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RESEARCH ARTICLE

The Usability of Cytological and Immunocytological Methods for Rapid Diagnosis of Encephalitic Listeriosis in Ruminants [1][2]

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Abstract

Although the clinical and pathological findings are important in the diagnosis of listeriosis, to isolation or to be shown the presence of the bacterium must be required for the definitive diagnosis. This study aims to investigate the availability of imprint cytological (IC) and immunocytochemical (ICC) methods in comparison with histopathological and immunohistochemical (IHC) methods for the rapid diagnosis of encephalitic listeriosis . In the study, the touching and smear preparations taken from the pons and medulla oblongata of 25 ruminants suspected with listeriosis by neurological symptoms were stained with modified giemsa and also with ICC technic for revealing antigens, as a new method. Same tissue sections were stained with Hematoxylin&Eosin and IHC methods too, and examined under light microscope by scoring. In IC examinations, there were intensive neutrophils in 14 cases and few neutrophils in 4 cases, and no neutrophils were observed in 7 cases. In histopathological examinations, 13 of these 14 cases revealed typical microabscesses and listeria positivity in IHC staining. ICC positivity was detected in 12 (92.3%) of the listeria positive 13 cases. A highly positive correlation was observed among cytology (14), ICC (12), histopathology and IHC (13) scores ($r2> 0.8$; $P<0.01$). In conclusion, the cytological examination of the pons and medulla oblongata of listeriosis-suspected ruminants revealed that a rapid pre-diagnosis could be made with the presence of intense neutrophils. Also, with ICC staining of cytological preparations, the diagnosis could be performed with 92.3% accuracy. Since ICC is an easy and fast method, it is concluded that it can be used safely especially in field studies, along with cytological examination.

Keywords: *Cytology, Histopathology, Immunocytology, Immunohistochemistry, Listeriosis*

Ruminantlarda Ensefalitik Listeriozisin Hızlı Tanısı İçin Sitolojik ve İmmunositolojik Yöntemlerin Kullanılabilirliğinin Araştırılması

Öz

Listeriozisin tanısında klinik ve patolojik bulgular önemli ise de, kesin teşhiste etken izolasyonu veya etkenin varlığının gösterilmesi gerekmektedir. Bu çalışmada ruminantlarda ensefalitik listeriozisin hızlı teşhisinde sitolojik ve immunositokimyasal (ICC) yöntemlerin, histopatoloji ve immunohistokimyasal (IHC) yöntemle kıyaslanarak rutinde kullanılabilirliklerinin araştırılması amaçlandı. Çalışmada, sinirsel semptomlarla listeriozisten şüphelenilen 25 adet ruminantın (1 sığır, 1 buzağı, 11 koyun, 9 kuzu, 1 keçi ve 2 oğlak) pons ve medulla oblongatasından alınan sürme ve kazıntı preparatlar modifiye Giemsa ile ve ayrıca ICC yöntemle boyandı. Yine aynı bölümlerden alınan doku örnekleri Hematoksilen-Eozin ve IHC yöntemleriyle de boyandı ve skorlanarak ışık mikroskobunda incelendi. Sitolojik muayenelerde 14 olguda yoğun, 4 olguda ise az sayıda nötrofil görülürken, 7 olguda hiç nötrofil gözlenmedi. Sitolojik olarak listeriozis olduğu değerlendirilen bu 14 vakanın 13'ünde histopatolojik incelemelerde tipik mikroapse ve perivaskuler hücre infiltrasyonu ile IHC boyamalarında listeria pozitifliği bulundu. IHC yöntemi baz alındığında, listeria pozitif 13 vakanın 12'sinde ICC pozitifliği (%92.3) belirlendi. Sitolojide az sayıda nötrofil görülen 4 olgu ile nötrofil görülmeyen 7 olguda ise mikroapseye rastlanmadı, IHC ve ICC de negatif olarak bulundu. Sitoloji (14), ICC (12) ile histopatoloji ve IHC (13) skorları arasında yüksek oranda ($r2>0.8$; $P<0.01$) pozitif korelasyon gözlendi. Sonuç olarak listeriozis şüpheli ruminantların pons ve medulla oblongatasının sitolojik muayenesinde, yoğun nötrofil görülmesiyle hızlı ön tanı konulabileceği, yine sitolojik preparatların ICC yöntemle boyanarak, %92.3 doğrulukla teşhisin yapılabileceği ortaya konuldu. ICC'nin kolay ve hızlı bir yöntem olması sebebiyle özellikle saha çalışmalarında sitolojik muayene ile birlikte güvenle kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: *Sitoloji, Histopatoloji, İmmunositoloji, İmmunohistokimya, Listeriosis*

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Introduction

Listeriosis causes meningoencephalitis and abortus in adult animals and septicemia characterized by milier visceral abscesses in youngs, while rarely can also cause mastitis, purulent conjunctivitis and keratitis in cattle. The causative agent of the disease, *Listeria monocytogenes* is also an important foodborne pathogen and infection source for humans [1-3]. *L. monocytogenes* is easily grown in contaminated water, straw, grass, beet pulp, wet feed, and especially in silages and then taken by animals. The disease sometimes causes important epidemics and economic losses [1].

