



RESEARCH ARTICLE

Determination of Apoptosis, Necroptosis and Autophagy Markers by Real-time PCR in Naturally Infected Pneumonic Pasteurellosis caused by *Pasteurella multocida* and *Mannheimia haemolytica* in Cattle

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ABSTRACT

Pneumonic pasteurellosis (PP) is defined as one of the pivotal infectious diseases caused by *Pasteurella multocida* and *Mannheimia haemolytica*. This study aimed to determine the levels of Bcl-2-associated protein X (Bax), B-cell lymphoma 2 (Bcl-2), caspase-3, autophagy related-5 (Atg5), beclin-1 and receptor interacting protein-3 (RIP3) in lung tissues with naturally infected PP caused by *P. multocida* and *M. haemolytica*, and to reveal their effects on the pathogenesis of *P. multocida* and *M. haemolytica* pneumonia. The material of the study consisted of 150 fibrinous pneumonia/pleuropneumonia and 10 healthy lung tissue samples. Relevant samples were examined by histopathological, immunohistochemical and real-time PCR methods. Immunohistochemically, 23 (15.3%) were positive for *P. multocida*, and 17 (11.3%) were positive for *M. haemolytica*. Subsequently, the processes of apoptosis, autophagy and necroptosis for *P. multocida* and *M. haemolytica* were evaluated by real-time PCR. *P. multocida* pneumonia increased Bax, Caspase-3, Atg5, Beclin-1, and RIP3 gene expressions (4.2, 3.8, 2.9, 2.1, 2.8-fold, respectively), whereas Bcl-2 gene expression was decreased (0.22-fold). While Bax, Caspase-3, Atg5, Beclin-1, and RIP3 gene expressions were increased in *M. haemolytica* pneumonia (2.3, 1.9, 1.7, 1.2, 4.2-fold, respectively), it was observed that Bcl-2 gene expression was reduced (0.52-fold). The results obtained in the study revealed the importance of necroptosis, apoptosis and autophagy processes in the pathogenesis of PP caused by *P. multocida* and *M. haemolytica* and contributed to the literature. In addition, we found that the processes of apoptosis and autophagy play a more active role in PP caused by *P. multocida*, and the process of necroptosis plays a more active role in PP caused by *M. haemolytica*.

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INTRODUCTION

Pasteurella multocida and *Mannheimia haemolytica* have an important place among the bacterial agents that cause pneumonia in cattle (Gaudino *et al.*, 2022). *P. multocida* and *M. haemolytica* pneumonia cause large amounts of productivity losses as well as economic losses due to their mortality (Abed *et al.*, 2020; Cengiz *et al.*, 2021). Pneumonic pasteurellosis (PP) is known as one of the pivotal infectious diseases caused by *P. multocida* and *M. haemolytica* (Abed *et al.*, 2020). The pathogenesis of PP involves the rapid proliferation of pathogens such as *P.*

multocida and *M. haemolytica* in the natural flora of the upper respiratory tract, causing disease as a result of stress factors, viral or parasitic infections suppressing the host immune system (Griffin *et al.*, 2010; Lopez and Martinson, 2017; Ciftci *et al.*, 2021). Stress factors such as housing animals from different origins, weaning, transportation, care and feeding errors, vaccination, crowded shelters and dehorning are known as predisposing factors for PP (Kabeta *et al.*, 2015; Ciftci *et al.*, 2021; Gaudino *et al.*, 2022). PP is characterized by fibrinous bronchopneumonia in cattle. Since PP lesions are mostly distributed at the lobe level, they are also

called lobar pneumonia (Tuzcu *et al.*, 2020; Ciftci *et al.*, 2021; Akcakavak *et al.*, 2023).

In PP, cranioventral lobar or lobular fibrinous bronchopneumonia, foci of coagulation necrosis and variable degrees of fibrinous pleuritis are observed macroscopically in the cranial and medial lobes of the lung, the caudal lobes may also be affected. The affected lung areas have a solid and firm consistency due to the alveoli being filled with fibrin. Histopathologically, fibrinous and suppurative bronchopneumonia constitute characteristic lesions in PP (Tuzcu *et al.*, 2020; Ciftci *et al.*, 2021). Necrosis of leukocytes and intraalveolar leukocytes called oat cells with a runny appearance and fusiform structure with pale basophilic chromatin are the characteristic features of *P. multocida* and *M. haemolytica* infections (Yavuz and Dinçel 2020a, 2020b; Ciftci *et al.*, 2021).

