Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
28 (5): 643-652, 2022
DOI: 10.9775/kvfd.2022.27945

Research Article

Comparison of Histopathological, Immunohistochemical and Real-Time PCR Methods for Diagnosis of Listeriosis in Ruminants with Encephalitis [1][2]

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[1] *This study was presented as a oral presentation at the 2nd International Congress on Biological and Health Science (ICBH), Afyonkarahisar/ Türkiye, 24-27 February 2022*

[2] *The study was supported by Selcuk University Scientific Research Projects Coordinatorship (BAP), Konya, TÜRKİYE (Project No: 15401064)*

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Article ID: KVFD-2022-27945 Received: 14.06.2022 Accepted: 28.09.2022 Published Online: 29.09.2022

Abstract: Encephalitic listeriosis is the most significant purulent encephalitis in ruminants and is a very common endemic problem in sheep, cattle, and goats. In this study, it was aimed to compare the presence of *Listeria (L.) monocytogenes* revealed by immunohistochemical (IHC) and Real-Time PCR methods with histopathological findings obtained from the archive materials. The study material consisted of pons and medulla oblongata paraffin tissue of 100 ruminants (9 cattle, 4 calves, 44 sheep, 38 lambs, and 5 goats). Positivity was obtained by the IHC method in 46 (46%) and by the Real-Time PCR method in 21 (21%) of 100 cases. In the *L. monocytogenesis* antigen IHC scoring, more severe staining was observed in sheep and goats (P>0.05). In the IHC positive cases, microabscess was more severe in sheep and goats than in cattle and lambs (P<0.05). In addition, 19 patients had *Coenurus cerebralis* cysts, and 3 of them were found to be positive for the IHC agent of *Listeria*. It was concluded that IHC and PCR methods can be used to detect *L. monocytogenes* from paraffin blocks, but the IHC method is a more effective method than PCR in revealing the presence of antigen from paraffin blocks stored for many years.

Keywords: *Histopathology, Immunohistochemistry, Listeriosis, Real-Time PCR, Ruminants*

Ensefalitisli Ruminantlarda Listeriyozisin Tanısı İçin Histopatolojik, İmmünohistokimyasal ve Real-Time PCR Yöntemlerinin Karşılaştırılması

Öz: Ensefalitik listeriozis, ruminantlardaki purulent ensefalitisler arasında en önemlisidir ve tüm dünyada koyun, sığır ve keçilerde oldukça yaygın görülen endemik bir problemdir. Bu çalışmada, immünohistokimyasal (IHC) ve Real-Time PCR yöntemleri ile ortaya konan *Listeria (L.) –monocytogenes*'in varlığının arşiv materyallerinden elde edilen histopatolojik bulgularla karşılaştırılması amaçlandı. Çalışma materyalini 100 ruminantın (9 sığır, 4 buzağı, 44 koyun, 38 kuzu ve 5 keçi) pons ve medulla oblongata parafin dokuları oluşturdu. İncelenen 100 olgunun 46'sında (%46) IHC yöntemi ile, 21'inde ise (%21) Real-Time PCR yöntemi ile pozitiflik elde edildi. IHC skorlamasında *L. monocytogenes* antijeni koyun ve keçilerde daha belirgin boyandığı gözlendi (P>0.05). IHC pozitif vakalarda, koyun ve keçilerde, sığır ve kuzulara göre mikroapse daha şiddetliydi (P<0.05). Ayrıca 19 hastada *Coenurus cerebralis* kisti vardı ve bunlardan 3'ünde IHC yöntemiyle *Listeria* etkeni pozitif bulundu. IHC ve PCR yöntemlerinin parafin bloklarından *L. monocytogenes* tespitinde kullanılabileceği, ancak IHC yönteminin uzun yıllar saklanan parafin bloklarından antijen varlığını ortaya çıkarmada PCR'den daha etkili bir yöntem olduğu sonucuna varıldı.

Anahtar sözcükler: *Histopatoloji, İmmünohistokimya, Listeriyozis, Real-Time PCR, Ruminant*

Introduction

Listeriosis caused by *Listeria (L.) monocytogenes* is a zoonotic disease progressing with three basic forms as meningo-encephalitis, abortion, and septicemia. Although rare, it also causes mastitis, purulent conjunctivitis, keratitis, and endocarditis [1,2]. Encephalitic listeriosis, which is seen as the most important of the purulent encephalitis in ruminants, is an endemic problem that is common all over the world. While encephalitic listeriosis is observed at a

How to cite this article?
Hatipoğlu F, Terzi F, Özdemir Ö, Ortatatli M, Çiftçi MK, Ateş MB: Comparison of histopathological, immunohistochemical and real-time PCR methods for diagnosis of listeriosis in ruminants with encephalitis. *Kafkas Univ Vet Fak Derg,* 28 (5): 643-652, 2022.
DOI: 10.9775/kvfd.2022.27945

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rate of 7.5 - 29.4% in ruminants in Europe, it is emphasized that it is the main cause of encephalitis observed in goats and sheep in Switzerland [2,3].

