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Evaluation of MMP-9 and iNOS expressions in sheep with encephalitic listeriosis

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ABSTRACT:

This study aimed to correlate Matrix Metalloproteinase-9 and iNOS expressions with the severity of histopathological findings in tissue samples taken from sheep with encephalitic listeriosis. Thus, the role of these molecules in the pathogenesis of the disease can be elucidated. After systemic necropsy, tissue samples of adult sheeps with meningoencephalitis were investigated by the culture, histopathological and immunohistochemical methods in the presence of *Listeria* spp. isolation from tissues was performed in accordance with the USDA-FSIS method with some modifications. Tissue samples were fixed in a 10% buffered formaldehyde solution. Following routine procedures, tissue sections at 5 µm were stained with Hematoxylin and Eosin, investigated under light microscope and photographed. Immunohistochemical staining was performed on the tissues using the avidin-biotin immune peroxidase complex method. *Listeria* spp. were obtained in 20 (83.3%) of 24 tissue samples with the presence of bright grey-black centred smooth colonies on Listeria Selective Agar and identified as *Listeria monocytogenes* through the phenotypically supportive tests. Liquefaction necrosis, purulent meningoencephalitis, perivascular cuffing, microabscesses and glial nodules were the most important histopathological findings. MMP-9 immunpositive reactions were observed in the cytoplasm of microglial cells and neurons in areas where inflammatory and necrotic areas are concentrated in medulla oblongata and pons. In perivascular cuffing areas, immune reactions in endothelial cells were detected. We detected iNOS positive reactions in the medulla oblangata and pons, especially in inflammatory cells in the microabscesses. Consequently, a positive correlation ($p < 0.05$) was found between MMP-9 expression and the severity of histopathological findings in sheep with encephalitic listeriosis. In addition, we found that iNOS expression increased in parallel with the increase in MMP-9 expression.

Ensefalitik listeriyozisli koyunlarda MMP-9 ve iNOS ekspresyonunun değerlendirilmesi

ÖZET:

Bu çalışmada ensefalitik listeriyozisli koyunlardan alınan doku örneklerinde gözlenen histopatolojik bulguların şiddeti ile Matriks metalloproteinaz-9 ve iNOS ekspresyonlarını korele etmeyi amaçladık.Böylece bu moleküllerin hastalığın patogenezindeki rolü açıklanabilecektir. Sistemik nekropsi sonrası meningoensefalitli erişkin koyunlardan alınan doku örnekleri *Listeria* spp. varlığı için kültür, histopatolojik ve immunohistokimyasal olarak incelendi. Dokulardan *Listeria* spp. izolasyonu bazı modifikasyonlarla USDA-FSIS yöntemine uygun olarak gerçekleştirildi. Doku örnekleri %10'luk tamponlu formaldehit solüsyonunda tespit edildi. Rutin işlemlerden sonra 5 µm kalınlığındaki kesitler Hematoksilen&Eozin ile boyandı, ışık mikroskobu altında incelendi ve fotoğraflandı. Dokulara immunohistokimyasal boya olarak avidin-biotin immunperoksidaz kompleks metodu uygulandı. *Listeria* spp. Listeria Selective Agar üzerinde parlak gri-siyah merkezli pürüzsüz koloniler bulunan 24 doku örneğinden 20'sinde (% 83.3) elde edildi ve fenotipik olarak destekleyici testler yoluyla *Listeria monocytogenes* olarak tanımlandı. Likefaksiyon nekrozu, purulent meningoensefalitis, perivasküler hücre infiltrasyonu, mikroapseler ve glial nodüller en önemli histopatolojik bulgulardı. MMP-9 immunpozitif reaksiyonları yangının ve nekrozun yoğun olduğu alanlardaki mikroglial hücreler ve nöronların sitoplazmasında gözlemledik. Perivasküler hücre infiltrasyonu alanlarında, endotelyal hücrelerde de immun reaksiyonu saptadık. iNOS pozitif reaksiyonları özellikle medulla oblongata ve pons bölgesinde yer alan mikroapselerdeki yangısal hücrelerde tespit ettik. Sonuç olarak ensefalitik listeriyozisli koyunlarda MMP-9 ekspresyonu ile histopatolojik bulguların şiddeti arasında pozitif bir korelasyon tespit ettik ($p<0.05$). Buna ek olarak MMP-9 ekspresyonundaki artışa paralel olarak iNOS ekspresyonun da artış gösterdiğini ortaya koyduk.