PP is reported to be responsible for 30% of total cattle deaths in feedlots, and the global economic impact of the disease is thought to be more than one billion dollars. In addition to the losses caused by the disease due to deaths, the cost of treatment is also very high. Morbidity and mortality are affected by factors such as age, location, previous exposure and immunity (Boudreaux, 2004; Abed *et al.*, 2020). Morbidity rates in the UK have been reported to be between 73-100%, with an average mortality rate of 4% (Andrews *et al.*, 2004).

Apoptosis, or programmed cell death, is the orderly breakdown of cells into parts that can be digested and eliminated. Apoptosis is initiated by the activation of a series of cysteine aspartic proteases known as caspases. Once cell damage is detected, initiator caspases such as Caspase-8 and Caspase-9 are activated by inactive procaspases. These activate executioner caspases such as caspase-3, caspase-6 and caspase-7 (D'Arcy, 2019; Sahoo *et al.*, 2023). For apoptosis to occur, pro-apoptotic proteins such as Bcl-2-associated X protein (Bax) and Bak directly cause the mitochondrial membrane to become permeable. These functions of these proteins are inhibited by antiapoptotic members of the same family, such as B-cell lymphoma gene-2 (Bcl-2) and Bcl-Xl (Morales-Martínez and Vega, 2022; Czabotar and Garcia-Saez, 2023).

Autophagy is defined as the destruction of intracellular macromolecules and organelles within the cell by enclosing them in a membrane and combining them with lysosomal enzymes. Autophagy basically consists of steps such as stimulation of autophagy and formation of phagophore, elongation of phagophore and formation of mature vesicle, and fusion of mature vesicle with lysosomes (Mizushima and Komatsu, 2011; D'Arcy, 2019; Cao *et al.*, 2021). The formation of autophagosomes is controlled by a set of evolutionarily conserved genes called autophagy-related (Atg) genes. The Atg12, Atg5, and Atg16 conjugation system plays a part in the formation of the double membrane (Ho *et al.*, 2017). Beclin 1, also known as Atg6, is found in the endoplasmic reticulum, mitochondria and perinuclear space. It is involved in the formation of double-membrane autophagosomes, which are necessary for autophagy and is defined as an autophagy marker (Mizushima and Komatsu, 2011; Prerna and Dubey, 2022).

Necroptosis is defined as a type of programmed necrosis that can be induced by stimuli that induce the

expression of death receptor ligands and/or death receptor ligands under conditions in which apoptotic death is insufficient (Pasparakis and Vandenabeele, 2015; Newton and Manning, 2016). Similar to caspases, which are essential cellular mediators of apoptosis, receptor-interacting protein kinases (RIPKs) are key mediators of necroptosis (Newton and Manning, 2016).

Molecular studies to evaluate apoptosis, autophagy, and necroptosis are very important in studies aimed at elucidating the pathogenesis of diseases. In this study, the levels of Bax, Bcl-2, Caspase-3, Atg5, Beclin-1 and RIP3 in lung tissues with PP caused by *P. multocida* and *M. haemolytica* were determined by real-time PCR method. Thus, it was aimed to reveal the effects of cell death mechanisms such as apoptosis, autophagy and necroptosis on the pathogenesis of *P. multocida* and *M. haemolytica* induced PP.

MATERIALS AND METHODS

Animal materials: The material of the study consisted of 150 bovine lung tissues in which fibrinous pneumonia/pleuropneumonia was detected during postmortem examination in slaughterhouses in the province of Yozgat (Türkiye) and its districts. Ten healthy bovine lung tissues, which were determined immunohistochemically as negative, were also used as controls. Relevant tissues were placed in both neutral formalin solution and sterile eppendorf tubes.