Encephalitic listeriosis is an endemic problem which is quite common in sheep, cattle and goats worldwide. In encephalitic listeriosis, the causative agent usually enters from the oral mucosal wounds, and comes to the trigeminal ganglion via the sensory axons and then arrives to the medulla oblongata [4,5]. In addition, it sometimes affects the cervical part of the medulla spinalis and thalamus, and causes encephalitis [6]. In encephalitic listeriosis, purulent encephalitis develops and meningitis occurs secondarily [7].

Macroscopically, there is usually no lesion in listeriosis, but sometimes leptomeningeal opacity and gray-white melting foci with a few mm diameter can be seen on the cross-sectional area of medulla oblongata [7,8]. Typical microscopic lesion of the disease is the microabscess where the melting areas are present in the brainstem (in pons and medulla oblongata). These microabscesses may be in the form of intense neutrophil leukocytes and macrophage infiltrations, or may be formed due to microglial reaction. In addition, white matter close to purulent foci has vasculitis and fibrin exudation. Perivascular inflammatory cell infiltration (perivascular cuffing) is severe. These include lymphocytes, histiocytes, plasma cells, and few neutrophils and eosinophil leukocytes [5,9].

Clinical findings, necropsy and histopathological examinations in ruminants cause to suspect encephalitic listeriosis, but there is a need to demonstrate the agent by bacteriological culture, PCR or immunohistochemical methods (IHC) for the definitive diagnosis of the disease [10,11]. These specific methods for the presence of the agent take time and require a specific workforce. Previously, the relationship between neutrophil granulocyte presence in cytology and microabscess formation was investigated for the prediagnosis of listeriosis, and it was reported that 90% of microabscess formation was observed in cases with intense neutrophils in cytology. However, no examination was performed to determine the agent in that study [10].

With this study, it was aimed to perform cytological and immunocytological examination of the touching and smear imprint preparations taken from the pons and medulla oblongata region in ruminants. Then, routine histopathological examination of the same regions was carried out and also the presence of the agent was investigated using IHC method. The findings were compared with cytological and immunocytological results. Thus, it was aimed to determine the usabilty of imprint cytology (IC) and immunocytochemistry (ICC) as a new method in the rapid diagnosis of listeriosis.

Material and Methods

In the study, 25 ruminants (1 cow, 1 calf, 11 sheep, 9 lambs, 1 goat and 2 kids), which have neural symptoms such as depression, leaning or lateral bending of the head, unilateral facial paralysis and suspected of encephalitic listeriosis in clinical examination, then sent for necropsy to our laboratory, were used. Each animal was originated from different herds located in Konya province and its districts. After systemic necropsies were performed routinely, the skulls were opened, firstly imprint cytological specimens were taken from the cut surface of the brainstem (pons and medulla oblongata) using the touching and/or scraping method and then tissue samples were taken for histopathological and IHC studies. For the study, ethics committee approval was obtained with the decision no. 2015/48 of the Selçuk University Veterinary Faculty Experimental Animals Production and Research Center Ethics Committee.

Imprint Cytological Examination

After the touching and smear preparations of pons and medulla oblongata region were air dried, they were stained with a rapid Modified Giemsa Technique [12]. For this purpose, 0.5 mL of Giemsa (Merck) solution was dropped on the imprint cytological preparations fixed in 96% alcohol for one minute. Tap water was added until it completely covered the preparation and it was stirred gently with a pipette and washed in the tap water after being stained for 2 - 3 min. The underside of the slides was dried on blotter paper and the wet top side was covered with coverslip and examined in terms of neutrophil content under a light microscope (Olympus BX 51, Tokyo, Japan). The averages of neutrophil numbers in 10 different areas in each specimen were accounted using 40× objective (×400 magnification) and results were scored as shown in *Table 1*.

Immunocytochemical Examination

After air drying, touching and smear preparations taken from the pons and medulla oblongata region were fixed in 96% alcohol for 10 min and washed in distilled water for 5 min. They were then stained according to the NovoLink™ Max Polymer Detection System (RE7280-K, Leica, Buffalo Grove,United States) kit procedure. Rabbit polyclonal Anti-*Listeria monocytogenes* antibody (ab35132, 1:100, one hour at room temperature, Abcam, Cambridge, United Kingdom) was used as primary antibody and 3,3'-diaminobenzidine (DAB) were used as chromogen. In negative controls, phosphate buffer saline (PBS) was

Table 1. *Scoring methods of cytological, histopathological, IHC and ICC results*

