Immunohistochemical Detection of Tissue Localization of Acute Phase Proteins in Cattle Pneumonic Pasteurellosis

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ABSTRACT

This study was aimed to determine by immunohistochemical methods the expressions of acute phase protein such as Haptoglobin, C-reactive protein and Serum Amyloid in the lungs of cattle suffering from pneumonic pasteurellosis (PP). The material of the study was the lungs of 8-18 month old cattle Holstein with Fibrinous Bronchopneumonia brought for necropsy to the Pathology Department of the Selcuk University Veterinary Faculty between 2019-2020. As a result of microbiological culture from the lungs of these animals, 6 lungs where *Pasteurella* spp. was isolated were used in the study. Additionally, 6 culture-negative healthy animals obtained from the nearby slaughterhouse were used for lung control. A total of 12 bovine lung samples were subjected to histopathological and immunohistochemical staining. In the histopathological examination, while no microscopic lesions were observed in the lungs of the control group, inflammatory hyperemia and red and grey consolidation stages of fibrinous pneumonia were detected in animals with pneumonic pasteurellosis. Immunohistochemically, immunoreactivity was very mild or without staining for primers in healthy lungs. Animals with pneumonic pasteurellosis showed immunopositivity in haptoglobin (Hp), oedema fluid and inflammatory cell infiltrations ($P>0.05$). C-reactive protein (CRP) staining showed expression in all criteria (epithelial, capillary, oedema and inflammatory cell infiltration) at all phases, and the highest mean score was found during the red consolidation phase ($P<0.01$). Serum Amyloid A (SAA) staining showed immunopositive staining in oedema fluid and inflammatory cell infiltrations, and the highest mean scores were during the red and grey consolidation phases ($P<0.01$). According to the results of the study, it can be considered that acute phase proteins may be important in the development process and/or pathophysiology of the disease, and SAA and CRP may provide more sensitive results for the diagnosis and prognosis of the disease.

Keywords: Serum Amyloid A; C-Reactive Protein; Haptoglobin; Immunohistochemistry; Pneumonic Pasteurellosis; Cattle: Holstein.

INTRODUCTION

Pneumonia is defined as pulmonary inflammation caused by infectious agents such as bacteria, viruses, fungi, parasites, mycoplasmas and non-infectious agents such as irritant gases and foreign substances. In Pneumonic Pasteurellosis (PP) the inflammation of the pulmonary parenchyma and bronchioles is accompanied by pleural inflammation and is a common respiratory system disease of cattle (1, 2) Pasteurellosis emerges as a multifactorial disease caused by many etiological agents. *Mannheimia haemolytica, Pasteurella multocida* and *Bibersteinia trehalosi* causing diseases in domestic animals (3). According to the appearance of the lung and the characteristics of the exudate during the course of PP, it is divided into 4 phases: inflammatory hyperemia, red consolidation, grey

consolidation and lysis. The lysis period is more common in humans (1).

Proteins whose levels in the blood fluctuate markedly at the onset of inflammation are defined as acute phase proteins (APP). Acute phase proteins are blood proteins used to evaluate the response of the immune system due to inflammation, infection and/or trauma (4, 5). The main production site of APPs is the liver. Also, other tissues and white blood cells are reported to play a role in their production (6). The acute phase response (APR) is regarded as the inflammatory response that occurs immediately after inflammation or infection complex but are non-specific (7). APR is induced by a number of proinflammatory cytokines (IL-1, IL-6, TNF-α) released by neutrophil granulocytes and monocytes to produce APP in response to tissue damage. After inflammation or infection, some blood proteins in the liver, such as Cortisol Binding Protein (CBG), Transferrin, Albumin and Prealbumin decrease over time and are called negative APPs. On the other hand, C-Reactive Protein (CRP), Serum Amyloid-A (SAA), Haptoglobin (Hp), Ceruloplasmin (Cp), Fibrinogen (Fb), Alpha 1-acid glycoprotein (AGP) increase and they are called positive APPs (4, 8). Basically, the task of AFPs is to act as part of the innate immune system to reshape homeostasis and promote the healing process (9).

Haptoglobin (Hp) is known as a major APP in ruminants. Hp has many functions, such as the binding of haemoglobin, bacteriostatic effects and the stimulation of angiogenesis (4, 10). Although it has been found at a very low level in normal homeostasis, when the immune system is stimulated, its total can increase by 100 times. Studies have reported that Hp is a useful parameter in clinically evaluating the severity of the inflammatory response in natural or experimental infections such as pneumonia, endocarditis, endometritis, enteritis, mastitis and abscess (4, 7, 11-13).

A major of APR is C-reactive protein (CRP) showing a reaction parallel to the severity of infection and may appear in many infectious processes (14, 15). CRP has functions such as complement activation and opsonization increment, monocyte and macrophage modulation, cytokine production inhibition and chemotaxis (10). In addition, CRP has proinflammatory and anti-inflammatory effects. The amount of CRP increases up to 50,000 times in cases of inflammation and/or infection (10, 16).

