeva K, Carattoli A. (2017) Klebsiella pneumoniae: a major worldwide source and shuttle for antibiotic resistance. *FEMS microbiol Rev.* 41, 252-275.

O'neill E, Day M, Hall E, Holden D, Murphy K, Barr F, Pearson G. (2006) Bacterial cholangitis/cholangiohepatitis with or without concurrent cholecystitis in four dogs. *J Small Anim Pract* 47, 325-35.

Pesavento P, Hurley K, Bannasch M, Artiushin S, Timoney J. (2008) A clonal outbreak of acute fatal hemorrhagic pneumonia in intensively housed (shelter) dogs caused by Streptococcus equi subsp. zooepidemicus. *Vet Pathol.* 45, 51-3.

Proulx A, Hume DZ, Drobatz KJ, Reineke EL. (2014) In vitro bacterial isolate susceptibility to empirically selected antimicrobials in 111 dogs with bacterial pneumonia. *J Vet Emerg Crit Care*, 24, 194-200.

Quinn P, Carter M, Markey B, Carter G. (1999) The streptococci and related cocci. *Clin Vet Microbiol.*. Mosby, Edinburgh, Scotland, 127-136.

Radaelli ST, Platt SR. (2002) Bacterial meningoencephalomyelitis in dogs: a retrospective study of 23 cases (1990-1999). *J Vet intern med.*, 16, 159-163.

Radhakrishnan A, Drobatz KJ, Culp WT, King LG. (2007) Community-acquired infectious pneumonia in puppies: 65 cases (1993–2002). *J Am Vet Med Assoc.* 230, 1493-7.

Roberts DE, McClain HM, Hansen DS, Currin P, Howerth EW. (2000) An outbreak of Klebsiella pneumoniae infection in dogs with severe enteritis and septicemia. *J Vet Diagn Invest*, 12, 168-73.

Rosendal S. (1978) Canine mycoplasmas: pathogenicity of mycoplasmas associated with distemper pneumonia. *J Infect Dis.* 138, 203-210.

Rosendal S. (1982) Canine mycoplasmas: their ecologic niche and role in disease. *J Am Vet Med Assoc.* 180, 1212-1214.

Sayin Z, Sakmanoglu A, Erganis O, Ucan US, Hadimli HH, Aras Z, Sanioglu G, Coskun AA. (2016) Genotypic Characterization of Bordetella bronchiseptica Strains Isolated from Stray and Pet Dogs. *Pak Vet J* 36, 482-486.

Schulz B, Kurz S, Weber K, Balzer H-J, Hartmann K. (2014) Detection of respiratory viruses and Bordetella bronchiseptica in dogs with acute respiratory tract infections. *Vet J.* 201, 365-369.

Seguin MA, Vaden SL, Altier C, Stone E, Levine JF. (2003) Persistent urinary tract infections and reinfections in 100 dogs (1989–1999). *J Vet Intern Med.*, 17, 622-631.

Songer JG, Post KW. (2004) *Veterinary microbiology-E-book: bacterial and fungal agents of animal disease*, Elsevier Health Sciences. 434 p.

Sykes JE, Kittleson MD, Pesavento PA, Byrne BA, MacDonald KA, Chomel BB. (2006) Evaluation of the relationship between causative organisms and clinical characteristics of infective endocarditis in dogs: 71 cases (1992–2005). J Am Vet Med Assoc. 228, 1723-34.

Taha-Abdelaziz K, Bassel LL, Harness ML, Clark ME, Register KB, Caswell JL. (2016) Cilia-associated bacteria in fatal Bordetella bronchiseptica pneumonia of dogs and cats. *J Vet Diagn Invest.* 28, 369-376.

Tuzcu M, Özmen M, Karakoç SR, Tuzcu N, Yoldaş A. (2012) Diagnosis of mycoplasmosis in chicks by pathological and Real Time-PCR methods. *Eurasian J Vet Sci.* 28, 82-86.

Tuzcu M, Tuzcu N, Akcakavak G, Celik Z. (2022) Diagnosis of Sarcina ventriculi-derived haemorrhagic abomasitis in lambs by histopathology and real-time PCR. *Acta Vet Brno*, 91, 227-233.

Tuzcu N, Tuzcu M, Akçakavak G, Başbuğ O, Kurul M. (2021) Köpeklerde canine distemper virüs enfeksiyonunun klinik, patolojik ve real-time RT-PCR ile teşhisi. *JAVST* 6, 57-64

Tuzcu N, Celik Z, Akcakavak G, Bulut A, Salik R, Oner M, Kaya V, Tuzcu M. (2023) Determination of Helicobacter heilmannii in cats by real time polymerase chain reaction and histopathology. *MAE Vet Fak Derg. 8, 69-73.*

Ulstad CR, Solheim M, Berg S, Lindbæk M, Dahle UR, Wester AL. (2016) Carriage of ESBL/AmpC-producing or ciprofloxacin non-susceptible Escherichia coli and Klebsiella spp. in healthy people in Norway. *Antimicrob Resist Infect Control*, 5, 1-11.

Vaissaire J, Remond M, Dufrêne M, Laroche M, Le Doujet C, Peigné F, Lebreton F. (1991) Stérilités, avortements et mortinatalité chez la chienne d'élevage dus à Streptococcus spp. du groupe G de Lancefield. Bull Acad Vet Fr. 144, 345-350.

Yoldaş A, Tuzcu M, Özmen E, Yığın A, Özmen M, Seval K, Tuzcu N. (2009) Sığırlarda Kuduz Hastalığının Teşhisi ve Kuduz Virüsünün Anatomik Lokalizasyonunun Real Time PCR ile BelirlenmesiÜzerine Karşılaştırmalı Çalışmalar. *Dicle Üniversitesi Vet Fak Derg,* 2, 42-50.