Scores	Cytology (× 400)	Microabscess	Perivascular Cuffing	IHC Staining (× 400)	ICC Staining (× 400)
Mild (+1)	1-2 neutrophil granulocytes	One small microabscess	1-2 layers of cells	Staining in 1-10 cells	Positivity in 1-3 cells or areas
Moderate (+2)	3-5 neutrophil granulocytes	Several small microabscesses	3-4 layers of cells	Staining in 11-20 cells	Positivity in 4-8 cells or areas
Severe (+3)	More than 6 neutrophil granulocyte	Medium-sized and some of them adjoining abscesses	5-6 layers of cells	Staining in more than 20 cells	Positivity in more than 9 cells or areas
Very severe (+4)	No scoring	Numerous and large micro-abscesses in the parenchyma	More than 6 layers of cells	No scoring	No scoring

used instead of primer antibody. All stained sections were examined under a light microscope (Olympus BX 51) and cytoplasmic brown staining was considered positive. Positivity scoring was calculated according to the number of stained cells in the whole slide area at ×40 objective magnification *(Table 1)*.

Histopathological Examination

After the cytological specimens were taken into the slides, tissue samples taken from especially the brain stem (pons and medulla oblongata), cerebral cortex and cerebellum longitudinally and horizontally were fixed in 10% formaldehyde solution for one day and then routine tissue processing procedures were performed. Five-micron thick sections were taken from the paraffin blocks and stained with Hematoxylin-Eosin (H&E). The changes observed in histopathological examinations were scored as shown in *Table 1*, similar to those performed by Oevermann, Di Palma [5].

Immunohistochemical Staining

The samples were cut to five microns thick and taken to polylisine slides. After deparaffinized in xyloles and rehydrated in graded alcohols, slides were stained according to NovoLinkTM Max Polymer Detection System (RE7280-K, Leica, Buffalo Grove, United States) kit procedure. Proteinase K was used in the antigen retrieval process, Rabbit polyclonal Anti-*Listeria monocytogenes* antibody was used as primary antibody (ab35132, 1:100, one hour at room temperature, Abcam, Cambridge, United Kingdom) and DAB was used as chromogen. In negative controls, PBS was used instead of primer antibody. All stained sections were examined in a light microscope (Olympus BX 51) and scored as shown in *Table 1* according to the number of cells stained positively at ×40 objective magnification.

Statistical Analysis

IHC and ICC staining results were compared using t test. IC, ICC, histopathological findings and IHC staining scores were compared using Pearson Correlations test (SPSS 13.0 for Windows/SPSS® Inc., Chicago, USA). Results were interpreted according to $P<0.05$ and r^2 values.

Results

Macroscopic Results

Macroscopically, there was no significant finding other than hyperemia in the meninges. Although a large melting and bleeding area was seen in the brainstem of only one sheep, this case was found to be negative for listeriosis in IHC staining.

Microscopic Results

Histopathological lesions, IC and IHC scores and ICC staining results are summarized in *Table 2*.

Histopathological examinations revealed microabscesses *(Fig. 1-A,C)* at different scores in the brainstem of 13 cases, and perivascular inflammatory cell infiltrations including neutrophil granulocytes were observed in 16 cases *(Fig. 1-C,D)*. In these microabscesses, there were few or more neutrophil granulocyte infiltrations according to the scores and glia cells, and sometimes necrotic neurons in the middle of them. In some areas, the microabscess foci were longitudinally extending along the axons. Besides, coenurosis, purulent meningoencephalitis and malacia were detected in 3 other lambs with perivascular inflammatory cell infiltration but not microabscess. In the lamb with purulent meningoencephalitis, severe purulent meningitis was seen and more widespread neutrophil granulocyte infiltrations, which were not typical micro-abscess-shaped, were noticed in brain tissue.

The definitive diagnosis of listeriosis was performed by IHC method in the study. According to this, of the 25 cases, 13 (52%) were found to be positive for *L. monocytogenes* antigen. Positive staining was observed in microabscesses at the brain stem, usually in the cytoplasm of neutrophils and glia cells *(Fig. 2-A,D)*. In addition, in some perivascular areas near the microabscess, positive staining was observed in the cytoplasm of few neutrophil granulocytes among the mononuclear cells *(Fig. 2-D)*.