Serum Amyloid A (SAA) is included in the classification of apolipoprotein and is produced by the liver (17). SAA presents functions such as inducing chemotaxis and anti-inflammatory effect. SAA is known as a major APP in cattle, and its secretion is observed during the acute phase of inflammation (10). In this context, it can be frequently used for the differentiation of acute and chronic inflammations (13). SAA levels in humans and experimental animals can increase 1000 times during APR (18, 19). Studies have reported that SAA levels increase with Hp in cases of respiratory diseases and are important in APR (11, 12, 20, 21).

Although the use in inflammation, infection and trauma biomarkers of APPs in human medicine has been recorded for many years, in veterinary medicine this is not the case. In recent years, its use as a biomarker in pet and farm animals has been increasing rapidly. In addition, in terms of creating a picture of the diagnosis and prognosis in cases of infection and parallel with pathophysiological studies, the use of APPs we predict will become widespread in the future (7, 10).

In our study, we aimed to determine by the immunohistochemical method the expression levels of Hp, CRP and SAA acute phase proteins in the lungs of Holstein cattle during the phases occurring during the course of pneumonia due to *Pasteurella* spp., and thus to obtain preliminary information about the course and prognosis of the disease. To the best knowledge of the authors, this is the first study in which the detection and evaluation of tissue APPs between the stages of *Pasteurella* spp. pneumonia have been demostrated.

MATERIAL AND METHODS

The material of the study consisted of 12 female Holstein Friesian bovine lung tissues, 6 of which were naturally infected with Pasteuella and 6 uninfected animals were used as controls. The infected lungs were obtained from 6 cattle (8-18 months) with Fibrinous Bronchopneumonia brought to the Pathology Department of Selcuk University Veterinary Faculty for routine necropsy between 2019-2020, whose culture was positive for *Pasteurella* spp. As a control, 6 healthy bovine (12-18 months) lungs taken from the slaughterhouse the culture of which was negative, were used as negative controls.

The compliance of the study with ethical rules was approved by SÜVDAMEK (2021/98).

Microbiological Analysis

Relevant lung samples were taken to the microbiology laboratory under cold chain conditions for bacterial analysis.

Samples were inoculated onto blood agar and MacConkey agar and incubated at 37˚C for 48 hours in an aerobic medium (22).

Histopathological Analysis

For the histopathological examination, the tissue samples taken from the lung were fixed in 10% neutral formalin for one day, and then routine tissue follow-up procedures were performed. Sections of 4-5 µm thickness were taken from the obtained paraffin blocks and stained with Hematoxylin-Eosin. A light microscope (Olympus BX51, Tokyo, Japan) was used to examine the preparations.

Immunohistochemical Analysis

For immunohistochemical analysis, paraffin sections of 4-5 µm thickness were placed on positively charged slides. An oven at 60˚C was used to keep the sections for 30 minutes. Sections were paraffin extracted and rehydrated. After washing the sections with PBS, the sections were kept for 20 minutes in 3% H_2O_2 in order to achieve inactivation of the endogenous peroxidase. To reveal the antigen in the tissues, the sections were treated with Tris-EDTA (pH; 9) antigen retrieval solution at 750 watts for 20 minutes. Following this, the sections were washed with PBS twice for 5 minutes, and treated with Ultra V block (TA-060-UB, Lab Vision, USA) for 5 minutes. Without washing the sections, they were incubated with Rabbit Anti-Hb (Polyclonal antibody, Proteintech, USA, Cat. No:16665-1-AP), Rabbit Anti-SAA (Polyclonal antibody, Proteintech, USA, Cat. No: 16665-1-AP) and Rabbit Anti-CRP (Polyclonal antibody, Proteintech, USA, Cat. No: 24175-1-AP) at a 1/200 dilution rate at 37˚C for 90 minutes. At the end of the incubation, the tissues were washed 4 times with PBS for 5 minutes and then were treated for 10 minutes with Biotinylated Goat Anti-Polyvalent (TP-060-BN, Lab Vision, USA) and Streptavidin Peroxidase (TS-060-HR, Lab Vision, USA), washing with PBS twice for 5 minutes between both treatments. As chromogen, 3.3 diaminobenzidine (DAB) was used. Mayer's hematoxylin was applied for counterstaining the sections after washing with distilled water. Sections were passed through alcohol (96%, 100%) and xylol (2 different series) solutions, respectively, and covered with coverslips. The preparations were examined under a light microscope (Olympus BX51, Tokyo, Japan). For the negative control, PBS was inoculated instead of the primary antibody, and for the positive control, all the primers were inoculated to liver tissue, with the cytoplasmic staining in hepatocytes considered positive.

