

*Research article*

# DETERMINATION BY IMMUNOHISTOCHEMISTRY OF ACUTE PHASE PROTEINS IN NATURALLY INFECTED SHEEP WITH LISTERIOSIS

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Listeriosis is an infectious and fatal disease affecting domestic mammals, poultry, and humans worldwide. The effectiveness of local tissue expression of acute phase proteins in listeriosis in domestic mammals is not yet clear. The aim of this study is to evaluate the local expression of acute phase proteins in 26 brainstem tissue samples according to the distribution and severity of inflammation due to natural Listeriosis disease in sheep. The study material consisted of 26 brainstem paraffin blocks, including 20 from listeriosis-infected cases and 6 from healthy controls. Sections obtained from the paraffin blocks were subjected to histopathological and immunohistochemical analyses. Histopathological examination revealed normal histological structures in the brainstems of the control group. In contrast, brainstem sections from listeriosis cases exhibited histopathological findings such as micro abscesses composed of neutrophil granulocytes and microglial cells, gliosis, meningitis, congestion, perivascular cuffs, neuronal degeneration, and neuronophagia. Based on the distribution and severity of inflammation, listeriosis cases were categorized into three groups: 5 mild cases (Group I), 8 moderate cases (Group II), and 7 severe cases (Group III). Immunohistochemical analysis demonstrated significantly increased expression levels of C-reactive protein (CRP), Haptoglobin (Hp), and Serum Amyloid A (SAA) in listeriosis groups compared to the control group, with the highest statistical scores observed in Group II and Group III ( $p<0.001$ ). The findings of this study suggest that acute-phase proteins may play crucial roles in the pathophysiological processes of naturally infected listeriosis and could express locally. Particularly, the increased expression of these proteins with the progression of inflammation may provide valuable insights into disease severity and the infection process.

**Keywords:** Listeriosis, acute phase proteins, immunohistochemistry

## INTRODUCTION

Listeriosis is recognized globally as an infectious and fatal disease affecting domestic mammals, poultry, and humans. The primary causative agent of the disease is *Listeria*

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*monocytogenes* (Lm), with other species such as *L. grayi*, *L. seeligeri*, *L. innocua*, and *L. ivanovii* contributing less frequently [1,2]. Notably, Lm is responsible for approximately 85% of listeriosis cases in animals, whereas this rate increases to nearly 98% in humans [3]. Moreover, Lm is a significant foodborne pathogen and infection source for humans, making it a critical public health concern [4]. Lm can easily grow in wet feed, hay, grass, beet pulp and especially silage and is easily taken up by ruminants [5,6]. Listeriosis is a disease that can occur in sporadic or epidemic forms in ruminant animals such as sheep, cattle and goats and causes significant economic losses [5,7]. The disease is generally detected in domestic animals during winter and early spring and is thought to be associated with the consumption of poorly prepared silage [5,6,8].

Clinically, listeriosis manifests in ruminants in three forms: meningoencephalitis, septicemia, and abortion, with the encephalitic form being the most frequently observed. Additionally, endocarditis, mastitis, purulent conjunctivitis, and keratitis may occasionally occur [5,9,10]. In the encephalitic form, histopathological findings such as multifocal micro abscesses, meningoencephalitis and perivascular cuffs are seen in the brainstem (pons and medulla oblongata). Micro abscesses, considered a hallmark histopathological feature of the disease, may result from microglial reactions or from intense neutrophil and macrophage infiltrations. In the septicemic form, numerous necrotic and abscess foci are observed in the liver [8,10,11].

The acute phase response (APR) is defined as a series of inflammatory responses by the host to trauma, tissue damage, and infection. Although nonspecific, APR plays a crucial role as part of the innate immune system [12-14]. It is induced by pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1beta (IL-1 $\beta$ ), and interleukin-6 (IL-6), which are secreted by active leukocytes at sites of tissue damage [12,13]. These cytokines regulate the primary response to tissue injury and infection and stimulate the production and plasma release of glycoproteins known as acute-phase proteins (APP) in the liver. APPs are blood proteins used to assess the immune system's response during tissue damage, trauma, and infection [12,15-17].