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1. Introduction

Listeriosis caused by members of the genus Listeria, is a ubiquitous, Gram-positive, facultative intracellular bacterium, which is responsible for sporadic and epidemic food-/feed- borne infections in ruminants and humans (12, 13, 26). *Listeria monocytogenes* is the primary pathogen in humans and animals cases, however, *Listeria ivanovii* was reported occasionally (11, 43). The other Listeria species such as *Listeria seeligeri*, *Listeria grayi* and *Listeria innocua* is found rarely in some cases with their unclear pathogenicity (33). Listeriosis causes significant economic losses in ruminants and serious health problems in humans (10, 16). Listeriosis is mostly detected in autumn, winter and early spring in temperate and cold climates and is thought to occur due to consumption of poorly prepared silage (17, 28, 44). This infection can lead to different clinical symptoms such as gastroenteritis with high fever, mastitis, encephalitis, septicemia and abortion (3, 20). Encephalitic listeriosis caused by only species-*L. monocytogenes* is more common especially in small ruminants and has high mortality rates (1, 37). Sheep are more sensitive to listeriosis than cattle (9). Encephalitic listeriosis causes anorexia, depression, excessive salivation, eye infections, keratitis, unilateral facial paralysis, motor incoordination and tremors. It also leads to tilting of the head and permanent circling motions (4, 30). Microabscesses, glial nodules and perivascular cell infiltration observed in the brainstem are diagnostic histopathological findings of encephalitic listeriosis (35).

Matrix metalloproteinases (MMPs) degrade all extracellular matrix (ECM) components and perform important tasks such as tissue remodeling and modulation of the immune system (39, 45). Increased expression of MMPs is known in many central nervous system diseases such as multiple sclerosis, experimental autoimmune encephalomyelitis, alzheimer's disease, stroke and meningitis (38, 42). The increase in MMP-9 expression, especially in samples from meningitis of viral and bacterial origin, suggests that this molecule may be an important factor in the pathogenesis of the disease (23). MMP-9, produced from activated microglia as a result of the effects of cytokines and reactive oxygen species, destroys the extracellular matrix of the brain and causes impaired neuronal function (5).

Nitric oxide (NO) is synthesized from L-arginine via nitric oxide synthase (NOS) (41). NOS enzyme responsible for nitric oxide formation exists in three forms. These are the two constitutive forms (neuronal NOS (nNOS) and endothelial NOS (eNOS)) and inducible NOS (iNOS), respectively (40). NO contributes significantly to the defense against viruses, bacteria, fungi and microbial agents such as protozoan and metazoan parasites. However, NO produced by iNOS-expressing cells contributes to a variety of disease symptoms ranging from immunosuppression to apoptosis and tissue damage. (36). There is a general consensus in the literature that the increase in iNOS expression is important in the pathogenesis of natural listeriosis in the brains of cattle and goats (41).

In this study, it was aimed to correlate MMP-9 and iNOS expressions with the severity of histopathological findings in tissue samples taken from sheep with encephalitic listeriosis.

2. Material and Methods

Animals:

The material of this study was consisted of 24 adult sheep that were brought to Pathology Department for systemic necropsy between 1998 and 2020. Some anamnestic findings were gathered accompanying to the sheep such as various neurological symptoms such as permanent circling movement, head pressing, unable to stand, hypersalivation, paralysis in the eyelid, sagging on the lower lip, blindness and torticollis. We used normal brain tissues of 4 sheep without any histopathological findings as negative controls.

Microbiological Examinations:

In this study, brain (medulla oblongata, pons and cerebellum) tissues from adult sheep were used for the isolation of Listeria agents. Isolation was performed in accordance with the United States Department of Agriculture - The Food Safety and Inspection Service (USDA-FSIS) method reported by McClain and Lee (27) with making some modifications. For this purpose, 2.5 g tissue sample was transferred to 22.5 ml Pre-Enrichment Broth (Trypticase Soy Broth (Merck 1.05459) containing 0.6% yeast extract and incubated at 30 °C for 24 hours under microaerobic

conditions. At the end of this period, 1 ml of this Pre-enrichment Broth was transferred into 9 ml Listeria Enrichment Broth (UVM formulation) (Oxoid CM0863) and incubated at 30 °C under the same atmospheric conditions. At the end of the period, Listeria Selective Agar (LSA) (Oxoid, CM0856) was plated with 25 µl aliquot of Listeria Enrichment Broth and incubated for 24 hours at 30 °C under microaerobic conditions. As a result of cultural process, the bright grey-black centred smooth colonies on the LSA medium were considered as *Listeria* spp.. Within the scope of identification, Gram staining characteristics, mobility at 25 °C, catalase and oxidase activities, carbohydrate (L-rhamnose, D-mannitol, D-xylose and α-methyl-mannosidase) fermentation capabilities and CAMP activities were evaluated. In the CAMP reaction, control strains of *Rhodococcus equi* (ATCC-33701) and *Staphylococcus aureus* (ATCC-25923) were used (2, 15).