For the immunohistochemical scoring of the sections, they were examined semi-quantitatively (0; absent, 1; mild staining, 2; moderate staining, 3; severe staining, 4; markedly severe staining). Averages were taken for the stages of *Pasteurella* spp. Pneumonia: Inflammatory hyperemia (epithelium, oedema, capillary vessel); Red consolidation (epithelium, oedema, inflammatory cell infiltration (neutrophil granulocyte), capillary vessel) and Gray consolidation (epithelium, inflammatory cell infiltration (neutrophil granulocyte), capillary vessel). In addition, to determining the prevalence and density of Hp, SAA and CRP antibodies, the Allred score was used (23). The total scores of staining intensity score (0; absent, 1; weak, 2; moderate, 3; strong) and staining degree (0; absent, 1; >0-1/100, 2; > 1/100-1/10, 3; >1/10-1/3, 4; 1/3-2/3 and 5; >2/3-1) were obtained.

Statistical Analysis

The immunohistochemical findings from the study were analyzed with the statistical program SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) and evaluated with ANOVA and post-hoc Duncan test. $P<0.05$ was regarded as the statistical significance limit.

RESULTS

Histopathological Findings

No pathological findings were observed in the microscopic examination of healthy lungs. In the cases of animals with *Pasteurella* spp. pneumonia, inflammatory hyperemia and consolidation, stages red and grey were observed in the lungs. In the period of inflammatory hyperemia, intense hyperemia in capillaries and other vessels and slightly red, homogeneous edematous fluid in alveoli were observed. In the red consolidation period, dense fibrin masses in the alveolar lumena, neutrophil granulocyte infiltrations and hyperemia in the capillaries were noted. In addition, the presence of oedema in the interlobular septa, the fibrin masses and thromboses in the lymphatic, drew attention. During the grey consolidation period, the hyperemia decreased, and dense neutrophilic granulocytic infiltrations were detected in the alveolar lumena. In all cases, oat cell formation was observed as the

Table 1. Statistical distribution of primers between phases (Mean ± SE).

N=6	Inflammatory hyperemia	Red consolidation	Gray consolidation	P value
Hp	0.55 ± 0.11[a]	0.67 ± 0.12[a]	0.61 ± 0.16[a]	>0.05
CRP	1.33 ± 0.21[b]	2.04 ± 0,20[a]	1.22 ± 0.07[b]	<0.01
SAA	0.22 ± 0.07[b]	0.58 ± 0.05[a]	0.55 ± 0.07[a]	<0.01

[a, b] Indicates statistical significance between values in the same row (P< 0.05). (CRP; C- reactive protein, Hp; Haptoglobin, SAA; Serum Amyloid A, N;number of animals).

Table 2. Allred scoring between primers (Mean ± SE).

N=6	Hp	CRP	SAA	P value
IHC Allred score	4.25 ± 0.33[b]	6.00 ± 0.58[a]	3.92 ± 0.44[b]	<0.05

[a, b] indicates statistical significance between values in the same row (P < 0.05). (CRP; C- reactive protein, Hp; Haptoglobin, SAA; Serum Amyloid A, N;number of animals).

oat and/or flow-like shape of the nuclei of some leukocytes in the alveoli. In addition, in some cases, necrotic foci were observed.

Immunohistochemical Findings

Statistical scores for the primary antibodies used are presented in Tables 1 and 2, and immunohistochemical scores are given in Table 3. For the negative control group, no immunopositivity was found in any edema fluid, epithelial, capillary and inflammatory infiltrations. The immunoreactivity staining was very mild to lacking in healthy lung tissues. In the lung tissues with pneumonic pasteurellosis, significant cytoplasmic staining was observed in the alveolar epithelium, the inflammatory oedema fluid, the alveolar macrophage and neutrophil granulocytes, in the inflammatory exudate in alveoli, bronchi and bronchiole lumens, inflammatory oedema in the interlobular interstitium was noted, as well as in neutrophil granulocytes and fibrin thrombi. In cases with necrosis, neutrophil granulocytes on the border of the necrosis were present.

No immunopositive staining was observed in epithelial and capillary vessels for the Hp-related staining. Immunopositivity for Hp was observed in the oedema fluid for the inflammatory hyperemia and red consolidation phases and in neutrophil granulocytes during the red and grey consolidation phases (Fig. 1. B-D). In addition, positive staining for Hp was observed in the inflammatory exudate in the bronchiolar lumens and the neutrophil granulocytes in the interlobular interstitium (Fig. 1. E-F). Although the highest scores between the phases regarding Hp were in the red consolidation phase, no statistically significant differences were found (Table 1, P>0.05).

In the stainings related to CRP, immunopositivity was determined in capillaries and epithelial cells in all phases (Fig. 2. C-D). Immunopositive staining was detected in oedema fluid in the inflammatory hyperemia and red consolidation phase, in neutrophil granulocyte infiltrations and the alveolar macrophages in the red and grey consolidation phases (Fig. 2. B-D). In addition, staining was observed in thrombi in the lymphatics in the interlobular interstitia and oat cell formations in the alveolar lumens (Fig. 2. E-F). The highest scores between phases regarding CRP were determined in the red consolidation phase and were statistically significant (Table 1, P<0.01).