In recent years, APPs have been widely evaluated as biomarkers for various disorders, including trauma, infection, and tissue damage, in both veterinary and human medicine. It is believed that monitoring APP levels provides a valuable tool for investigating innate immune responses to different non-neoplastic as well as neoplastic diseases [12,17,18]. This study aims to evaluate the local immunohistochemical expression of acute phase proteins such as C-reactive protein (CRP), Haptoglobin (Hp), and Serum amyloid A (SAA) according to the distribution and severity of inflammation in naturally infected sheep with Listeriosis.

# MATERIAL AND METHODS

## Animal materials

The material for this study consisted of brainstem paraffin blocks from 20 *Listeria monocytogenes* immunohistochemically positive sheep (male, 12–24 months old) obtained from the tissue archive of the Department of Pathology, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, between 2018 and 2024. Additionally, brainstem samples from 6 healthy sheep (male, 12–24 months old) collected from various nearby slaughterhouses were included. The study was approved by the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (16.08.2024-56).

## Histopathological examination

Following necropsy, brainstem tissue samples were fixed in a 10% neutral formalin solution 24-48 hr. After fixation, the samples were processed through graded alcohol (70%, 80%, 90%, 100%) and xylene solutions before being embedded in paraffin. Sections obtained from the paraffin blocks were stained with hematoxylin and eosin (H-E) and examined under a light microscope (Olympus BX51, Tokyo, Japan). Histopathological evaluation was performed according to the method described by Oevermann et al. [11]. Micro abscess were graded as follows: score 0: None, score 1: A single small microabscess, score 2: Several small to medium-sized microabscesses, score 3: Widespread medium-sized microabscesses, score 4: Multiple and extensive microabscesses in the parenchyma. Perivascular cuffing was scored as: 0: None, score 1: 1–2 layers, score 2: 3–4 layers, score 3: 5–6 layers, score 4: More than 6 layers. Afterwards, the scores for microabscesses and perivascular cuffing were then combined into a single score. Based on the distribution and severity of inflammation, the total scores were categorized as follows: 0: None, Mild (Group I): Total score of 1–3, Moderate (Group II): Total score of 4–5, Severe (Group III): Total score greater than 6.

## Immunohistochemical examination

Paraffin extraction and rehydration procedures were performed on tissue sections from paraffin blocks. Immunohistochemical staining was performed using a commercial kit according to the previously reported method [19]. Primary Anti-Lm, CRP, Hp, SAA antibodies were used as shown in Table 1.

**Table 1.** Primers used in the study

| Primers                 | Manufacturer             | Dilution |
|-------------------------|--------------------------|----------|
| Anti-L. monocytogenesis | Thermo fisher, PA1-73128 | 1/100    |
| Anti-CRP                | Affbiotech. DF6027       | 1/400    |
| Anti-Hp                 | Affbiotech, DF6467       | 1/400    |
| Anti-SAA                | Affbiotech, DF7899       | 1/400    |

CRP; C – reactive protein, Hp – Haptoglobin, SAA – Serum Amyloid A

Negative control sections were inoculated with PBS. 3, 3'-diaminobenzidine (DAB) was used as a chromogen and counterstaining was done with Mayers hematoxylin. The sections were then passed through alcohol and xylene sections and cover slipped using entellam and examined under a light microscope (Olympus BX51, Tokyo, Japan). Immunohistochemical staining was evaluated semi-quantitatively by a blinded pathologist at x20 magnification (0; none staining, 1; mild staining; 2; moderate staining, 3; severe staining) [20].

### **Statistical Analysis**

Data were analyzed using SPSS 25 (Inc., Chicago, USA). All immunohistochemical data was assessed utilizing the Levene test for homogeneity of variances and the Shapiro-Wilk test for normality from the parametric test assumptions before the significance testing. Immunohistochemical data were then evaluated using the nonparametric Kruskal-Wallis test, and intergroup comparisons were evaluated using the Mann-Whitney U test.  $p < 0.05$  was accepted as the significance level.