In the examination of imprint cytological brainstem preparations prepared during necropsy, intensive neutrophils (+ 2 and + 3 scores = mean 3 - 5 or more) were observed in 14 patients *(Fig. 3-A,C)*, and in 13 of them, purulent

Table 2. *Distribution of cytological, histopathological, immunohistochemical and immunocytochemical findings in animal species*

Findings		Scores	Animals						
			Calf (n:1)	Cow (n:1)	Sheep (n:11)	Lamb (n:9)	Goat (n:1)	Kid (n:2)	TOTAL (n:25)
Histopathological findings	Microabscess scores*	0	1	1	1	8	-	1	12
		+1	-	-	3	-	-	-	13
		+2	-	-	-	-	-	-	
		+3	-	-	2	-	1	1	
		+4	-	-	5	1	-	-	
	Perivascular cuffing scores **	0	1	1	1	5	-	1	9
		+1	-	-	-	2	-	-	16
		+2	-	-	-	-	-	-	
		+3	-	-	2	-	-	-	
		+4	-	-	8	2	1	1	
	IHC staining scores ***	0	1	1	1	8	-	1	12
		+1	-	-	1	-	1	-	13
		+2	-	-	1	-	-	1	
		+3	-	-	8	1	-	-	
Cytological/immuno-cytological findings	Neutrophil scores ****	0	-	-	1	5	-	1	7
		+1	1	1	-	2	-	-	18
		+2	-	-	3	-	-	-	
		+3	-	-	7	2	1	1	
	ICC staining scores *****	0	1	1	2	8	-	1	13
		+1	-	-	1	-	1	1	12
		+2	-	-	2	-	-	-	
		+3	-	-	6	1	-	-	

Scores: ** 0 = no lesions, +1 = one small microabscess, +2 = several small microabscesses, +3 = Medium-sized and some of them adjoining microabscesses, +4 = Numerous and large microabscesses in the parenchyma; ** 0 = no lesions, +1 = Perivascular cell infiltration in 1-2 layers, +2 = Perivascular cell infiltration in 3-4 layers, +3 = Perivascular cell infiltration in 5-6 layers, +4 = Perivascular cell infiltration forming more than 6 layers; *** 0 = No staining, +1 = positive staining in 1-10 cells, +2 = positive staining in 10-20 cells, +3 = positive staining in more than 20 cells; **** 0 = No neutrophil, +1 = 1-2 neutrophils (mild), +2 = 3-5 neutrophils (moderate), +3 = 6 and more neutrophils (severe); ***** 0 = No staining, +1 = positivity in 1-3 cells or areas, +2 = positivity in 4-8 cells or areas, +3 = positivity in more than 9 cells or areas*

encephalitis characterized by microabscess and perivascular cell infiltration were identified in histopathological examinations. In IHC staining of these 14 cases, listeria positivity in accordance with microabscess was found in 13 (92.8%) of them. In one lamb with neutrophils at +3 score in the imprint cytology, there was no IHC or ICC positive staining and typical microabscess formation, but there was severe purulent meningoencephalitis. No microbscess formation and IHC positive staining were observed in none of the cases with 1 - 2 neutrophils (+ 1 score, 4 cases, *Fig. 1-A)* or no neutrophils (7 cases) in IC examination.

In the study, 12 cases (48% of total number of animals) were positive in ICC staining of brainstem tissue imprint preparations. Based on the IHC method in this study (13 cases), a positivity of 92.3% was determined in ICC. ICC scoring was similar to that of IHC. It was noted that positive staining was usually found in neutrophil granulocytes and in the cytoplasm of round or oval nucleated cells, which were considered to be glia cells, and have a diffuse-dense staining pattern that was sprinkled in granular or sometimes filled cytoplasm *(Fig. 4)*. While the cases showing strong positivity (+3 scoring) in the preparation can be evaluated more easily and quickly during the ICC examination, the entire preparation had to be screened laboriously and patiently in order to reach a final decision in negative cases.

On the basis of animal species, listeriosis was found in 10 of the 11 sheep and in only one of the 9 lambs, and only one positive case was found in goats and kids. One cattle and one calf were negative for listeriosis.

In this study, very high (r^2>0.8; P<0.01) positive correlation was determined between microabscess formation (13 cases), perivascular cuffing (16 cases), IHC (13 cases), intense neutrophils in imprint cytology (14 cases) and ICC (12 cases) positivity *(Table 3)*.