Histopathological Examinations:

After systemic necropsy of sheep, tissue samples (cerebrum, cerebellum, etc.) were fixed in a 10% buffered formaldehyde solution. Following routine procedures, tissue sections at 5 µm were stained with Hematoxylin and Eosin (H&E), investigated under light microscope (Olympus Bx53) and photographed with Cell ^P Program (Olympus Soft Imaging Solutions GmbH, 3,4).

Immunohistochemical Examinations:

Immunohistochemical staining was performed on the tissues using the avidin-biotin immune peroxidase complex method. For immunohistochemical staining, the sections of 4 µm in thickness taken to poly-L-lysine coated slides were deparaffinized and rehydrated in graded alcohols. In order to prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide solution in Phosphate Buffered Saline (PBS) for 15 minutes. For antigen retrieval, the sections were boiled in Citrat Buffer Solution (pH 6) for 25 min in the microwave oven (at 800 watt). In order to prevent nonspecific staining, the sections were incubated for 30 min with non-immune serum (Genemed Biotechnologies REF 54-0003) at room temperature. Diluted antibodies MMP-9 (Santa Cruz, sc-393859, Dilution Ratio: 1/100) and iNOS (Santa Cruz, sc-7271, Dilutio Ratio: 1/100) were incubated for one hour at room temperature. The sections were washed 3 times in PBS solution for 5 minutes, and the biotinylated secondary antibody (Genemed Biotechnologies REF 54-0003) was applied to them at room temperature for 30 minutes. After washing in PBS (3-5 min), all sections were incubated with peroxidase-bound Streptavidin (Genemed Biotechnologies REF 54-0003) for 30 minutes at room temperature. A solution of 3.3-diaminobenzidine tetra hydrochloride (DAB) (Genemed Biotechnologies REF 10-0048) was used as a chromogen for 15 minutes. The sections were treated with Mayer's Hematoxylin for 30 second and washed in running water for 5 min, dehydrated in graded alcohols, cleared in xylene and coated with entellan. Primary antibody was omitted from the negative brain control sections and were treated with diluted normal serum. The slides prepared after the covering were examined under a light microscope and photographed via the Cell^P program. Analyzes of the images were done with Image J Program.

Statistical Analysis:

Histopathological changes (meningitis, perivascular cuffings, microabscesses and necrosis) MMP-9 immunepositive expressions and iNOS scoring were evaluated under a light microscope and scored as absent (-), mild (+), moderate (++) and severe (+++). Correlation tests were used to determine the relationship between the MMP-9 expression and the severity of histopathological changes and between the MMP-9 expression and iNOS variables. In comparing the average of data belonging to the groups where the sample size is less than 20, The Paired Samples T-test was used. The Pearson's Correlation Test was used to calculate the correlation coefficient between the variables. The One-Way Analysis of Variance (ANOVA) was used to test the homogeneity of the variables. Statistical Package for Social Sciences (SPSS) 20 Program was used in statistical tests.