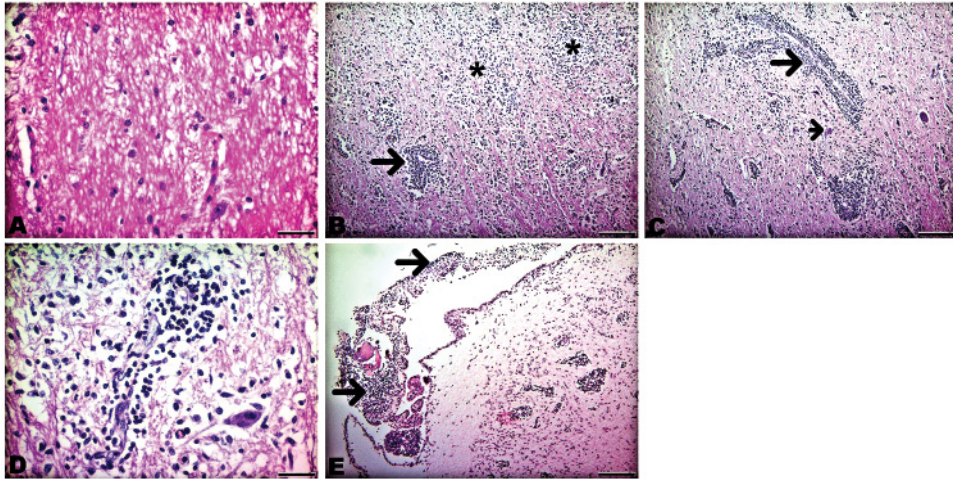
## **RESULTS**

### **Histopathological findings**

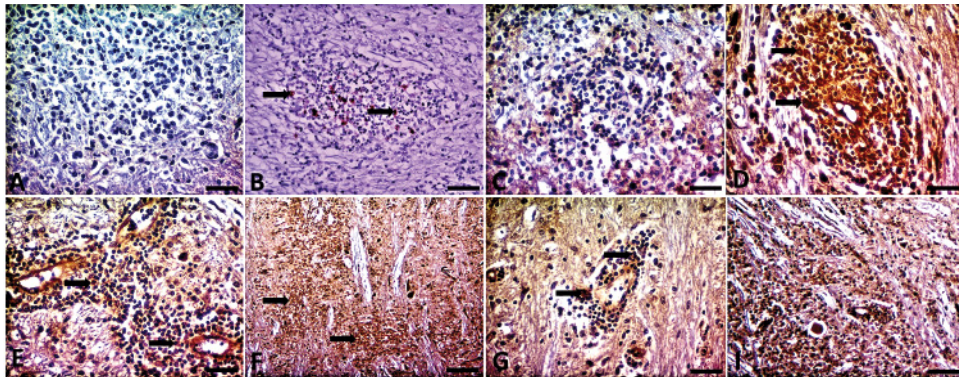
Based on the distribution and the severity of inflammation, the cases were categorized into three groups: 5 cases as mild (Group I), 8 cases as moderate (Group II), and 7 cases as severe (Group III). The brainstems (pons and medulla oblongata) of the control group animals exhibited normal histological structures (Figure 1.A). Histopathological findings in *Listeria monocytogenes* (Lm)-positive sheep included micro abscesses composed of neutrophilic granulocytes and microglial cells, gliosis, meningitis, congestion, and perivascular cuffs predominantly consisting of plasma cells, histiocytes, lymphocytes, and a smaller proportion of neutrophilic granulocytes (Figure 1.B-E). Additionally, neural degeneration and neuronophagia were detected in some inflammatory regions. No evidence of malacia was observed in the examined cases.