No immunopositive staining related to SAA was observed in any phase in epithelial and capillary vessels. Immunopositive staining was observed in the oedema fluid during the inflammatory hyperemia and red consolidation phases and in the neutrophil granulocyte infiltrations during the red and grey consolidation phases (Fig. 3. B-D). In addition, inflammatory cell infiltration in the interlobular interstitium and immunopositive staining in the oat cells were observed (Fig. 3. E-F). Regarding SAA, the highest scores between the phases were determined in the red and grey consolidation phases, and were statistically significant (Table 1, P<0.01).

DISCUSSION

P. multocida and *M. haemolytica* are known to be relevant respiratory pathogens that can cause pneumonia outbreaks in domestic ruminants such as calves, cattle, sheep and goats (24, 25). The presence of these agents is natural in the upper respiratory tract and nasopharynx of healthy ruminants (26).

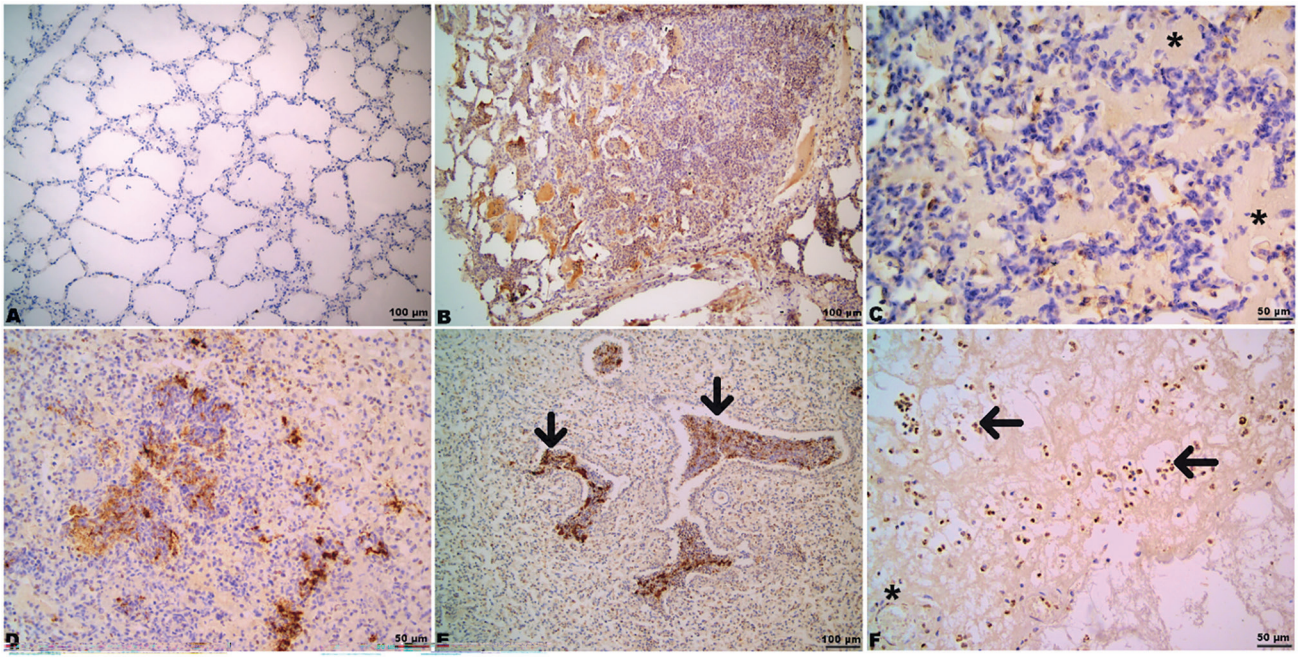

Figure 1. Hp, IHC staining (DAB). **A.** Healthy control, x200. **B.** View of phases together, x200. **C.** Immunopositivity in inflammatory edema (stars) and neutrophils, inflammatory hyperemia period, x400. **D.** Immunopositivity in neutrophils, gray consolidation period, x400. **E.** Immunopositivity in exudate in bronchiole lumens (arrows), x200. **F.** Edema in interlobular interstitium (star) and immunopositivity in neutrophils (arrows), x400.

In the pathogenesis of the disease, in cases where the defence mechanisms are impaired due to stress factors, and viral or parasitic infections, these agents may colonize the lower respiratory tract and subsequently generate PP (24, 27). PP is common in cattle worldwide and causes substanital economic losses (2). It has been reported that APPs are associated with the severity of the disease during bacterial pneumonia. In addition, they are important proteins due to their biomarker roles and functions (28). Studies on APPs focus on changes in blood concentrations and the course of the disease (11, 29). In this study, local specific expressions of Hp, CRP and SAA proteins were detected immunohistochemically for the first time during the inflammatory phases occurring in the course of *Pasteurella* spp. pneumonia.