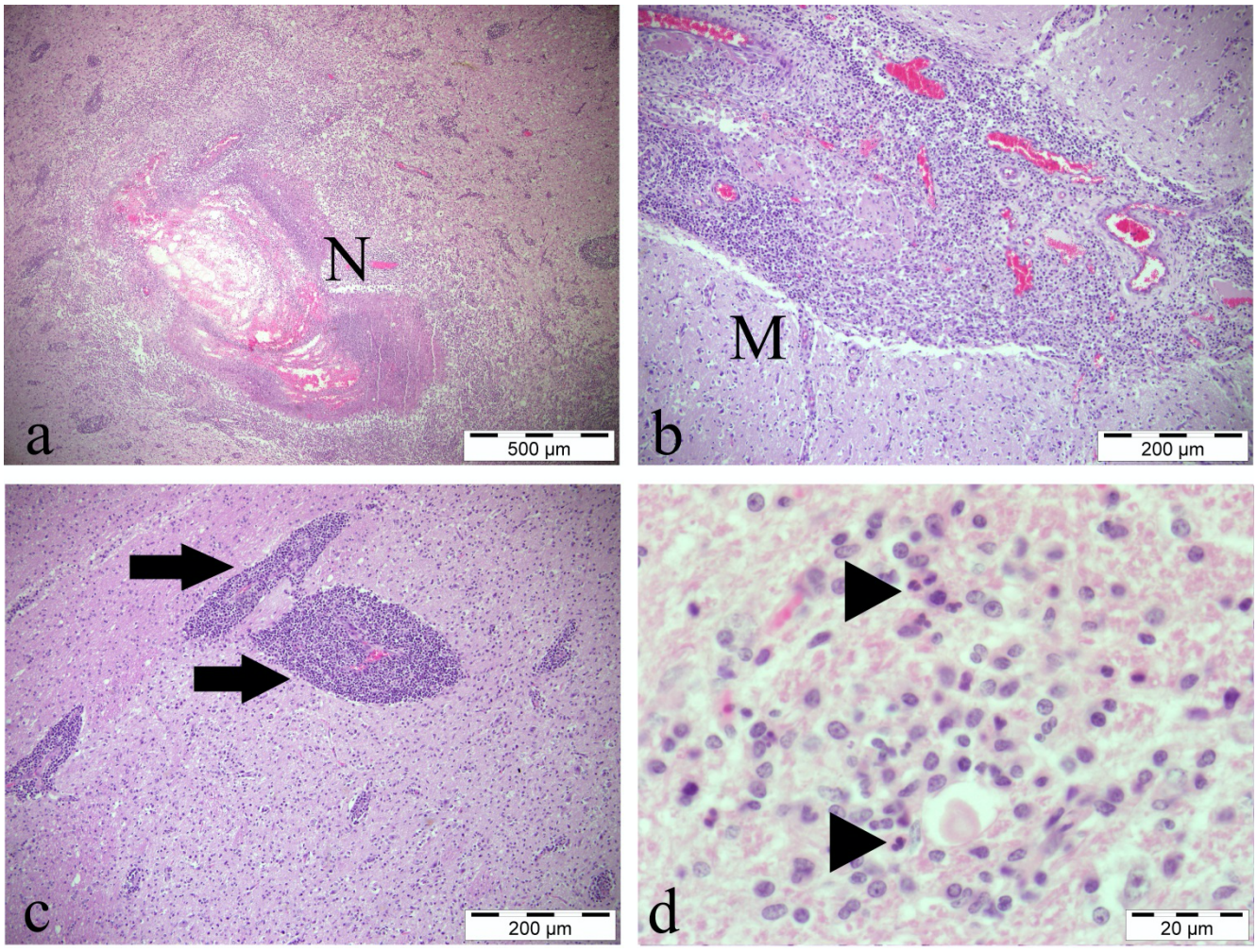

Figure 1: (a) Brainstem, Liquefaction necrosis (N), bar = 500 µm, **(b)** Nonpurulent meningitis (M), bar = 200 µm, **(c)** Pons, Perivascular cuffings (arrows), bar = 200 µm, **(d)** Pons, Microabscess (arrowheads), bar = 20 µm, Hematoxylin & Eosin

Şekil 1: (a) *Beyin kökü, likefaksiyon nekrozu (N), bar = 500 µm,* ***(b)*** *Nonpurulent meningitis (M), bar = 200 µm,* ***(c)*** *Pons, Perivasküler hücre infiltrasyonu (oklar), bar = 200 µm, (d) Pons, Mikroapse (okbaşları), bar = 20 µm, Hematoksilen & Eozin*