### **Immunohistochemical findings**

No immunopositivity was seen in the negative control sections (Figure 2.A). Immunohistochemical analysis revealed cytoplasmic immunopositivity for Lm in neutrophilic granulocytes and microglial cells within micro abscesses (Figure 2.B). However, no immunopositivity was generally observed in the cell infiltrates of the perivascular cuffs. In a few cases, sparse immunopositivity was detected in neutrophilic granulocytes within perivascular cuffs located near micro abscesses. Additionally, Lm immunopositivity was more widespread in Group II and Group III compared to Group I.



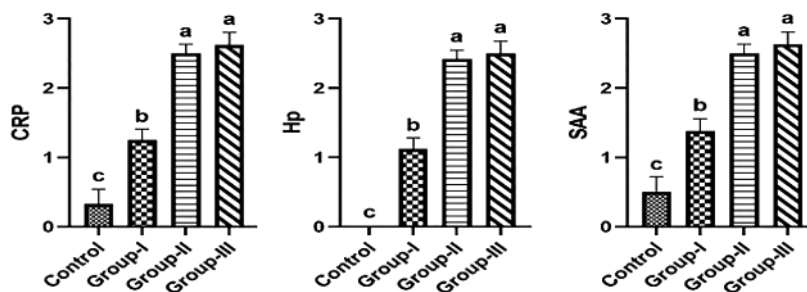
**Figure 1.** Histopathological examination of Listeriosis in sheep, brainstem, Hematoxylin-Eosin, **A.** Normal histological appearance of healthy control animals, bar; 50  $\mu$ m. **B-C.** Appearance of microabscess (asterisk), perivascular cuff (arrow) and neurophagia (arrowhead) in cases in Group III, bar; 200  $\mu$ m. **D.** Appearance of the perivascular cuff in a case in Group II, bar; 50  $\mu$ m. **E.** Appearance of meningitis in a case (arrows), bar; 200  $\mu$ m.



**Figure 2.** Immunohistochemical examination in Listeriosis sheep (Chromogen DAB was used), **A.** Appearance of immunohistochemical staining of negative control section bar; 50  $\mu$ m **B.** Appearance of *Listeria monocytogenes* agent-positive neutrophil granulocytes (arrows), bar; 200  $\mu$ m **C.** Mild CRP immunoreactivity in the perivascular cuff and surrounding micro abscess in one case in Group II. bar; 50  $\mu$ m, **D.** Severe CRP immunoreactivity (arrows) in the perivascular cuff in a case in Group III. bar; 50  $\mu$ m. **E.** Appearance of moderate Hp immunoreactivity (arrows) in the perivascular cuff in one case from Group II. bar; 50  $\mu$ m. **F.** Severe Hp immunoreactivity (arrows) in neutrophil granulocytes and microglia cells in microabscesses in one case from Group III. bar; 50  $\mu$ m, **G.** Appearance of mild perivascular cuff SAA immunoreactivity (arrows) in one case from Group I. bar; 50  $\mu$ m, **H.** Appearance of moderate SAA immunoreactivity in a case in Group II. bar; 50  $\mu$ m (CRP; C-reactive protein, Hp; Haptoglobin, SAA; Serum Amyloid A).

The statistical scores for the CRP, SAA, and Hp antibodies used in the study are presented in Figure 3. In Lm-positive sheep, immunopositivity for CRP, SAA and Hp was significantly increased compared to the control group, with the highest scores

observed in Group II and Group III ( $p < 0.001$ ). The CRP immunoreactivity was seen cytoplasmically localized in microglial cells, neutrophil granulocytes, plasma cells, histiocytes and lymphocytes (Figure 2.C-D). The Hp immunoreactivity was detected cytoplasmically localized in microglial cells, neutrophil granulocytes and plasma cells (Figure 2.E-F). The SAA immunoreactivity was seen cytoplasmically localized in microglial cells, neutrophil granulocytes, histiocytes and plasma cells (Figure 2.G-I).



**Figure 3.** Statistical scores between groups according to the distribution and severity of inflammation in *Listeria* infected sheep. **a–c** letters in vertical columns indicate statistical significance ( $p < 0.05$ ). (CRP; C – reactive protein, Hp – Haptoglobin, SAA – Serum Amyloid A).