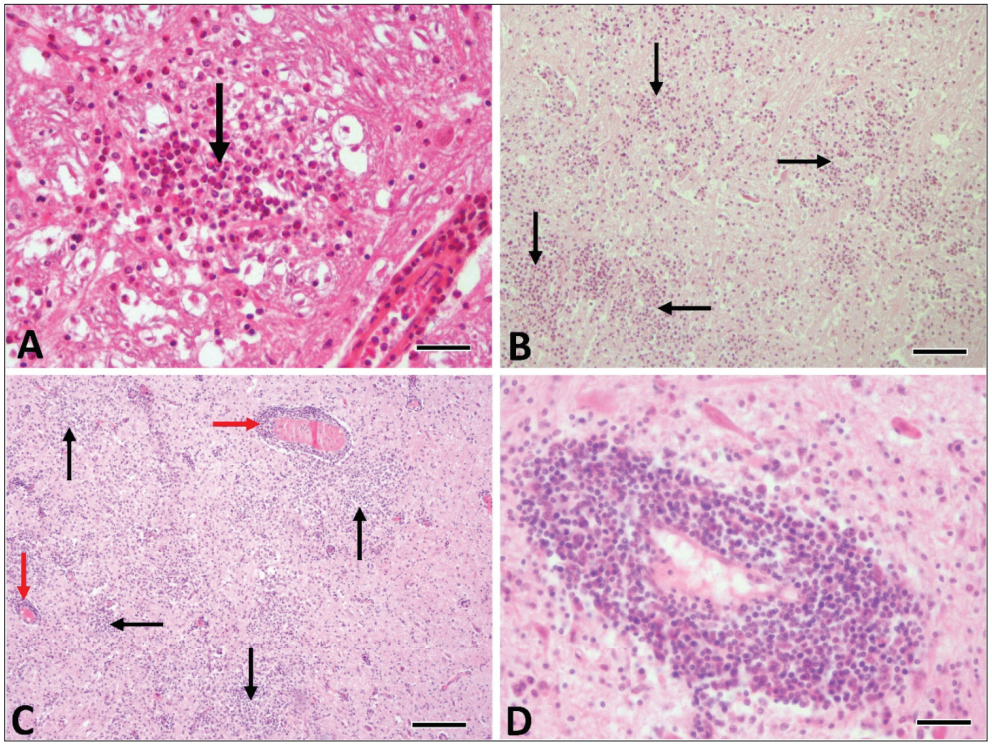

Fig 1. Microabscess and perivascular cell infiltrations in brain stem. **A.** Mild (+1) microabscess *(arrow),* sheep, **B.** Severe (+3) (Large, small and confluent) microabscesses *(arrows),* sheep, **C.** Perivascular cell infiltration (+3) *(red arrows)* and numerous, common microabscesses (+4) *(black arrows),* kid, **D.** Intensive perivascular cell infiltration (+4), sheep, H&E. Scala bar = 50 µm (A,D) and 100 µm (B) and 200 µm (C)

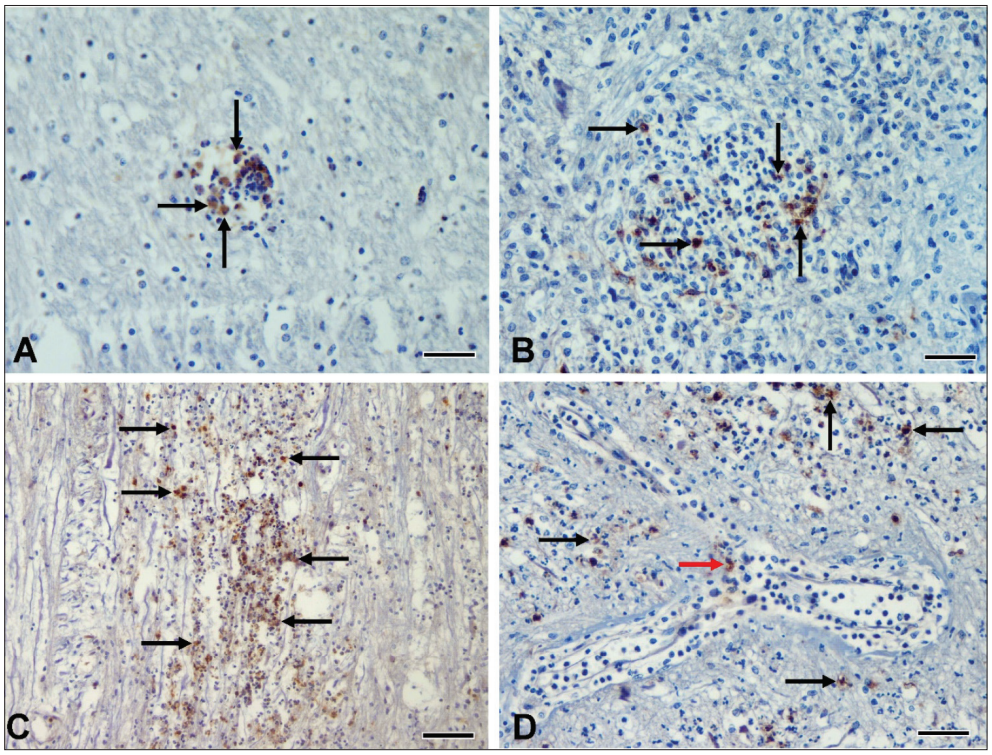

Fig 2. *L. monocytogenes* positive IHC staining in the cytoplasm of neutrophils and glia cells in the brain stem *(black arrows).* **A.** Mild (+1), lamb, **B.** Moderate (+2), kid, **C.** Severe (+3), microabscess along the longitudinal line (parallel to the axon length) in the brain stem, lamb, **D.** Positive staining in parenchyma (Severe (+3) and in perivascular cells in the area close to the microabscess *(red arrow),* sheep, IHC (DAB). Scala bar = 50 µm (A-B,D) and 100 µm (C)