Patient or asymptomatic animals scatter the agent with feces, urine, runny nose, milk, and placenta [4,5]. Contaminated straw, grass, pulp, water, and especially poorly fermented silages cause the disease to occur in herds as epidemics [6-8]. Encephalitic listeriosis is mostly seen in late winter and early spring, in indoor silage fattening [6,9]. Disease agent enters the body through abrasions in the buccal mucosa, teething wounds, and portantres in the intestinal mucosa [7]. Macroscopic lesions are often unremarkable in encephalitic listeriosis. However, sometimes thickening of the membranes covering the medulla oblongata due to greenish gelatinous edema, and hemorrhage with gray-colored melting foci with a diameter of a few millimeters on the cross-sectional surface of the medulla oblongata may be observed. Besides, turbidity can be determined in the cerebrospinal fluid (CSF). Lesions begin in the brain parenchyma and meningitis forms as secondary. The typical histopathological finding of the disease is microabscesses formed in the parenchyma of the pons and medulla oblongata. Microabscesses can be formed due to too many neutrophils and macrophage infiltrations, as well as due to microglial reaction [10-12]. Lymphocyte, histiocyte, plasma cells, and less commonly neutrophil and eosinophil granulocytes are seen around the vessels (perivascular cuffing) near the microabscesses [13-15]. In leptomeningitis, exudate accumulation consisting of macrophages, lymphocytes, plasma cells, and very few neutrophil granulocytes is seen and is often severe [11,16].

In our previous studies [17,18], we have revealed that brainstem cytology and immunocytological methods are significant and can be used in the rapid diagnosis of listeriosis. In the present study, it was aimed to compare the histopathological findings with the positivity of encephalitic listeriosis determined by IHC and RT-PCR methods from the archive materials (pons and medulla oblongata in paraffin blocks) of the cases, which were necropsied with the suspicion of listeriosis between 2000 and 2015 and encephalitis was detected.

Material and Methods

Ethical Statement

This study was approved by Ethics Committee of Selcuk University, Faculty of Veterinary Medicine, Experimental Animal Production and Research Center (Approval no: 2015/49).

Cases and Samples

In the study, the brainstems of 100 ruminants (9 cattle, 4 calves, 44 sheep, 38 lambs, and 5 goats) brought to Selcuk University, Faculty of Veterinary Medicine, Department of Pathology between 2000-2015 for necropsy were used. The archive material of pons and medulla oblongata of these animals, which were reported to have neurological symptoms in anamnesis and encephalitis was found in necropsy, were evaluated.

Histopathology

Five micron-thick sections were taken from paraffin blocks of the pons and medulla oblongata and stained with hematoxylin&eosin (H&E) and examined under a light microscope (Olympus BX51, Tokyo, Japan). Changes observed in histopathological examination of sections were evaluated as per scoring criteria of Oevermann et al.[19]. Accordingly; none (0); no microabscess, mild (+1); 1 small microabscess, moderate (+2); several small to medium-sized microabscesses, severe (+3); diffuse microabscesses of medium size, some of which coalesce, very severe (+4); multiple and extensive microabscesses in the parenchyma. Perivascular cell infiltration (perivascular cuffing) histopathological scoring; none (0); no lesion, mild (+1); 1-2 layers, moderate (+2); 3-4 layers, severe (+3); 5 to 6 layers, very severe (+4); more than 6 layers.

Immunohistochemistry

After the samples were cut into the size of 5 micron-thick and taken to polylysine slides, were deparaffinized, rehydrated, and stained as per the NovoLinkTM Max Polymer Detection System (RE7280-K) kit procedure. Antigen retrieval was performed with Proteinase K for 15 minutes at room temperature. Then, 3% hydrogen peroxide solution was dripped to remove endogenous peroxidase activity. The Protein Block was dripped and then incubated with the primary antibody (Rabbit polyclonal Anti-*L. monocytogenes* antibody - ab35132) for 1 hour at room temperature. Then, NovoLink Post Primer Block and NovoLink Polymer were incubated for 30 min at room temperature, respectively. Finally, the DAB solution was dripped onto the sections and incubated for 3-5 min at room temperature, then counterstained with Hematoxylin and closed with entellan. All stained sections were examined under a light microscope (Olympus BX 51) and scored as per the number of positively stained cells at x400 magnification. IHC staining scores: none (0); no staining, mild (+1); IHC positive staining in 1-10 cells, moderate (+2); IHC positive staining in 11-20 cells, severe (+3); IHC positive staining in more than 20 cells.

Real-Time PCR

Deparaffinization of Samples: Paraffin-blocked medulla oblongata, pons, and cerebellum tissues were cut in microtome of 5 µm-thick and taken into 1.5 mL Eppendorf tubes, and 1000 µL of xylene was added to remove the paraffin and shaken slowly in a vortex device (Drogan Lab). Then, eppendorf tubes were kept in the heat block (Dry

Bath) brought to 56°C. Eppendorf tubes were centrifuged at 13200 rpm and the supernatant was discarded. This process was repeated two more times. Then, 500 µL of xylene was added to the tubes and vortexed and kept in a 56°C heat block. By vortexing again, spinning was done and 500 µL of ethanol was added. The tubes were vortexed and kept in a 56°C heat block centrifuged at 13200 rpm and the supernatant was discarded. The tubes were centrifuged at 13200 rpm by adding 1000 µL of ethanol, and the same process was repeated once more by discarding the supernatant. The deparaffinization process was completed by allowing the ethanol to evaporate for 10 min in the 56°C heat block with the lids of the eppendorf tubes open.