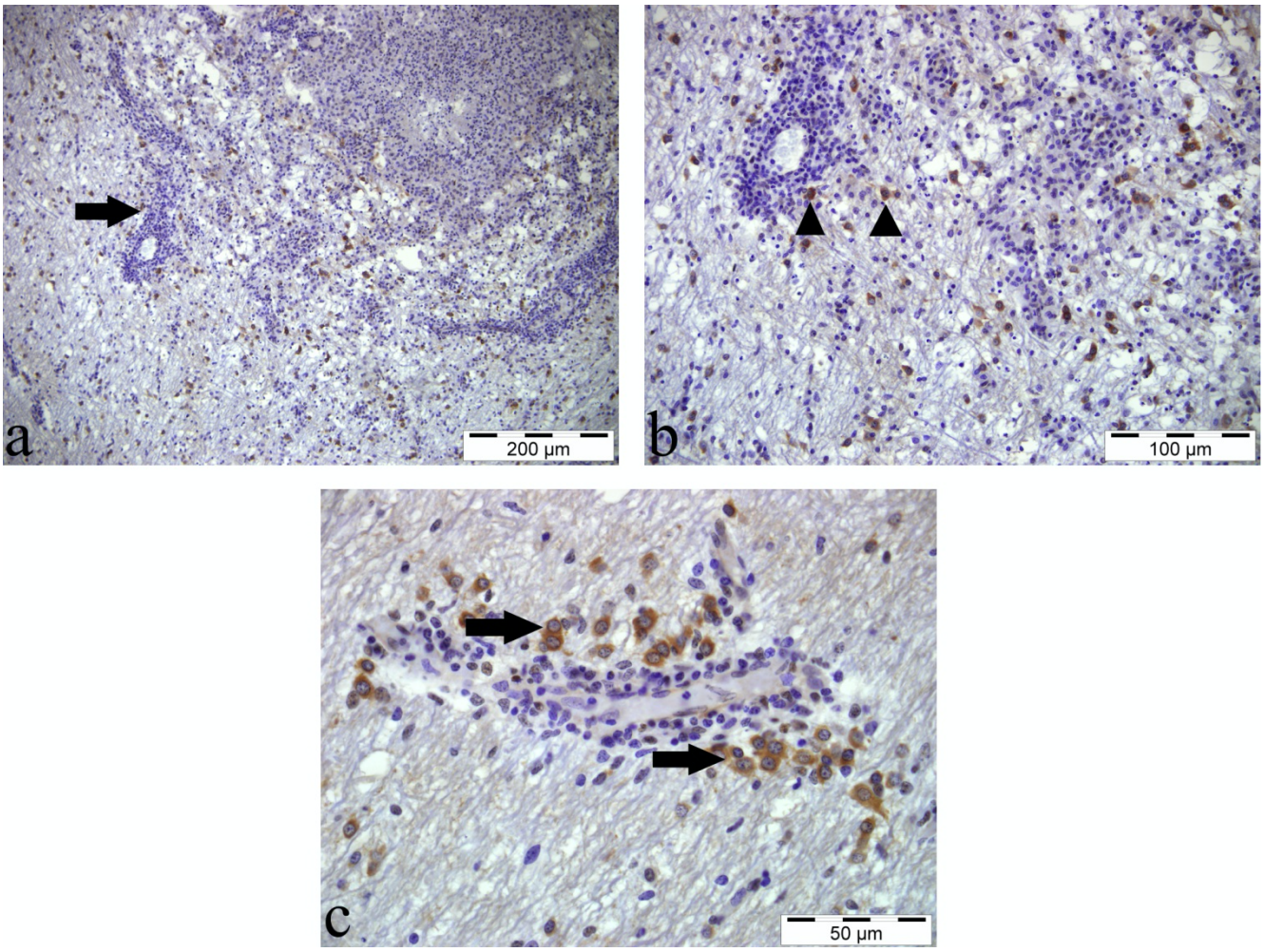

Figure 2: (a) Pons, Intense positive reaction around liquefaction necrosis (N) and perivascular cuffing (arrow), bar = 200 µm, **(b)** Higher magnification, immune positive cells (arrowheads) around perivascular cuffing, bar = 100 µm, **(c)** Immun positive reactions in microglial cells (arrows) around perivascular cuffing and endothelial cells, bar = 50 µm, Immunohistochemistry

Şekil 2: (a) *Pons, Likefaksiyon nekrozu (N) ve perivasküler hücre infiltrasyonu (ok) etrafında yoğun pozitif reaksiyon, bar = 200 µm,* ***(b)*** *Daha yüksek magnifikasyon, perivasküler hücre infiltrasyonu etrafındaki pozitif hücreler (okbaşları), bar = 100 µm,* ***(c)****Perivasküler hücre infiltrasyonu etrafındaki mikroglial hücreler (oklar) ve endotel hücrelerde immun pozitif reaksiyonlar, bar = 50 µm, İmmunohistokimya*