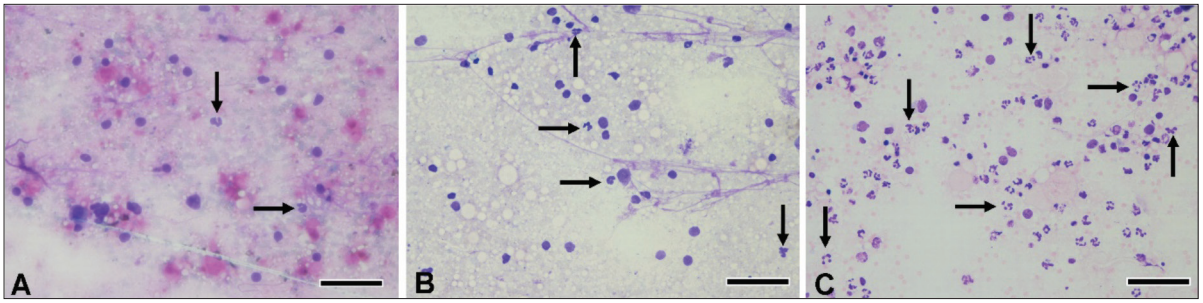

Fig 3. Neutrophil *(arrows)* density in brain stem cytology, sheep, Modified Giemsa, **A.** Mild (+ 1), **B.** Moderate (+ 2), **C.** Severe (+ 3). Scala bar = 20 µm (A-C)

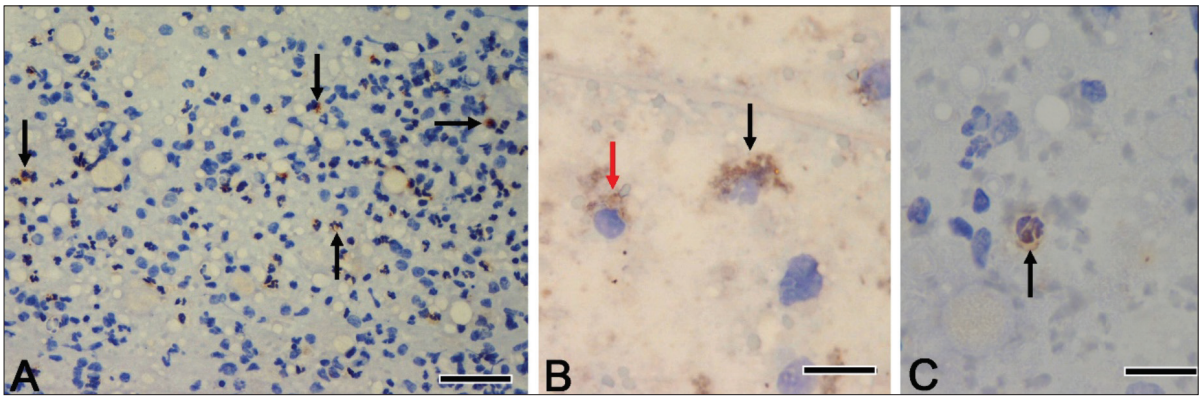

Fig 4. A-C. *L. monocytogenes* positive ICC staining in cytoplasm of neutrophil *(black arrow)* and glia cells *(red arrow),* **A-B:** kid, **C:** lamb, ICC (DAB). Scala bar = 50 µm (A) and 20 µm (B-C)

Table 3. *Degree of correlation between groups. Pearson Correlation Test*

Findings	Perivascular Cuffing	IHC	Neutrophil in Cytology	ICC
Microabscess	$r^2 = +0.90$ P<0.001	$r^2 = +0.97$ P<0.001	$r^2 = +0.90$ P<0.001	$r^2 = +0.92$ P<0.001
Perivascular cuffing		$r^2 = +0.92$ P<0.001	$r^2 = +0.96$ P<0.001	$r^2 = +0.94^*$ P<0.001
IHC			$r^2 = +0.92$ P<0.001	$r^2 = +0.94$ P<0.001
Neutrophil in cytology				$r^2 = +0.91$ P<0.001

Discussion

Listeriosis is not only an important problem in animal husbandry due to the fact that it causes widespread deaths in farm animals and is difficult to diagnose and treat, but also a very important foodborne infection for people. The disease occurs in single cases in cattle and as herd problems in sheep and goats [1,10,13]. It is stated that the disease is less common in goats than sheep and this may be due to the fact that goats are usually grown in mountainous areas and may have different dietary habits due to their free movements [14]. Similarly, it was attracted attention, in this study, that 11 of 13 cases diagnosed as encephalitic listeriosis were sheep while only 2 of them were goat.