Histopathological examination: After the lung samples were fixed in 10% neutral formalin solution for 24-48 hours, they were subjected to routine pathological tissue follow-up procedures and paraffin blocks were obtained. Sections taken from paraffin blocks were stained with the Hematoxylin-Eosin method. The preparations were evaluated under light microscopy (Olympus, BX51, Tokyo, Japan).

Preparation of hyperimmune serum: Rabbit polyclonal anti-*P. multocida* and anti-*M. haemolytica* hyperimmune serums were obtained from Selçuk University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology.

Immunohistochemical examination: To determine the presence of *P. multocida* and *M. haemolytica* in lung samples 5µm thick sections were taken from paraffin blocks on polylysine slides. Immunohistochemical staining was performed with the Ultravision Detection System Anti-Polyvalent, HRP (TP-060-HL, Lab Vision, USA) kit in accordance with the recommendations of the manufacturer. Polyclonal anti-*Pasteurella multocida* (1/5000 dilution, 1-hour incubation) and anti-*Manheimia haemolytica* (1/5000 dilution, 1-hour incubation) hyperimmune sera were used as primary antibodies. Negative control sections were inoculated with PBS instead of antibodies. 3,3'-Diaminobenzidine was used as chromogen, counterstained with Mayer-Hematoxylin, coated with entellan and examined under a light microscope (Olympus, BX51, Tokyo, Japan).

Real-time PCR (qPCR) Analysis: qPCR analysis was performed on the cases in which *P. multocida* and *M.*

haemolytica were identified in the immunohistochemical staining of samples taken from bovine lungs with fibrinous pneumonia/pleuropneumonia using specific hyperimmune sera and on eppendorf tubes. Five cases in which *P. multocida* and *M. haemolytica* were detected together were not taken into consideration. RNA extraction was performed using the High Pure RNA Isolation Kit (Roche, Germany, Cat. No: 11828665001) following the protocol recommended by the manufacturer. The quantity and purity of RNA in each sample was determined using the NanoDrop spectrophotometer. cDNA synthesis from RNA samples were carried out using the High Fidelity Transcriptor cDNA synthesis kit (Roche Germany; Cat. No: 0508995001) according to the protocol recommended by the manufacturer. qPCR was performed with the TaqMan probe method on the Roche Light Cycler 2.0 device, and Roche Light Cycler TaqMan Master (Roche, Germany; Cat No: 04735536001) was used for this purpose. Gene sequences of the Bax, Bcl-2, Caspase-3, Beclin-1, Atg5, RIP3 and β -actin (housekeeping) primers used in the study are given in Table 1. qPCR reaction conditions consisted of 10s of denaturation at 95°C, 30s of annealing at 54°C, 3s of extension at 72°C, and a 30s cooling step at 40°C. The expression levels of the genes investigated in the study were calculated by the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001; Akcakavak and Ozdemir, 2023).

Statistical analysis: The findings obtained in qPCR analysis were assessed with the SPSS 25.0 (Inc., Chicago,

USA) statistical program. Before the analyses, the findings were subjected to a normal distribution test and then assessed with the One-way ANOVA and Duncan test as a post-hoc test. The data obtained are offered as mean \pm standart deviation (Mean \pm SD). $P < 0.05$ was accepted as the limit of statistical significance.

RESULTS

A total of 150 fibrinous pneumonia/pleuropneumonia cases, which constitute the material of the study, were obtained at 18 different cutoff periods. Fibrinous pneumonia/pleuropneumonia was detected in 150 (10%) of 1430 animals in 18 different cutoff periods.

Macroscopic results: In the study, it was observed that in cattle with pneumonia induced by *P. multocida* and *M. haemolytica*, macroscopically dark red/grey-coloured, viscous, hepatized areas in the lungs were located in the cranial lobes, and in all cases, the lesions were separated from the healthy lung by a clear border. When the cross-sectional surfaces of the affected areas were examined, it was observed that they contained hepatized areas with a pale grey-brown colour and uniform structure (Fig. 1). Particularly, the lesions in the pleura were quite evident. In these, it was determined that the lung pleura had a thick, matte and rough appearance, no exudate leaked from the cross-sectional surfaces, and the lung had a dry structure. The cross-sectional surfaces of some of them had a mottled appearance.