DNA Isolation: After deparaffinization, 180 µL of tissue lysis buffer and 70 µL of Proteinase K were added to each tube. The tubes were vortexed and incubated at 56°C and 90°C for 1 h in a dry heat block. After the tubes were brought to room temperature, 200 µL of DNA Binding Buffer was added and kept at 15-20°C for 10 min. Spin (Scilogex) was performed by adding 100 µL of isopropanol to each tube. The lysate (average 550 µL) in the tubes was taken into Spin Filter tubes and centrifuged at 8000 rpm for 1 min, and the collective tubes at the bottom were changed after each procedure. 500 µL Wash Buffer I and II were added to each tube, respectively, and centrifuged (8000 rpm x 1 min.). Filtered tubes were taken into new Eppendorf tubes, 35 µL of DNA Elution buffer was added, kept at 15-20°C for 5 min, and centrifuged (8000 rpm X 1 min.) DNA was obtained. 1 µL Probe, 1 µL Primer Forward, 1 µL Primer Reverse, 10 µL Master, 2 µL H_2O; 5 µL of the sample (total volume 20 µL) was added into each well using DNA Master Hydrolysis Probes. Base sequences (LM1: CCTAAGACGCCAATCGAA, LM2: AAGCGCTTGCAACTGCTC) determined by Border et al.[20] were used as primers. In the Roche 96 Cycle device, the plates were set for pre-incubation at 95°C 600 sec-1 cycle, for amplification at 95°C 15 sec and 64°C 45 sec-45 cycles, and cooling at 37°C 30 sec-1 cycle.

Statistical Analysis

IBM SPSS Statistics 25.0 software was used for the comparison of histopathology, immunohistochemical, and Real-Time PCR results. Histopathological scores of immunohistochemically positive cases and immunohistochemical scores among ruminates were analyzed using the Kruskal-Wallis test using non-parametric statistics, followed by the Mann-Whitney U test as a post-hoc analysis between the two groups. Chi-square test was applied to IHC and PCR scores of *L. monocytogenes* among ruminant groups between 2000 and 2015. The value of $P<0.05$ is considered statistically significant. Pearson correlation analysis was applied to determine the significant and positive relationship between microabscess, perivascular cuffing, and IHC ($P<0.01$).

Results

Macroscopic Results

In the macroscopic examination, hyperemia and edema were found in the meninges, and diffuse hyperemia and melting areas of substantia alba on the cross-sectional surface of the brainstem were seen in 4 lambs and 1 calf. Besides some melting areas with a 1x2 mm size and yellowish-green consistency exudate in the brainstem of 2 lambs and 1 sheep were noticed. Also, a *Coenurus cerebralis* cyst was detected in 8 lambs and 11 sheep.

Histopathology Results

The number and scores of cases for which microabscess and perivascular cuffing were observed in the study are given in *Table 1* by the ruminant species. When microscopic findings were evaluated, microabscess was observed in a total of 57 cases. In 13 of these 57 cases, 1 small microabscess (+1) with diffuse neutrophil infiltration and glia cells was found in the brainstem. In 11 cases, microabscess (+2 and +3) *(Fig. 1-A)* was observed, from small to medium-sized, and some of them even united.

Table 1. *Histopathological, IHC and Real-Time PCR results according to animal species*

Parameters	Histopathological Changes										IHC and Real-Time PCR Results					
	Microapse					Perivascular Cuffing					IHC				Real-Time PCR	
Lesion score	0	+1	+2	+3	+4	0	+1	+2	+3	+4	0	+1	+2	+3	Positive	Negative
Cattle	4	2	1	1	1	2	0	3	0	4	4	3	0	2	2	7
Calf	1	2	0	1	0	2	1	1	0	0	4	0	0	0	0	4
Sheep	17	3	3	3	18	8	4	2	2	28	19	6	4	15	13	31
Lamb	20	6	2	0	10	10	2	4	3	19	25	4	1	8	6	32
Goat	1	0	0	0	4	1	0	0	0	4	2	0	0	3	0	5
Total	43	13	6	5	33	23	7	10	5	55	54	13	5	28	21	79
		57					77					46				

Histopathological and immunohistochemical score; 0; none lesion, +1; middle, +2; moderate, +3; severe, +4; very severe