In our study, inflammatory hyperemia and red and grey consolidation phases were observed histopathologically in cattle with PP. In the period of inflammatory hyperemia, intense hyperemia in capillaries and other vessels and oedema fluid in alveolar lumens were detected. During the red consolidation phase, dense fibrin masses, neutrophil granulocyte infiltrations and hyperemia were observed in the alveolar lumens. In the grey hepatization phase, the hyperemia in the vessels decreased, and dense neutrophil granulocyte infiltration was observed in the alveolar lumens. In addition, oedema in the interlobular septum, fibrin masses and thromboses in the vessels were detected. Oat cell formations, which were reported to occur due to the effects of bacterial toxins on leukocytes (1), were found in all the cases. The histopathological findings of our study show parallels with the findings of the studies mentioned in the literature (30, 31).

In the study conducted by El-Deeb and Elmoslemany (11), it was reported that in pneumonic sheep, the Hp concentration in serum increased 34 times, showing a major response to the infection. In the study by Akgul *et al.* (12) it waas stated that, in comparison with the control group, calves with pneumonia presented a significant increase in serum concentrations of Hp. Dörtkardeş and Şahinduran (21) reported increased levels of Hp in serum in calves with pneumonia compared to healthy controls. Coskun *et al.* (32) registered that haptoglobin levels at BALF and serum levels were increased significantly in pneumonic calves in comparison with the control, and APP were useful for detecting lung infection. Studies in the literature show that increased Hp concentrations can be induced by the tissue damage caused by the inflammatory process and/or infection. In the

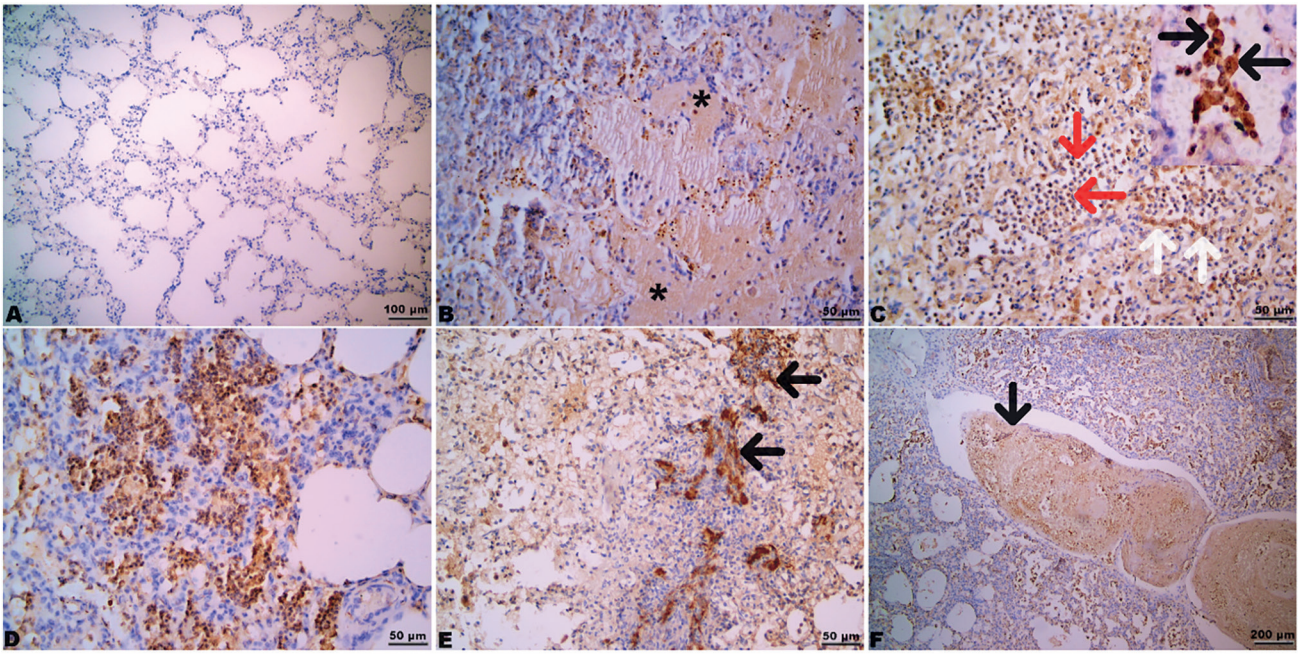

Figure 2. CRP, IHC staining (DAB). **A.** Healthy control, x200. **B.** Inflammatory edema (stars) and immunopositivity in neutrophils, inflammatory hyperemia, x400. **C-D.** Strong positive staining in alveolar epithelium (white arrows), lumen neutrophils (red arrows) and alveolar macrophages (small image, black arrows), red consolidation. x400. **E.** Strong (arrows) Immunopositivity in oat cell, gray consolidation. x400. **F.** Immunopositivity in thrombi in the lymphatics and in the interlobular interstitium (arrow), x100.