Table 1: Bax, Bcl-2, Caspase-3, Beclin-1, Atg5, RIP3 and β -actin primers gene sequences.

Bax	F	5'-TCT CCC CGA GAG GTC TTT TT-3'
	R	5'-TGA TGG TCC TGA TCA ACT CG-3'
Bcl-2	F	5'-ATG TGT GTG GAG AGC GTC AA-3'
	R	5'-CTA GGG CCA TAC AGC TTC AC-3'
Caspase-3	F	5'-GAAGATGCTCCCAAGGC-3'
	R	5'-CCAAGCGTCAAGTAAGAAGT-3'
Beclin-1	F	5'-GACACTCAGCTCAACGTCAC-3'
	R	5'-GCTTCCTCCTGATCCAACCT-3'
Atg5	F	5'-CCACAAAGTTACTGGGCACATA-3'
	R	5'-CACTTTGTGTCAGTTACCAACGTCA-3'
RIP3	F	5'-GAAGATCTGTGACATGTCGTGCGTCAAGTTATG-3'
	R	5'-CCGCTCGAGGCCGCCGCTTATTTCCCGCTATGATTAT-3'
β -actin	F	5'-GCCCTGAGGCTCTC TCCA-3'
	R	5'-GCGGATGTGACGTCACA-3'

(Bax; Bcl-2-associated protein X, Bcl-2; B-cell lymphoma 2, Atg5; Autophagy related-5; RIP3; Receptor interacting Protein-3, F; Forward, R; Reverse)

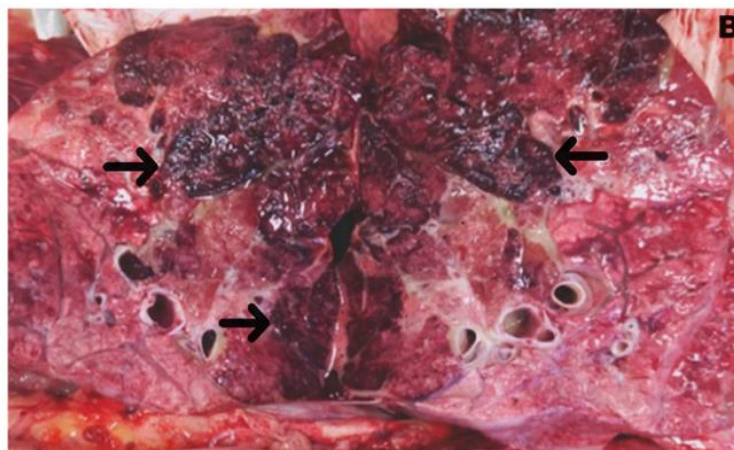
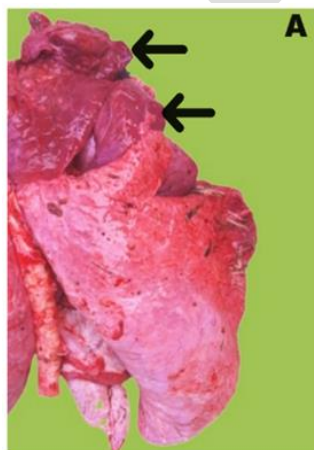


Fig. 1: A. Hepatized areas (arrows) in the apical lobes, *Pasteurella multocida*, B. Necrosis and hemorrhage (arrows) on the cross-sectional surface of the lung, *Mannheimia haemolytica*.

Microscopic results: In *P. multocida* and *M. haemolytica* pneumonia periods of inflammatory hyperemia, red hepatization and grey hepatization at different intensities were detected microscopically. The main change in these cases was perceived as intense fibrin strands, neutrophil granulocyte infiltrations, oedema and alveolar macrophages in the alveolar lumens. There was necrotic bronchiolitis in some bronchioles and hyperplasia in the bronchi and bronchiolar epithelium. Inflammatory exudate consisting of neutrophils, lymphocytes, plasma cells, macrophages, and necrotic cells along with sloughed epithelial cells was also observed in the bronchi and bronchiole lumens (Fig. 2A-B). Again, capillary thrombosis and oat cells with elongated nuclei and a flowing appearance were noted (Fig. 2C). It was noted that necrotic areas were more common in cases with *M. haemolytica* (Fig. 2D). In addition, no findings of interstitial pneumonia such as

thickening of the interalveolar septum, bronchiolitis obliterans, inclusion bodies, lymphoid hyperplasia, etc. were found in the tissues positive for the relevant agents in histopathological examination.