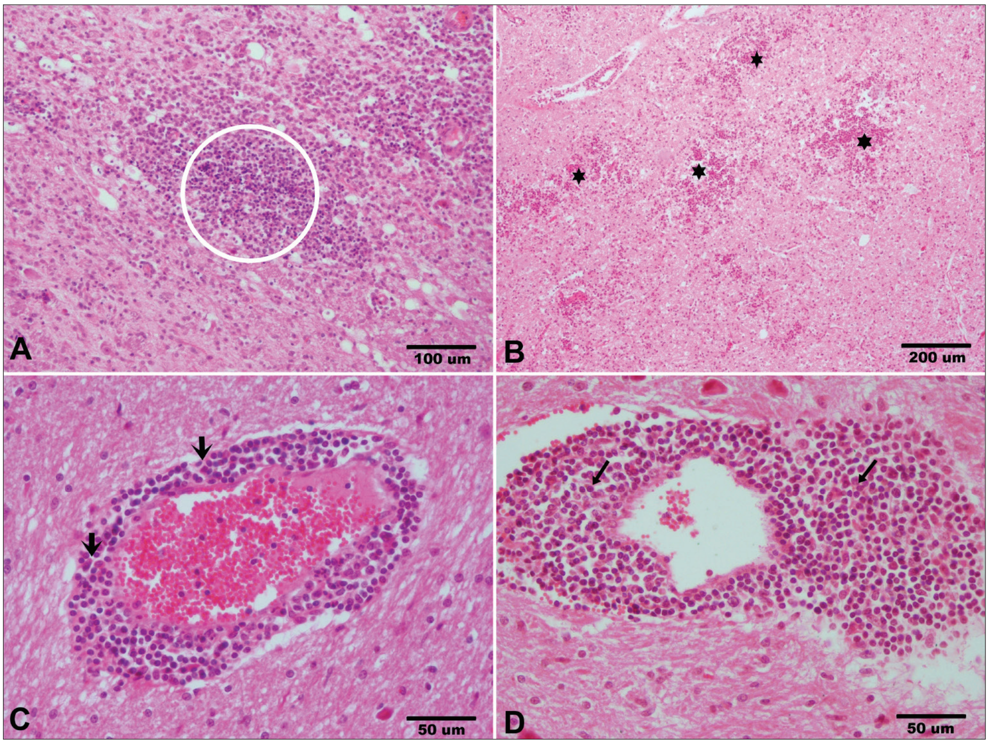

Fig 1. A-D. Brainstem, H&E. **A.** Microabscess *(circle)* (moderate), Bar, 100 µm, **B.** Numerous and widespread microabscesses *(stars)* (very severe), Bar, 200 µm, **C.** Perivascular mononuclear cell and neutrophil infiltrations *(arrows)* (moderate) and hyperemia, Bar, 50 µm, **D.** Perivascular mononuclear cell and neutrophil infiltration (very severe) *(arrows),* Bar, 50 µm

Large microabscesses (+4) and *(Fig. 1-B)* were detected in the parenchyma in 33 cases. Besides, perivascular cuffing consisting of lymphocytes, histiocytes, and plasma cells and neutrophils in the Virchow-Robin spaces around the vessels in the brainstem parenchyma was observed in a total of 77 cases (+1 in 7 cases, +2 in 10 cases *(Fig. 1-C),* +3 in 5 cases, and +4 in 55 cases *(Fig. 1-D).* No microabscess was found in 20 of these 77 cases in which perivascular cell infiltration was observed in the study. In cases with *L. monocytogenes* antigen IHC positive, microabscess was observed histopathologically in sheep and goats ($P<0.05$), and more severe in cattle and lambs *(Fig 3-A).* Perivascular cuffing was found at a similar rate ($P>0.05$) in cattle, sheep, goat, and lambs, while it was most severe in goats *(Fig. 3-B).* In the cases with *Coenurus cerebralis* cyst, it was determined that the cyst walls and foreign body giant cells formed against them and an inflammatory zone in which eosinophil granulocytes were also found.

Immunohistochemical Results

Listeria monocytogenes antigen in the cytoplasm of neutrophils found in microabscesses in the brainstem was stained by IHC method and its severity was scored. IHC staining results, lesion scores, and general distribution by the ruminant species are given in *Table 1.* When the IHC findings were evaluated collectively, 46 of 100 cases (5 cattle, 25 sheep, 13 lambs, and 3 goats) were found to be positive for *L. monocytogenes* antigen *(Fig. 2-A,D). L. monocytogenes* antigen was detected mild in 13 cases *(Fig. 2-A),* moderate in 5 cases *(Fig. 2-B)* and severe staining in 28 cases *(Fig. 2-C,D)* by IHC method. *Listeria* antigens were determined by IHC in 46 (80.7%) of 57 cases with microabscess in histopathological examinations.

While there was no statistical difference between ruminant species in *L. monocytogenes* antigen IHC scoring ($P>0.05$) *(Fig. 3-C),* more intense staining was observed in sheep and goats. A significant difference ($P<0.05$) was determined between the positivity and negativity of *L. monocytogenes* in sheep and no significant difference ($P>0.05$) was seen in cattle, goats and lambs *(Table 2).* According to pearson correlation analysis, a strong, positive and significant relationship was found between microabscess, perivascular cuffing, and IHC *(Table 3).* In addition, the incidence of *L. monocytogenes* was determined to be highest in spring and winter months, respectively *(Fig. 4).* When the findings were evaluated according to years, the highest *L. monocytogenes* positivity was detected in 2012 with 8 cases, while IHC positivity was not detected in 2002 and 2006 *(Fig. 5).*

Real-Time PCR Results

The distribution of Real-Time PCR results by animal species is given in *Table 1.* Accordingly, *L. monocytogenes* positivity was found in 21 of 100 cases *(Fig. 6)* (2 cattle,