## DISCUSSION

Listeriosis is a seasonal disease primarily caused by *Lm*, which poses a significant problem in livestock farming due to its challenging diagnosis and treatment in ruminants [5,6,8,21]. Additionally, listeriosis is recognized as a critical foodborne infection in humans [4]. Factors such as animal health and hygiene, farm management practices, feed quality, and storage conditions play a significant role in the emergence of the disease [7,21,22]. One of the most important pathological findings in ruminants with listeriosis is encephalitis. Encephalitis in listeriosis is reported to occur most commonly in sheep among ruminants [5,7,8]. To the best of our knowledge, there are no reports evaluating the local immunohistochemical expression of the acute phase proteins CRP, SAA, and Hp within encephalitis of sheep naturally infected with *Listeria*. This study aims to evaluate the local immunohistochemical expression of CRP, SAA, and Hp acute-phase proteins based on the distribution and severity of inflammation in naturally infected sheep with listeriosis.

Listeriosis is more commonly observed during winter and early spring and is associated with the consumption of poorly prepared silage [10,11]. Clinically, listeriosis presents in three forms: meningoencephalitis, septicemia, and abortion. In the encephalitic form, it is hypothesized that the agent reaches the brain via the branches of the trigeminal nerve. Clinical symptoms of listeriosis arise from lesions in the brainstem and, although individual cases may vary, the affected animals generally exhibit fever, depression, septicemia, abortion, anorexia, blindness, conjunctivitis, nystagmus, facial and tongue paralysis, head tilting or turning, and circling behavior [10,23,24]. The

anamnesis findings recorded in this study were consistent with those reported in the literature. Furthermore, 15 (75%) of the cases were sheep brought to the department during the winter, consistent with the seasonal trend described in the literature, and 12 (60%) of these cases were reported to have been fed silage.

C-reactive protein (CRP) is a major acute-phase protein primarily produced in the liver and is recognized as an important component of the innate immune response. CRP exhibits various functions, including the modulation of monocytes and macrophages, inhibition of cytokine production, activation of the complement system, enhancement of opsonization, and promotion of chemotaxis [12,14]. CRP is regarded as a marker of inflammation, with levels increasing during bacterial infections [14,17,25]. Specifically, CRP's ability to bind to phosphorylcholine is associated with the clearance of infectious agents and damaged cells at the site of infection. Both experimental and natural infections demonstrate that CRP accumulates in areas of inflammation and tissue damage. In the present study, it was determined that CRP expressions were increased in sheep with naturally infected listeriosis compared to the control group, consistent with the findings of previous infectious studies, and the highest scores were found in groups II and III, where the severity and distribution of inflammation increased (Figure 2 and Figure 3). In addition, the relevant CRP expressions were found to be predominantly in inflammatory cells in the perivascular cuffs. The current findings indicate that CRP may play significant roles in the pathophysiology of the disease and can be locally expressed in naturally infected listeriosis.

Some evidence suggests that CRP is not only a marker of inflammation and infection but also plays a protective role against bacterial infections by activating the complement system and subsequently facilitating pathogen opsonization [26,27]. In an experimental study on *Streptococcus pneumoniae* infection, CRP was shown to protect mice against infection by binding to a phosphorylcholine determinant on the pathogen's cell wall and activating the complement pathway. Pre-treatment of mice with 200 µg of CRP prior to infection significantly increased survival rates (28). Similarly, Marnell et al. [27] reported that CRP conferred protection against *Haemophilus influenzae* infection by binding to pneumococcal C-polysaccharide and opsonizing bacteria for phagocytosis. In the present study, the intense CRP immunoreactivity observed in groups II and III was thought to be associated with the immune response to the listeriosis infection and the phagocytic activity involved. In addition, the fact that agent positivity is more common in groups II and III supports this view.