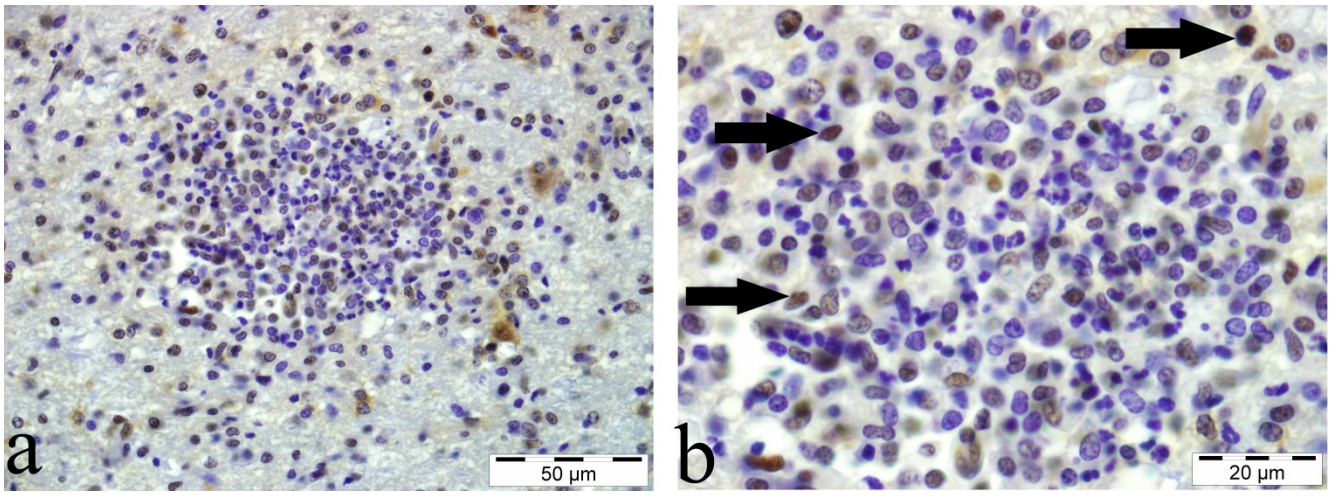

Figure 3: (a) Pons, iNOS immunepositive reactions in inflammatory cells in microabscess, bar = 50 µm,(**b)** Higher magnification, iNOS immunepositive reactions in inflammatory cells (arrows), bar = 20 µm, Immunohistochemistry

Şekil 3: (a) *Pons, mikroapsedeki yangısal hücrelerde iNOS immun pozitif reaksiyonlar, bar = 50 µm, (**b)** Daha yüksek magnifikasyon, yangısal hücrelerde iNOS pozitif reaksiyonlar (oklar), bar = 20 µm, İmmunohistokimya*

3. Results

Microbiological Results:

In this study, *Listeria* spp. were isolated from 20 (%83.3) tissue samples with the presence of specific colonies on LSA, typical microscopic morphologies (0.4-0.5 µm wide and 1-2 µm long, non-spore forming Gram positive bacilli), catalase positive and oxidase negative properties and mobility at 25 °C. All of the isolates were identified as *L. monocytogenes* as the result of L-rhamnose and α-methyl-mannosidase activities and positive CAMP reaction with *S. aureus*.

Hematoxylen & Eosin Results:

Liquefaction necrosis infiltrated by neutrophils (Figure 1a), nonpurulent meningitis (Figure 1b), perivascular cuffing consisted of mostly lymphocytes and fewer histiocytes and plasma cells (Figure 1c), varying sizes and multifocal microabscesses (Figure 1d) (including a small number of neutrophil granulocytes, mostly macrophages, histiocytes and plasma cells), glial nodules, neuronal necrosis and neuronophagia were the most important findings observed in histopathological examination of the medulla oblongata and pons.

MMP-9 and iNOS Results:

In immunohistochemical examinations, we did not detect any MMP-9 expression in normal brain tissues. MMP-9 positive reactions were found in the cytoplasm of microglial cells and neurons in areas where inflammatory and necrotic areas are concentrated (Figure 2-a,b). Immune reactions were detected in endothelial cells in perivascular cuffing areas in the pons and medulla oblongata (Figure 2c). We observed iNOS positive reaction in very few neurons in normal brain tissue. Especially, in cases of encephalitic listeriosis, we detected iNOS immunereactivity in the inflammatory cells in the microabscess foci located in the pons and medulla oblongata (Figure 3-a,b).