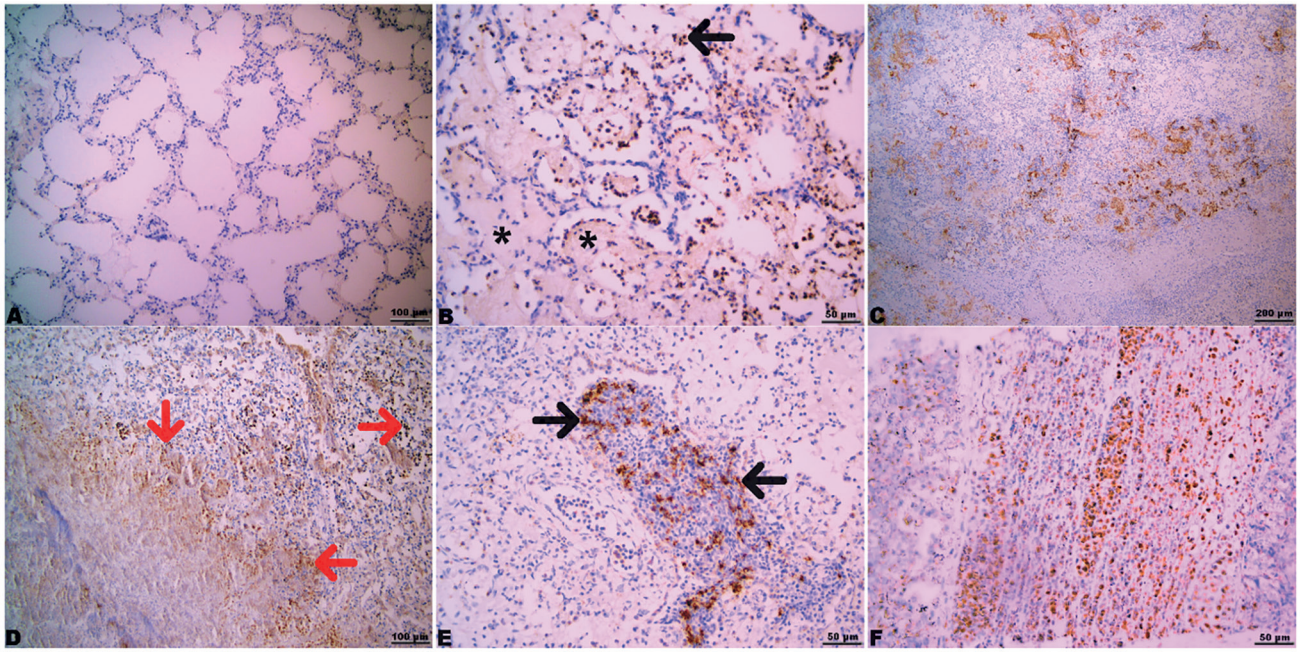

Figure 3. SAA IHC staining (DAB). **A.** Healthy control. **B.** Inflammatory edema (stars) and immunopositivity in neutrophils (arrow), red consolidation, x400. **C-D.** Positive staining of neutrophils (red arrows) in the alveolar lumen and around necrosis, gray consolidation. (C: x100, D:X200). **E.** Strong (arrows) Immunopositivity in oat cell, gray consolidation, x400. **F.** Immunopositivity in the interlobular interstitium, x400.

Table 3. Distribution of immunohistochemical scores of AFPs according to pneumonic pasteurellosis inflammatory phases.

	Hp				CRP				SAA			
	Ep.	Ed.	Cap.	Ng.	Ep.	Ed.	Cap	Ng.	Ep.	Ed.	Cap	Ng.
Inflammatory Hyperemia	0	2	0	0	0	2	1	0	0	1	0	0
	0	1	0	0	1	1	1	0	0	0	0	0
	0	3	0	0	1	2	1	0	0	0	0	0
	0	2	0	0	3	3	1	0	0	1	0	0
	0	1	0	0	2	1	1	0	0	1	0	0
	0	1	0	0	1	1	1	0	0	1	0	0
,Red consolidation	0	1	0	2	3	2	3	3	0	1	0	2
	0	1	0	1	2	2	1	4	0	1	0	1
	0	2	0	2	2	2	1	3	0	1	0	2
	0	2	0	2	2	3	1	3	0	2	0	0
	0	0	0	1	1	1	1	3	0	1	0	1
	0	1	0	1	1	1	1	3	0	1	0	1
Gray consolidation	0	0	0	1	1	0	1	2	0	0	0	1
	0	0	0	2	1	0	1	2	0	0	0	2
	0	0	0	3	1	0	0	3	0	0	0	2
	0	0	0	2	1	0	0	3	0	0	0	2
	0	0	0	3	1	0	0	2	0	0	0	2
	0	0	0	0	1	0	0	2	0	0	0	1