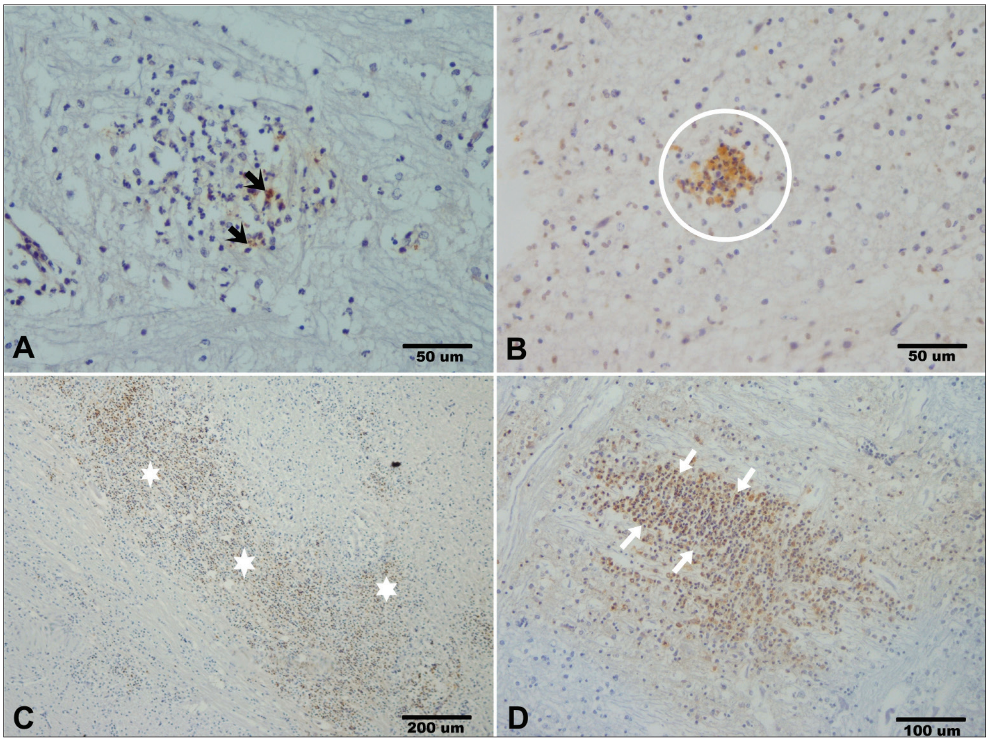

Fig 2. A-D. Microabscess in the brainstem and *L. monocytogenes* antigen in the cytoplasm of neutrophils, IHC positive staining. DAB. **A.** Mild *(arrows)*, Bar, 50 µm, **B**. Moderate *(circle)*, Bar, 50 µm, **C.** Severe *(stars)*, Bar, 200 µm, **D.** Severe *(arrows)*, Bar, 100 µm

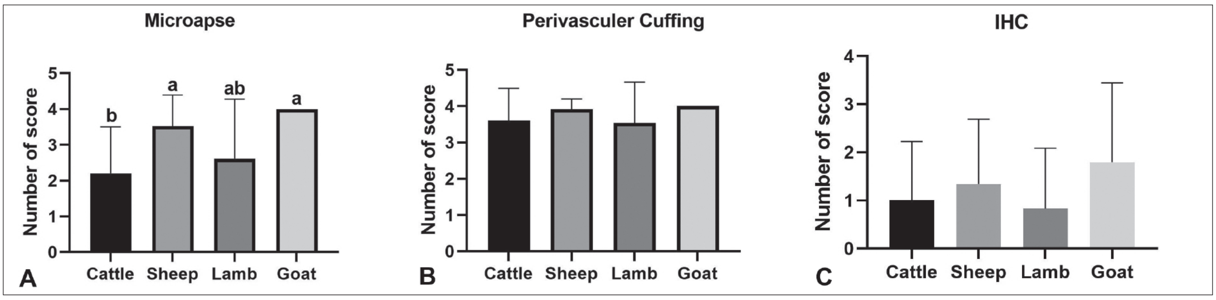

Fig 3. A. Statistical expression of microabscess severity among *L. monocytogenes* positive cattle, sheep, lamb and goat, (Data presented mean value ± standard deviation, significant differences (p<0.05) marked with different superscripts), **B.** Statistical expression of perivasculer cuffing severity among *L. monocytogenes* positive cattle, sheep, lamb and goat, (Data presented mean value ± standard deviation (P>0.05), **C.** Statistical expression of IHC scores of *L. monocytogenes* antigen in ruminants. Data presented mean value ± standard deviation (P>0.05)

Table 2. *IHC and Real-Time PCR results and ratios of L. monocytogenes in ruminant species*

Animals	IHC			PCR		
	Number (%) Negative Cases	Number (%) Positive Cases	x^2 (P Value)	Number (%) Negative Cases	Number (%) Positive Cases	x^2 (P Value)
Cattle	4 (44.4%)	5 (55.6%)	6.496 (0.167)	7 (77.8%)	2 (22.2%)	5.774 (0.417)
Sheep	19 (43.2%)	25 (56.8%)	23.183 (0.004*)	31 (70.5%)	13 (29.5%)	20.104 (0.013*)
Lamb	25 (65.8%)	13 (34.2%)	6.902 (0.920)	32 (84.2%)	6 (15.8%)	8.490 (0.782)
Goat	2 (40.0%)	3 (60.0%)	5.000 (0.172)	5 (100%)	0 (0.00%)	-