The rate of listeriosis in ruminants clinically presenting neurological symptoms and suspected of listeriosis was 52% (13/25 cases) in this study. It is seen that this rate is 55% (11/20) for sheep plus lamb and 90.9% (10/11) for sheep only. Thus, as stated by other researchers [15,16], more than half of the sheep showing neurological symptoms in the winter and spring periods may be related to listeriosis. Therefore, it would not be wrong to evaluate this kind of events as listeriosis from the beginning to save time for treatment.

L. monocytogenes is considered to be the most effective neuroinvasive bacterium and it has been reported that it has the potential to overcome the blood-brain barrier or blood-placental barrier within infected leukocytes or free bacteria in the blood can enter the brain tissue by invading vascular endothelium directly [1,4,17]. Alternatively,

the bacterium reaches the brain through neuronal pathway with centripedal movement via axons [18,19]. In a neuropathogenesis study [5], the researchers reported that in most cases the agents reached the brain via axonal migration through 12th (hypoglossal), 7th (facial) and 5th (trigeminal) cranial nerves, and rarely the 3rd, 6th, 8th and 10th nerves. The same researchers [5] also showed that the infection spread to other regions axially within the brain, and that the microabscess and the active antigens were longitudinally aligned with the axon. Similarly, in this study, it was found that some microabscesses *(Fig. 2-C)* showed longitudinal alignment in parallel direction to axons, and there was significant degeneration in these axons. In addition, because it was determined that there was usually no IHC staining in perivascular areas, it was evaluated that as the researchers reported, the disease reached the brain through the nerves.

Typical neuropathological lesions in listeriosis are the microabscesses that are sometimes accompanied by melting areas in the brainstem. These microabscesses consist of different numbers of neutrophil granulocyte clumps and different amount of macrophage (microglia) infiltrations. Perivascular cell infiltrations containing lymphocytes, histiocytes, plasma cells, and few neutrophil and eosinophil leukocytes in the disease may be severe [1,5,9]. Oevermann, Di Palma [5] scored microabscess and perivascular cuffing in the disease and considered the microabscess in which neutrophils were dense as acute, the microabscess dominated by macrophages as chronic, and the cases where they were both together as subacute. In our study, microabscesses were observed in all of the cases with listeriosis (13 cases). According to the scoring, 3 of them had mild (+ 1; small, single microabscess, *Fig. 1-A),* and 10 of them had many wide-reaching microabscesses (scores + 3 and + 4, *Fig. 1-B,C)*. In all cases, intense presence of neutrophils were observed in microabscesses and were interpreted as acute according to the way the researchers' categorization [5]. As reported in the above literature, microabscesses are the most prominent and specific histopathological findings of encephalitic listeriosis. Severe perivascular inflammatory cell infiltrations were observed in 16 cases *(Fig. 1-C,D),* and 13 of them had listeriosis, and the other three were due to different inflammatory causes (purulent meningoencephalitis, malacia and coenurosis) and had no typical microabscess. Thus, it was seen that perivascular inflammatory cell infiltrations, including neutrophils, were significant in terms of demonstrating a purulent encephalitis but had no specific significance when they were not associated with microcabscesses.

Although the standard bacteriological culture method, which has been used for the definitive diagnosis of encephalitic listeriosis for years, is important in the isolation of the agent, it has negative aspects such as long duration of the method, the need for costly additional applications and the lack of growth in cases where antibiotics are used [5,20].

It is seen that IHC method has been used effectively in brain tissue in the etiological diagnosis of the disease in recent years and bacterial antigens can be detected although antibiotics have been used in animals [5,15,20-22]. In a study [23] listeria antigen was found positive in IHC staining method in 34 (80.9%) of 42 ruminants but determined 28.5% growth in culture. Campero, Odeon [21] reported that bacterial isolation could be performed in only 10 of IHC positive 17 cases with typical histopathological findings. Similarly, in another study the researchers [20] also reported positive results with IHC in patients with negative culture. As a result of these studies, it was emphasized that IHC was a faster and more specific technique in the diagnosis of encephalitic listeriosis in ruminants. In this study, *L. monocytogenes* positivity was found in IHC staining (52%) in 13 of 25 ruminants showing neurological symptoms and the IHC method was used as the base in confirmation of the diagnosis. It was observed that listeriosis was determined in all cases with typical microabscess (13 cases) and the result was fully compatible with IHC. Thus, it was considered that in cases where etiological diagnosis was not possible, a high rate diagnosis of listeriosis could be made by the detection of typical microabsscess histopathologically in the brain stem as observed in the study.

In IHC staining, generally no positive staining of the agent was observed in the perivascular areas and meninges, except few positive staining in some neutrophils in the perivascular areas, only adjacent to the microabscess or in very close *(Fig. 2-D)*. They were thought to be caused by transporting the neutrophils in the microabscess to the adjacent perivascular areas. In this case, it can be said that the agent reaching the brain by neuro-axonal way in accordance with the generally accepted pathogenesis of encephalitic listeriosis creates a lesion in the parenchyma first and then tends to spread towards the periphery and meninges.