* Inter-phases were analyzed semi-quantitatively and mean scores were obtained (0; absent, 1; mild staining, 2; moderate staining, 3; severe staining, 4; very severe staining) (CRP; C- reactive protein, Hp; Haptoglobin, SAA; Serum Amyloid A, Ep; Epithelial, Ed; Edema, Cap; Capillary, Ng; Neutrophil granulocyte)

immunohistochemical evaluation of Hp in our study, immunopositive staining was observed in the oedema fluid and neutrophil granulocytes during inflammatory phases (Fig. 1). No positivity was found in epithelial cells and capillaries. Although the highest staining score of Hp was detected in the red consolidation phase, no statistical differences were found between the groups (Table 1, $P > 0.05$). Our findings revealed that Hp was present, especially in the oedema fluid and neutrophil granulocyte infiltrations, in specific phases (inflammatory hyperemia, red and grey consolidation). It also showed that Hp was a locally important APP for PP in cattle. Hiss *et al.* (33) reported that they detected immunohistochemically Hp expressions in bronchial and bronchiole epithelium in their study of pigs with bronchopneumonia. They also stated that there was no immunopositivity in the alveolar epithelium. In our study, it was determined that alveolar, bronchial and bronchiole epithelial cells did not play a role in Hp expression in PP in cattle. In the present study, the increased expression of Hp in animals with pneumonia compared to healthy animals also concurred with the findings of previous clinical studies.

Haptoglobin has many functions, such as binding of haemoglobin, bacteriostatic effects and stimulation of angiogenesis (10). Hp specifically binds free haemoglobin, which results in the deprivation of the iron necessary for bacterial growth. In cases of inflammation and/or infection, hyperemia and dilation are important factors that facilitate blood cells' extravasation. Extravasation of blood cells, which occurs in many respiratory tract infections, is considered a result of the host response (10, 34). In addition, after haptoglobin binds to haemoglobin, phagocytosis occurs by macrophages via CD163 receptors (9, 35). Abdullah *et al.* (36) reported that the local expression of Hp and CD163 during lung infection in humans operates as a functional pulmonary defence element of the respiratory system and a part of the inflammatory immune response.

From the findings of our study, it may be interpreted that the immunopositive determination of Hp, without a

statistical difference, in all phases, modulates phagocytosis as a host response, stabilizes proinflammatory effects, reduces oxidative damage, and subsequently participates in the attempt of tissue remodelling. In addition, the presence of immunoreactivity in the early period (inflammatory hyperemia) in the local area of infection suggests that Hp contributes to the pathophysiology of the disease, suggesting that it is an early biomarker of acute inflammatory reaction. On the other hand, it should be considered that it might result from systemic APR since only neutrophil granulocytes and the oedema fluid were immunopositive. In the study conducted by Theilgaard-Mönch *et al.* (37), it was stated that, in areas of inflammation or tissue damage, Hp was produced and released into the environment in a form specific to neutrophil granulocytes. Although the significant level of expression in neutrophil granulocytes during the grey and red consolidation phases in our study strengthens this conclusion, more molecular studies are needed in the future.

CRP in cattle is reported to be associated with lactation rather than being synthesized in the liver. In addition, it has not been fully clarified that it is an APP (4). While CRP is considered an important APP in humans, pigs and dogs, it has been stated that in case of inflammation and/or infection in ruminants, no change in the serum concentrations is observed (38). Currently, blood concentrations of CRP are frequently evaluated as a marker in lung infections in ruminants, and significant increases are reported compared to control animals (11, 12, 29). Although in this study on lung tissue, we have no evidence of blood concentrations, local tissue accumulation of CRP was demonstrated in the PP of cattle.

Kozat and Özkan (15) and Akgul *et al.* (12) reported that serum CRP concentrations were increased in calves with pneumonia compared to control animals. In the immunohistochemical study by Haligur and Ozmen (20), it was reported that CRP was significantly increased in sheep with pneumonia compared to control animals. In the CRP immunohistochemical evaluation in this study, varying degrees of staining were observed in all criteria at all phases, especially in inflammatory infiltrates (neutrophil granulocyte), severe immunopositivity was registered (Fig. 2). It may be considered that the increase in immunohistochemical staining scores, especially during the red consolidation period, was associated with the severity of the infection and the increase in oedema and inflammatory infiltration (neutrophil granulocyte) (Table 1, $P<0.01$). In addition, it was understood that the decrease in immunohistochemical scores in the grey consolidation phase was due to the absence of hyperemia and inflammatory oedema fluid in this period because of the progression of the fibrinous inflammatory process. In this study, the increased expression of CRP in animals with pneumonia compared to healthy animals was similar to the findings of previous clinical studies. Another finding in the study of Haligur and Ozmen (20) was that there was a negative correlation between the severity of the infection and the level of expression of CRP. The fact that this situation does not concur with the findings of our study may be due to the differences in the materials used and etiological factors and the evaluation of specific local tissue expressions in inflammatory phases in our study.