** Statistically significant test (Chi square, P<0.05)*

13 sheep, 6 lambs). In sheep, a significant difference was found between positive and negative aspects with the *L. monocytogenes* PCR method, and no statistically significant difference was found in cattle, goats and lambs *(Table 2)* between 2000 and 2015. When PCR findings were evaluated by years, PCR positivity could not be detected in

Table 3. Degree of correlation between histopathological results and IHC method. Pearson Correlation Test			
Findings	**Microabscess**	**IHC**	**Perivascular Cuffing**
Perivascular cuffing	$r^2 = +0.74$ P<0.01*		
Microabscess		$r^2 = +0.92$ P<0.01*	
IHC			$r^2 = 0.66$ P<0.01*
** P<0.05*			

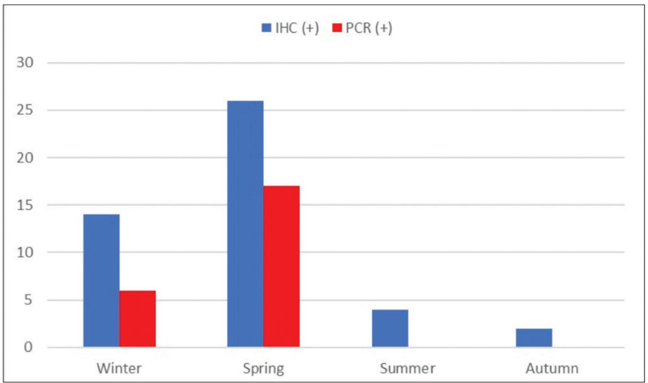

Fig 4. Distribution of *L. monocytogenes* IHC and Real-Time PCR positive cases by season

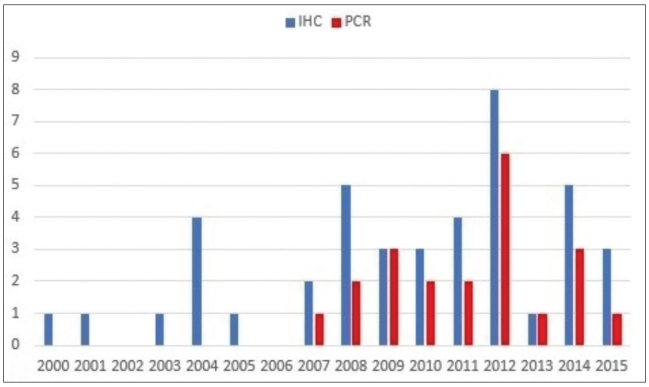

Fig 5. IHC and Real-Time PCR results of listeriosis-positive cases in ruminant between 2000 and 2015

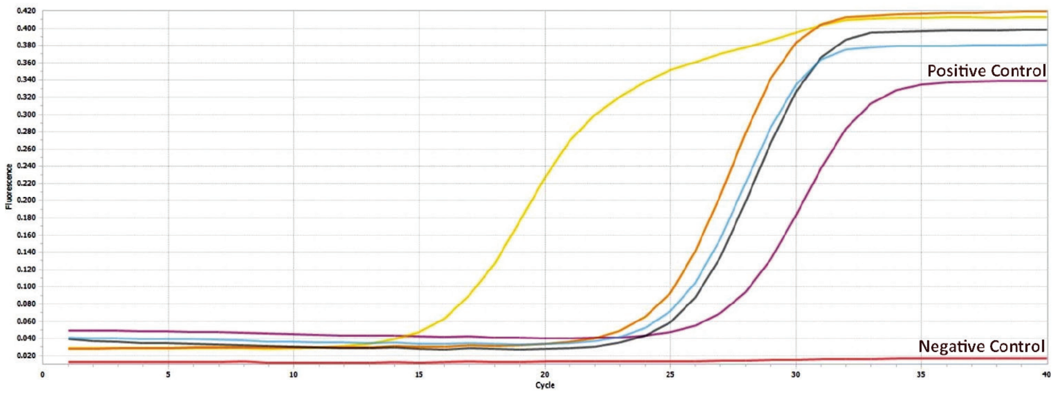

Fig 6. Real-Time PCR reactions: positive control, negative control and positive cases

cases between 2000 and 2006, *L. monocytogenes* was found positive between 2007 and 2015, the most positivity was detected in 2012 with 6 cases *(Fig. 4)*. It was determined that the incidence of *L. monocytogenes* increased in the in spring and winter months by PCR method, respectively, similar to IHC findings *(Fig. 3)*.