A group of our researchers [10] previously researched the relationship between the presence of neutrophils in the brain stem imprint cytology and microabscess formation in suspected cases of listeriosis in sheep and cattle and they reported that neutrophil granulocytes were found in the cytological examinations in 9 of 10 cases with microabscess, and that the presence of neutrophil granulocytes in the cytology could be evaluated as "positive" in the preliminary diagnosis of encephalitic listeriosis. In this study, neutrophil granulocytes were found in 18 cases in the IC preparations prepared by touching and scraping method. While neutrophils were seen in 14 of them with significant and severe score (+2 and +3), few neutrophils were observed in 4 of them. Both IHC and ICC were found negative for listeriosis in cases with few neutrophils and no microabscess was observed in histopathological examination. In these cases, neutrophil granulocytes in the vessels associated with other septicemic diseases could be taken into cytological preparations during scraping/

rubbing and were therefore interpreted as being seen in cytology. IHC positivity was found in 13 out of 14 cases with intense neutrophil granulocytes (+2 and +3 intensity) and the rate of listeriosis compliance was determined as 92.8% with IC examination. In one case, intense (+3) neutrophil granulocytes were detected, but IHC and ICC stainings were negative. Severe purulent meningoencephalitis not associated with listeriosis was detected in this case and this infection was interpreted as the source of neutrophil granulocytes in imprint cytology.

Marco, Ramos [24] and Liu, van Kruiningen [25] each conducted a study on the diagnosis of listeriosis with immunocytochemical method, however these studies had been carried out in the brain tissues with the known IHC method not in cytological preparations, but this method was called "immunocytochemical" examination by the authors. Apart from this, a study truely carried out with ICC method in the cytological preparations is not present previously. Therefore, in this study conducted with ICC staining technic as a novel method, 92.3% (12 of 13 cases) positivity was determined when compared to IHC. ICC staining was not seen in only one case with listeria positive, but it was thought that this could be related to staining error or that possible slight positive staining may be overlooked in the laborious and sensitive evaluation process.

Despite the material number was low in the study, histopathological, IC, IHC and ICC values were not statistically different from each others (t test; $P>0.05$), and IC (92.8%) and ICC (92.3%) methods were considered to be an alternative in the rapid diagnosis of listeriosis. In addition, the presence of neutrophil in imprint cytology, microabscess formation, perivascular cuffing, and a high positive correlation ($r^2 >0.8$; $P<0.01$) between ICC and IHC positivity support this conclusion.

With ICC method, touching and/or scraping preparations taken from the brainstem can be stained after a few minutes of alcohol fixation and evaluated in the same day without any procedure requiring several days, such as tissue fixation, washing, paraffin embedding and sectioning-staining stages. It was seen that the cases showing intense positivity in IC and ICC examinations (+3 scoring cases) were more easily evaluated and the result could be reached easily. On the other hand, it was understood that all the preparation should be scanned and examined thoroughly in order to reach the final decision of negative cases and weakly stained or low scored (+1) cases since there was no tissue integrity in the imprint cytology and no lesion localization in a specific area. In addition, it was noticed that DAB staining precipitates and artifacts, which might be present in ICC preparations, could adversely affect the evaluation process and would cause erroneous results if attention is not paid. Therefore, the advantages of ICC staining were that it was easier and took shorter time than IHC, the disadvantages were that errors could be made frequently, positivity was more subjective than IHC and it required a patient microscopic examination. On the other hand, lesioned tissue fresh cytology may have some risks on the contamination of possibly living organisms, through the bench, microscope, and staining set, even for the alcohol fixed smear slides. Therefore, care should be taken to reduce the risk of contamination to clean areas and the staff while performing fresh tissue cytology during necropsy.

The availability of ICC method, which had a staining procedure in a much shorter time than culture and IHC methods, was investigated in this study, and it was concluded that immunocytology could be recommended as a fast, reliable and effective new method in the diagnosis of encephalitic listeriosis. In addition, it was suggested that the studies about the detection of the agent from CSF taken from live animals showing the clinical findings of encephalitic listeriosis could be performed with ICC method based on the idea that *Listeria monocytogenes* went through CSF due to lesions in the brain.

Conflict of Interest

The authors have no conflict of interest.

Authors Contributions

Ö. Özdemir, M. Ortatalı, F. Terzi, F. Hatipoğlu, M. K. Çiftçi, and M. B. Ateş made the experiment and the histological and immunohistochemical interpretation, and wrote the manuscript. Ö. Özdemir and M. Ortatatlı planned methodology. F. Terzi and M. B. Ateş investigated resources. Ö. Özdemir, F. Hatipoğlu, M. K. Çiftçi, M. Ortatatlı, F. Terzi, and M. B. Ateş wrote and review and editing the manuscript. All authors discussed the results and contributed to the final manuscript.

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