Haptoglobin (Hp), recognized as a significant acute-phase protein in ruminants, performs various functions, including hemoglobin binding, angiogenesis stimulation, and bacteriostatic activity [12,14]. Recent evidence indicates that Hp concentrations can be induced by tissue damage resulting from inflammatory processes and/or infections [17,29]. The present study revealed that Hp expression was significantly increased in sheep naturally infected with listeriosis compared to the control group (Figure 2 and Figure 3), suggesting that Hp can be locally expressed.

Some research report that Hp serum concentrations increase by 3-8 times during burns, injuries, bacterial infections, and ischemic lesions, highlighting its anti-inflammatory role [30,31]. Hp contributes to the regulation of local and systemic activation of various cells and cytokine expression through the induction of prostaglandin synthesis [31]. Additionally, following Hp binding to hemoglobin (Hb), macrophages mediate phagocytosis via CD163 receptors. Hp-Hb binding also regulates the activity of Th1 and Th2 lymphocytes and modulates the secretion of IL-6, IL-10, and TNF- $\alpha$  [32]. Morimoto et al. [33] reported that Hp ameliorates inflammation and brain injury by binding to HMGB1 and regulating macrophage/microglial polarization. In the present study, Hp immunoreactivity showed significant increases in animals with listeriosis, especially in groups II and III, where the prevalence and severity of inflammation increased. This was interpreted as a severe inflammatory process continuing and also severe tissue damage occurring. The high Hp immunoreactivity observed in groups II and III may also indicate ongoing efforts to eliminate the causative agent and counteract inflammation.

Some research suggests that damaged brain tissue can synthesize Hp, and elevated CSF Hp levels have been observed in various pathologies [34,35]. A number of studies have reported activated astrocytes and Hp-producing oligodendrocytes after ischemia-reperfusion injury and intracranial hemorrhage injury [34,36]. Another study suggested that Hp is produced and released in a neutrophil granulocyte-specific form in areas of inflammation or tissue damage [37]. In the present study, no Hp immunopositivity was detected in the brainstem of healthy sheep. In contrast, notable Hp immunoreactivity was observed in neutrophil granulocytes and microglial cells in the brainstem of sheep with listeriosis, consistent with the literature [34,36,37].

Serum amyloid A (SAA) is an apolipoprotein-like acute-phase protein produced by the liver. SAA secretion is observed during the acute phase of inflammation and is frequently used to differentiate between acute and chronic inflammatory processes. SAA can exhibit both pro-inflammatory and anti-inflammatory effects [14,38]. Recent evidence suggests that SAA enhances chemokine release from monocytes and peripheral blood mononuclear cells by inducing granulocyte colony-stimulating factor (G-CSF) production in macrophages [39,40]. In the current study, SAA immunoreactivity was significantly elevated in sheep with listeriosis compared to the control group (Figure 2 and Figure 3). Particularly in groups II and III, where the distribution and severity of inflammation were higher, this increase might be related to the amplification of the inflammatory process. Especially in groups II and III, the high expression of SAA may result from the rapid migration of inflammatory cells to the region. It also suggests that the significant increase in group II and group III may be due to the response of the host defense and may express locally according to the amplification of the inflammation process and contribute to the exacerbation of the process in listeriosis.

One study has reported that SAA accumulates in high concentrations in the brain tissue under pathological conditions of the central nervous system [41]. A different

study showed that SAA is produced, induced, or released by inflammatory cells during inflammation [42]. Increased SAA expression may enhance the number of pro-inflammatory cytokines and microglial cells, intensifying the inflammatory process. In present study, SAA immunoreactivity was observed in microglial cells, neutrophil granulocytes and other inflammatory cell infiltrates in the brainstem of sheep with listeriosis. This finding suggests that, apart from hepatic upregulation, SAA may also be expressed in the damaged brain and could be a critical protein associated with neuroinflammation in listeriosis.