Statistical Results:

Initially, the homogeneity test of variances, which is the basic assumption of one-way analysis of variance (ANOVA), was confirmed since p value ($p = 0.016$ for HP/MMP-9 and $p = 0.000$ for MMP-9/iNOS) are greater than 0.01 (Table 1 and Table 2). Due to the sample size is less than 20 and the data of histopathological (HP) analysis, MMP-9 and iNOS groups obtained from the same samples, paired samples t-test was used, and the means of the samples were compared. Additionally, correlation analyzes were conducted between the HP and MMP-9 variables and the MMP-9

and iNOS variables. As the results of paired samples t-test, the average of the HP variables was found as 2.15 and the MMP variables as 1.85. A significance value was detected below 0.05 (p = 0.010 for 2-tailed and p = 0.000 for correlation analysis) with 95% confidence interval (Table 1). Indeed, a statistically high relationship was detected between these two variables and it can be said that the MMP-9 expression has increased in parallel with the increase in HP findings. Although there was no statistically significant value between the averages of MMP-9 and iNOS variables (p = 1.000 for 2-tailed), the correlation value between iNOS and MMP-9 variables was found to be 0.781 (p = 0.000), so a statistically high relationship was found between these two variables at 95% confidence interval. Thus, it can be said that the iNOS expression has increased in parallel with the increase in MMP-9 findings (Table 2).

Table 1: A comparative statistical analysis of HP and MMP-9 findings

Tablo 1: *HP ve MMP-9 bulgularının karşılaştırılmalı istatistiksel analizi*

Case number	HP findings	MMP-9 scores	Correlations				Test of homogeneity of Variances (ANOVA)			
					HP	MMP				
4, 6, 7, 13, 14, 15	+++	+++	HP	Pearson Correlation	1	.867**	HP			
1, 3, 10, 20	+++	++		Sig. (2-tailed)		.000	Levene Statistic	df1	df2	Sig.
17	++	++	MMP	Pearson Correlation	.867**	1				
8, 9	++	+		Sig. (2-tailed)	.000					
2, 5, 11, 12, 16, 18, 19	+	+	** Correlation is significant at the 0.01 level (2-tailed)				5.286	2	17	.016

Table 2: A comparative statistical analysis of MMP-9 and iNOS findings

Tablo 2: *MMP-9 ve iNOS bulgularının karşılaştırmalı istatistiksel analizi*

Case number	MMP-9 scores	iNOS scores	Correlations				Test of homogeneity of Variances (ANOVA)			
					HP	MMP				
4, 6, 15	+++	+++	MMP-9	Pearson Correlation	1	.781**	HP			
7, 13, 14	+++	++								
1, 10	++	+++		Sig. (2-tailed)		.000	Levene Statistic	df1	df2	Sig.
3, 1, 20	++	++	iNOS	Pearson Correlation	.781**	1				
19	+	++		Sig. (2-tailed)	.000					
2, 5, 8, 9, 11, 12, 16, 18	+	+	** Correlation is significant at the 0.01 level (2-tailed)				15.362	2	17	.000

4. Discussion and Conclusion

Clinical findings, bacteriological analysis and histopathological changes in the brain are used in the diagnosis of encephalitic listeriosis (9, 25). Characteristic lesions of listerial encephalitis are microabscesses, focal gliosis and perivascular cuffing (8). Typical lesions of the disease are observed in the brainstem (rhombencephalitis), especially in the pons and the medulla oblongata (6, 16). In this study, it was determined the presence of *L. monocytogenes* in 20 of 24 sheep that showed various neurological symptoms such as permanent circling movement, head pressing, unable to stand, hyper salivation, paralysis in the eyelid, sagging on the lower lip, blindness and torticollis similar to the literature

data (1, 3, 4, 28) by bacteriological methods (4, 10, 44). As reported in previous studies, we observed microscopically large areas of liquefaction necrosis (3, 8, 34), nonpurulent meningitis (7, 19, 31), mostly lymphocyte-containing perivascular cuffing (8, 9, 32) varying sizes of multifocal microabscesses (20, 25, 35) (a small number of neutrophil granulocytes in the middle part) in the brainstem.