Infectious agents and damaged cells clearance at the site of infection is based on the ability of CRP to bind phosphorylcholine (10). It also has functions such as inhibiting chemotaxis, inducing cytokines, increasing opsonization and activating complement. It has been reported that CRP contributes considerably to innate immunity as a system of early defence against infections (4, 10). In our study, the presence of immunohistochemically high-score expression values during the red consolidation phase, as well as the immunoreactivity in alveolar macrophages, can be considered as a positive correlation with phagocytosis due to the progression of the inflammatory process in relation to the host response. In addition to the immunoreactivity of CRP in the period of inflammatory hyperemia, which confirmed that it is an early marker of acute inflammatory response in PP, its immunoreactivity, especially in epithelial cells and capillaries, demonstrated that it was released by extrahepatic cells in PP in cattle. Regarding the APPs examined, the highest mean value in the Allred scoring was found to belong to the CRP antibody ($P<0.05$). This may be due to the release, especially by extrahepatic cells, of CRP. It may be stated that CRP is, in terms of density and prevalence in the lesioned area, is an important protein in the pathophysiological process of Pneumonic Pasteurellosis infections.

Akgul *et al.* (12) reported that pneumonic calves presented with significantly higher serum SAA levels compared to controls. In the study conducted by El-Deeb and Elmoslemany (11), it was reported that the serum SAA level in pneumonic sheep was significantly higher than in control

animals. In the study conducted by Haligur and Ozmen (20), it was stated that the immunoreactivity of SAA increased in sheep with pneumonia. In our study, no positivity was found in the immunohistochemical evaluation of SAA in the inflammatory phases in the epithelial cells and capillaries of the lung. On the other hand, immunopositivity was found in oedema fluid and inflammatory cell infiltrations (neutrophil granulocytes) at different phases ($P<0.01$). It can be considered that the increase in the average scores, especially in the red and grey consolidation phases, was related to the increase in the severity of the inflammation. In this study, the increased expression of SAA in animals with pneumonia compared to healthy animals was in line with the findings of previous clinical studies.

It has been reported that in respiratory system diseases, SAA gave faster and more sensitive responses compared to Hp (39). At the evaluation of the findings in our study, the statistically significant increase in SAA in parallel with the increased severity of inflammation compared to Hp revealed that it was more specific and sensitive in PP. In addition, alveolar, bronchial and bronchiolar epithelial cells were not found to play a role in SAA expression in PP. Hatanaka *et al.* (40) reported that SAA induced the release of (IL)-1β, TNF-α and IL-8 in neutrophil granulocytes in humans. In addition, functions of SAA such as neutrophil degranulation, phagocytosis, and induction of cytokine release from monocytes and neutrophils have also been reported (40). In this study, the increase in immunoreactivity during the red and grey consolidation period, when the inflammation intensified, may possibly be due to the response of the host defence, suggesting that it may accumulate locally according to the amplification of the inflammation process and contribute to the exacerbation of the process in PP.

Proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), Interleukin-6 (IL-6) and IL-1β are the main inducers of APPs synthesized from the liver. While IL-1 and TNF-α are effective in extrahepatic cases, IL-6 is more effective in hepatic APR (4, 10). It has been hypothesized that the extrahepatic production or local expression of APPs may reduce the tissue damage caused by inflammation and infection and, therefore, contribute to maintaining homeostasis. Furthermore, the local extrahepatic secretion of APPs has stabilizing effects on proinflammatory cytokines and may cause some systemic effects due to its contribution to their circulating concentrations (42). Although we did not have any findings relating to blood concentrations of PP in cattle in our study, the increased tissue expression levels of the related APPs in the early stages of the disease suggest that the acute phase reaction is not only a systemic reaction but also an important part of the possible local immune response. In this context, we propose that extrahepatic tissues somewhat provides the necessary transcription factors for acute phase proteins in PP.

This study focused on the evaluation of expression levels of acute phase proteins (Hp, CRP, SAA) between inflammatory stages in naturally infected PP. The fact that the material of our study consisted of cattle brought to autopsy and the absence of blood results are among the important factors limiting our study in terms of the diagnosis and prognosis (clinical) of the disease. Although it may seem difficult *in vivo* due to the material used, we suggest comparing the blood and tissue levels of the relevant markers together in future studies. Thus, we believe that valuable results could be obtained for the diagnosis and prognosis of PP and will make an important contribution to the knowledge of this disease.

In conclusion, the expression levels of Hp, CRP and SAA in PP in cattle were examined immunohistochemically and comparatively between the inflammatory phase of the disease, and it was concluded that all the inflammatory acute phase proteins might be important in the pathophysiology of the disease. Additionally, it was evaluated that SAA and CRP may provide more sensitive results in terms of diagnosis and prognosis of the disease. In this study, the value as diagnostic biomarkers of local tissue expressions of Hp, CRP, and SAA acute phase proteins of naturally occurring PP in cattle was emphasized.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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