APPs may play a role in neutralizing pro-inflammatory effects within damaged tissue through local extrahepatic production. It has been suggested that measuring the local levels of APPs in infectious or inflammatory conditions enhances diagnostic accuracy [14,43]. Khafaga et al. [44] reported increased serum and CSF concentrations of Hp and SAA in sheep with encephalitic listeriosis compared to the control group. In the present study, we identified immunohistochemical increases in the local expression of CRP, Hp, and SAA in sheep with listeriosis, supporting the findings of Khafaga et al. [44]. Our findings suggest that the increase in local APP expression in listeriosis parallels the distribution and severity of inflammation and may contribute to systemic circulation. This suggests that CRP, Hp, and SAA acute-phase proteins may provide sensitive results for the diagnosis and prognosis of listeriosis in sheep. Indeed, the local extrahepatic secretion of APPs exerts modulatory effects on pro-inflammatory cytokines and may lead to certain systemic effects due to their contribution to circulating concentrations [45]. Moreover, the increased tissue expression levels of the relevant APPs in all groups, ranging from mild to severe (Group-I, II, III), indicate that the acute-phase response is not merely a systemic reaction but also a crucial component of the local immune response. In this context, we suggest that in listeriosis, the necessary transcription factors for acute-phase proteins in brain tissue are provided in some manner.

## CONCLUSION

This study highlights the diagnostic value of local tissue expression of acute-phase proteins, such as C-reactive protein, haptoglobin, and serum amyloid A, in naturally occurring listeriosis in sheep. Notably, the increased expression of these proteins in parallel with the distribution and severity of inflammation may provide insight into disease severity and the progression of infection.

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### Authors' contributions

OK and GA designed the study. OK performed the laboratory procedures. Both authors read and approved the final version of the manuscript.

### Declaration of conflicting interests

The author(s) reported no potential conflicts of interest concerning the research, authorship, or publication of this article.

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## **IMUNOHISTOHEMIJSKA DETERMINACIJA PROTEINA AKUTNE FAZE KOD OVACA PRIRODNO ZARAŽENIH LISTERIOZOM**

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Listerioza je zarazna i smrtonosna bolest koja pogađa životinje i ljude širom sveta. Efikasnost lokalne tkivne ekspresije proteina akutne faze kod listerioze kod domaćih sisara još nije jasna. Cilj ovog istraživanja bio je da se proceni jačina inflamacije kod prirodne listerioze ovaca prema proteinima akutne faze u 26 uzoraka tkiva moždanog stabla. Materijal za proučavanje sastojao se od 26 parafinskih blokova moždanog stabla, uključujući 20 iz slučajeva zaraženih listeriozom i 6 iz zdravih kontrola. Isecci dobijeni iz parafinskih blokova podvrgnuti su histopatološkoj i imunohistohemijskoj analizi. Histopatološkim pregledom utvrđene su normalne histološke strukture u moždanom stablu kontrolne grupe. Nasuprot tome, delovi moždanog stabla iz slučajeva sa listeriozom pokazali su histopatološke nalaze kao što su mikroapscesi sastavljeni od neutrofilnih granulocita i mikroglijalnih ćelija, gliozna, meningitis, kongestija, perivaskularni infiltrati, degeneracija neurona i neuronofagija. Na osnovu distribucije infiltrata i stepena inflamatornih promena, slučajevi listerioze su kategorisani u tri grupe: 5 slučajeva niskog intenziteta (I grupa), 8 umerenih slučajeva (II grupa) i 7 slučajeva visokog intenziteta (III grupa). Imunohistohemijska analiza je pokazala značajno povećan nivo ekspresije C-reaktivnog proteina (CRP), haptoglobina (Hp) i serumskog amiloida A (SAA) u grupama listerioze u poređenju sa kontrolnom grupom, sa najvišim statističkim rezultatima uočenim u Grupi II i Grupi III ( $p < 0,001$ ). Rezultati ove studije sugerišu da proteini akutne faze igraju ključnu ulogu u razvoju promena kod prirodne infekcije listerioze i da se mogu lokalno eksprimirati. Posebno, povećana ekspresija ovih proteina sa progresijom inflamacije može pružiti vredan uvid u težinu i razvoj infekcije.