Matrix metalloproteinases (MMPs) are a family of 28 zinc-dependent endopeptidases; which are subdivided into collagenases, gelatinases, stromelysins, matrilysin, membrane-type metalloproteinases and metalloelastase (23, 24). MMPs cause to cleavage of ECM and modulate the pathological processes such as inflammation and innate immune defenses (24). MMPs are thought to play an important role in the pathogenesis of meningitis, especially since they perform functions such as the breakdown of the blood-brain barrier (BBB) (typical histopathological feature) and the accumulation of blood-derived immune cells (23, 42). MMP-9 is mainly secreted by monocytes, which are central cells in developing an immune response to infectious diseases. The production of MMP-9 by monocytes is interesting in the context of facilitating leukocyte infiltration into infected areas by breaking down type IV collagen in vascular basement membranes (39). In addition, microglia cells are a remarkable source for the secretion of MMPs (38). Due to the destruction caused by MMP-9 in the extracellular matrix of the brain, a disorder in neuronal functions may occur (5). In many infectious diseases, MMP-9 level has been found to increased (39). MMP-9 activity increases in BBB as a result of bacterial meningitis. This increase in concentration is due to the damage that occurs after meningitis (29). In our study, we found that MMP-9 expressions increased significantly in cases where histopathological findings such as meningitis, microabscesses, necrosis and perivascular cuffings were more severe. Therefore, in line with the data obtained from our study, we concluded that there may be a serious relationship between neuronal dysfunction and MMP-9 expression.

There is only one study in which MMP-9 expression is evaluated immunohistochemically in Listeriosis in sheep (21). In this study conducted by İlhan et al. (21), MMP-9 immunoreactivity was reported in the endothelial cells, microglial cells and neurons especially in inflammatory areas. Sulik and Chyczewski (42) was also reported MMP-9 immunoreactivity in brain endothelial cells, an important factor of the BBB. In the present study, MMP-9 expressions were detected in brainstem (neurons, microglial and endothelial cells in inflammatory and necrotic areas are concentrated) were compared to the previous investigation (5, 21, 23, 42). In this study, it was statistically revealed that there is a positive correlation ($p < 0.05$) between MMP-9 expression and the severity of histopathological findings. The MMP-9 expression has increased in parallel with the increase in HP findings (Table 1). Yamada et al. (45) suggested that MMPs inhibitors increase host resistance in *L. monocytogenes* infection.

The excessive NO production, mainly produced by iNOS, has been indicated as a mediator of cellular damage in inflammatory areas. Under these conditions, nitric oxide reacts with molecular oxygen or superoxide and produces reactive nitrogen species that can modify bioorganic molecules and mediate many biological processes, including ECM proteolysis (18). Oxidants such as superoxide, NO and peroxynitrite are critical to MMP activation (14). MMP-9, produced from activated microglia as a result of the effects of cytokines and reactive oxygen species (especially nitric oxide), destroys the extracellular matrix of the brain and causes impaired neuronal function (5, 14). Similar to the previous studies (22, 36, 40, 41), we observed that iNOS immunereactivity in the inflammatory cells in the microabscess foci located in the pons and medulla oblongata. As a result of our statistical analysis, we revealed that the increase in iNOS and MMP-9 expressions were parallel to each other. We interpreted that NO synthesized by iNOS contributes to the MMP-9 activity and this activation may lead to degradation in the ECM of the brain.

In conclusion, in this study, it was found that there was a positive correlation between MMP-9 expression and histopathological findings in encephalitic listeriosis cases in sheep caused by *L. monocytogenes*. Based on the data obtained from this study, it was believable that MMP-9 plays an important role in the pathogenesis of the disease. In addition, we thought that the activation of iNOS expression increases MMP-9 levels. The given characteristic of MMP-9 can be exploited by the researchers as a marker of prognosis and diagnosis of the disease, or a specific structure targeted by the anti-MMPs for preventing of brain damage. Further investigations focused on the MMP-9 activity in the central nervous system is required.

Conflict of Interest

The author declared no conflict of interest.

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Authors' Contributions

Emin KARAKURT contributed to the evaluation of histopathological and immunohistochemical analysis and to write the publication. Fatih BÜYÜK, Özgür ÇELEBİ, Doğan AKÇA and Elif ÇELİK contributed to microbiological analysis. Enver BEYTUT contributed to the evaluation of histopathological and immunohistochemical analysis. Serpil DAĞ contributed to the evaluation of histopathological and immunohistochemical analysis. Hilmi NUHOĞLU contributed to the gross pathology and laboratory procedures of samples taken from animals for histopathological and immunohistochemical analyzes. Ayfer YILDIZ contributed to the gross pathology and laboratory procedures of samples taken from animals for histopathological and immunohistochemical analysis.

Ethical Approval

The ethics committee report of this study was obtained from Kafkas University, Local Ethics Committee of Animal Experiments (Authorization number: KAU-HADYEK-2020/065).

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