Discussion

Encephalitic listeriosis causes loss of productivity and sometimes death in farm animals, causing economic losses in the world. Also, listeriosis is very important for human health, since it is a foodborne infection in humans. While encephalitic listeriosis is sporadic in cattle, it occurs as epidemics in sheep and goat herds [6]. Encephalitic listeriosis is usually seen as a result of feeding with grass, straw, which are contaminated with *L. monocytogenes*, especially silage that are not well fermented, juicy beet, and malt pulps [21]. Nightingale et al.[5] and Wesley et al.[22] reported that they encountered listeriosis in the winter and spring months (December-May). In the study presented, it was determined that 14 of 46 cases diagnosed with encephalitic listeriosis came in the winter months and 26 at the beginning of spring. The conclusions were considered that the disease caused epidemics in the herds in Konya and the surrounding provinces. The reason for this could be in these region, sheep and goat breeding are common, during the periods when the animals are not taken to the pasture, due not pay attention to the cleaning of the barns and water, and the incorrect storage of wet feeds such as silage and pulp, and/or the feeding with spoiled silage.

Disease agents enter the body through abrasions in the buccal mucosa, teething wounds, and portantres in the intestinal mucosa and come to the trigeminal ganglia and from there to the brain (medulla) by motor branches and cause purulent encephalitis. When moved the animals are seen circular movements clinically, and tongue and facial paralysis due to paralysis of the 7th nerve [6-8]. Since similar findings can also be seen in parasitic infestations such as *Coenurus cerebralis*, it makes it difficult to distinguish clinically encephalitic listeriosis and coenuriasis. However, macroscopically, the appearance of *Coenurus cerebralis* cysts in the cerebral hemispheres or the cerebellum provides a macroscopic diagnosis of coenuriasis. In the study presented, macroscopically *Coenurus cerebralis* cysts were determined in the brain hemispheres of 8 lambs and 11 sheep, and histopathologically, it was determined that there is an inflammatory zone with eosinophil granulocytes and foreign body giant cells formed against the *Coenurus cerebralis* cyst walls. In 3 of these 19 cases, in which a *Coenurus cerebralis* cyst was detected, *Listeria* was also found to be positive by IHC. The results obtained in the study presented showed that ruminants found to have *Coenurus cerebralis* cyst should also be investigated for encephalitic listeriosis and the coexistence of both diseases should not be ignored.

In encephalitic listeriosis, it was stated that microabscesses formed due to a large number of neutrophil and macrophage infiltrates and a small number of microglial reactions are the characteristic findings, histopathologically [11,23]. Oevermann et al.[19] scored histopathologically microabscess and perivascular cuffing formation in 220 ruminants (59 goats, 89 sheep, and 72 cattle) to determine encephalitic listeriosis and reported that they found microabscess in 59 goats (100%), 89 sheep (100%), and 69 cattle (95.8%). In the presented study, microabscess formation to varying degrees in the brainstem was detected in the histopathological examinations of 57 out of 100 ruminants with neurological findings. The presence of varying degrees of microabscess in all 46 cases (80.7%) confirmed to have listeriosis by IHC reveals that although microabscess finding is not pathognomonic in terms of listeriosis, it is a very important histopathological finding. In cases with intense microabscess (moderate, severe, and very severe), this rate increases to 90.9% (40/44). In sheep and goats, microabscess formation was found to be statistically more severe (P>0.05) in *L. monocytogenes* positive cases by IHC method. These findings were evaluated as acute according to the scoring made by Oevermann et al.[19], and the fact that acute cases were more common in small ruminants in the study supports the view that encephalitic listeriosis progresses more severely.

Lymphocyte, histiocyte, plasma cells, and less commonly neutrophil and eosinophil granulocytes are seen around the vessels (perivascular cuffing) near the microabscesses [11]. Histopathologically, perivascular cell infiltration was detected in 17 cases with mild and moderate scores *(Fig. 1-C)*, and in 60 cases with severe and very severe scores *(Fig. 1-D)* in our study. In the study, perivascular cuffing scores were found to be similar in cattle, sheep, goats, and lambs in the *L. monocytogenes* positive cases, while it was most severe in goats (P>0.05). In addition, microabscess was not detected in 20 cases with perivascular cuffing in the brain parenchyma. Since perivascular cell infiltration can also be seen in parasitic, viral, and other bacterial infections in histopathological examinations [24,25], it was concluded that it is more appropriate to evaluate the cases in which microabscess is observed with perivascular cell infiltration in terms of encephalitic listeriosis. Although microabscess and perivasculer cuffing is not pathognomonic for listeriosis, the presence of a strong, positive, and significant correlation between microabscess, perivascular cuffing, and IHC reveals that it is a very important histopathological finding.

Although clinical, macroscopic, and histopathological findings are important in the diagnosis of encephalitic listeriosis, it is also necessary to demonstrate the agent with methods such as bacteriological culture, IHC, and PCR in the definitive diagnosis of the disease [26,27]. On the other hand, although culture is shown as the gold standard in the diagnosis of the disease, there are disadvantages such as inability to produce bacteria, especially in animals using antibiotics, long duration, and high cost [28,29]. In addition to this method, molecular methods such as IHC and PCR have been developed, which are more specific, less costly, and give results in a shorter time. In the presented study, *L. monocytogenes* antigen was detected as positive in 46 cases (46%) by the IHC method from paraffin blocks *(Fig. 2A-D)*. This result supports the studies.

Immunohistochemistry is an effective method for the detection of bacteria and their antigens, since the morphological features of tissues and organs are preserved [30]. Allen et al.[31] and Loeb [32] reporting that IHC can be used as an alternative to the culture method in the *L. monocytogenes* diagnosis. In the current study, a strong and positive correlation was determined between the histopathological and immunohistochemical findings of *L. monocytogenes* in archival paraffin block tissue of small ruminates. Besides, it showed parallelism with the studies [33,34] showing that it is possible to determine the causative agent from archive materials by IHC method, and supported the view that it can be used safely in the diagnosis of diseases from previous years' cases.

We determined the presence of *L. monocytogenes* in cases with encephalitic listeriosis findings by using immuno-histochemical and Real-Time PCR methods in archival paraffin block tissues. In recent years PCR technology has been instrumental in identifying infectious agents and therefore in some cases complements or even surpasses conventional methods in terms of sensitivity [35]. Mygind et al.[36] found a good correlation between IHC and PCR methods in their study for the detection of *Chlamydia pneumoniae* in mouse lung paraffin block tissues. In the study, the IHC method is a safe method in archival paraffin block tissues and it is thought to affect the sensitivity of the Real-Time PCR method depending on the age of the paraffin block tissue.

Tissue samples that are fixed with formalin or embedded in paraffin are considered a suitable source for DNA analysis as their structures and proteins are preserved. Besides, the high sensitivity and specificity provided by the PCR method are important in terms of its applicability to a wide variety of samples. By PCR method in animals, *Listeria* agents were determined in brain tissue [37], in cerebrospinal fluid [38] and colonies formed as a result of the culture of brain samples taken. In the literature that could be examined, PCR studies to determine *Listeria* agents from paraffin block tissues were not encountered. In the present study, *Listeria* agent was detected as positive by Real Time-PCR method in 21 (21%) of archive brain tissues in paraffin blocks *(Fig. 6)* belonging to 100 ruminants who showed neural findings and were diagnosed with encephalitis between 2000-2015. Since positive results were found in cases after 2007 in the study, it was concluded that positive results could not be obtained using the PCR method from paraffin block tissues, belonging to 8-10 years ago or older, in the determination of *Listeria* agent, and therefore paraffin blocks older than 10 years were not suitable for PCR method.

When IHC and PCR results were evaluated together, PCR was found to be positive in only 1 of 13 IHC positive cases with lesion score of mild, but PCR was positive in 19 of 33 cases with IHC scores moderate and severe. On the other hand, in only 1 lamb, while IHC was found to be negative, it was positive by the PCR method. From the results obtained, it was concluded that IHC and PCR methods can be used in archive paraffin block tissues, but the IHC method is more sensitive and specific for old tissues in the diagnosis of encephalitic listeriosis. It was noted that the majority of PCR positive cases were IHC positive cases with moderate and severe, and this situation made us think that for the safe use of the PCR method, the presence of more intense agents in the paraffin-embedded tissue is needed. On the other Szafranska et al.[39] showed the inability to completely remove inhibitory substances such as paraffin, alcohol, or xylene as the reason for the inability to obtain pure DNA from archive tissues in the paraffin block by PCR method. The fact that positivity could not be determined by PCR in cases where the number or density of agents was low in the study supports the opinion of the researchers, considering that the inhibitory substances in question may have suppressed the detection of a small number of agents. Based on this idea, it was predicted that such inhibitory substances should be removed more carefully and adequately in similar studies to be carried out in the future.

It was concluded that IHC and PCR methods can be used to detect *L. monocytogenes* from paraffin blocks, but in the detection of thick-walled bacteria such as *Listeria* sp. in paraffin-embedded archival materials, due to the difficulties in the disintegration of the bacterial wall, the low amount of the agent, and/or the residue of inhibitory substances. It was evaluated that IHC method is a more effective method than PCR in revealing the presence of antigen from paraffin blocks stored for many years. The fact that IHC was found to be positive in 3 of 19 cases with a macroscopically and microscopically seen *Coenurus cerebralis* cyst showed that encephalitic listeriosis may occur together with coenuriasis and it was concluded that the cases in which the *Coenurus cerebralis* cysts were determined should also be examined in terms of *Listeria*. It was also noted that both diseases that cause herd problems can progress together, and it is recommended to take the necessary precautions for both diseases in the treatment of such cases. Also, it was considered that prospective studies on live animals or fresh materials could be useful to more clearly determine the effectiveness of the PCR method and to compare it with IHC.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author (F. Hatipoğlu) on reasonable request.

Ethical Statement

This study was approved by Ethics Committee of Selcuk University (Approval no: 2015/49).

Author Contributions

All authors contributed to the study conception and design. FH: Conceptualization, Methodology, Writing - Original Draft. FT: Formal analysis, Resources. ÖÖ: Investigation, Resources. MO: Writing - Review & Editing, Validation. MKÇ: Writing - Review & Editing. MBA: Resources, Investigation.

Funding Support

The study was supported by Selcuk University Scientific

Research Projects Coordinatorship (BAP), Konya, TÜRKİYE (Project No: 15401064).

Conflict of Interest

The authors declared that there is no conflict of interest.

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