Immunohistochemical results: In the immunohistochemical examination of these cases using *P. multocida* and *M. haemolytica* specific antibodies, staining was determined in 23 cases with *P. multocida* (15.3%), in 17 cases with *M. haemolytica* (11.3%) and in 5 cases with both *P. multocida* and *M. haemolytica*. In the current study, immunopositive stainings for *P. multocida* and *M. haemolytica* were detected in the epithelium of bronchi, bronchioles and alveoli, in neutrophils, macrophages and sloughed epitheliums of the alveolar lumens (Fig. 3). No immuno-positive staining was detected in the negative control sections.

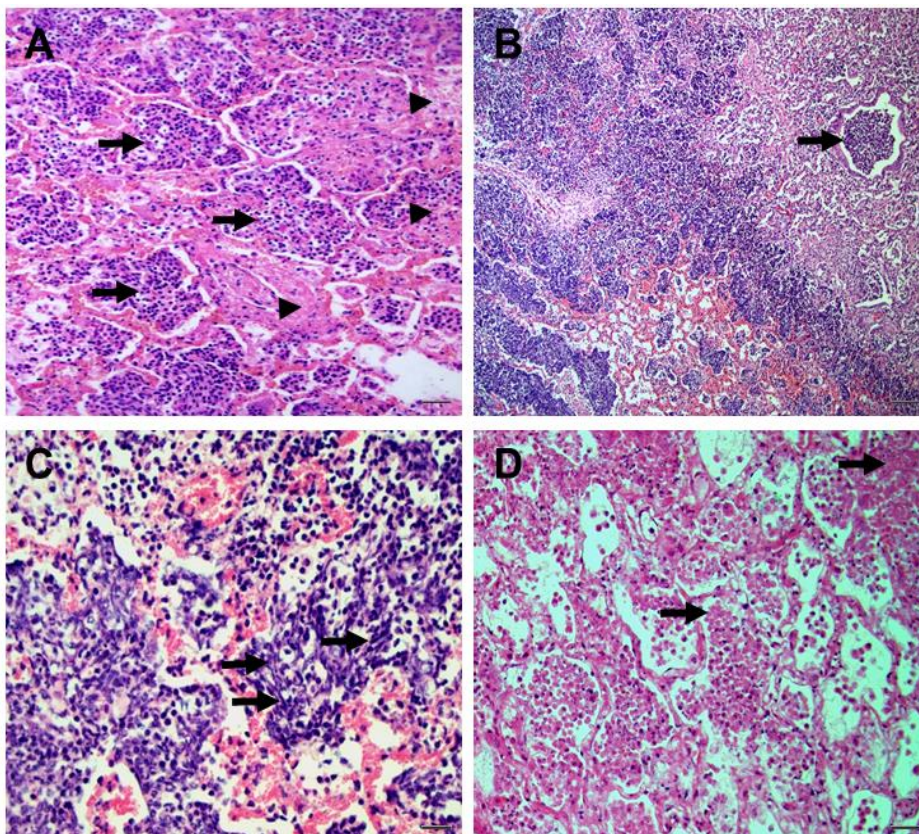


Fig. 2: Histopathological examination, Hematoxylin-Eosin, in cases of fibrinous pneumonia/pleuropneumonia, A. Numerous neutrophil granulocyte and macrophage infiltration in the alveolar lumen (arrows), fibrin-containing edema fluid in the alveoli (arrowheads), *P. multocida*, bar; 100 μ m, B. Inflammatory exudate (arrow) in the bronchiolar lumen, *P. multocida*, bar; 200 μ m. C. Appearance of oat cells (arrows), *M. haemolytica*, bar; 50 μ m. D. Areas of necrosis (arrows), *M. haemolytica*, bar; 100 μ m.

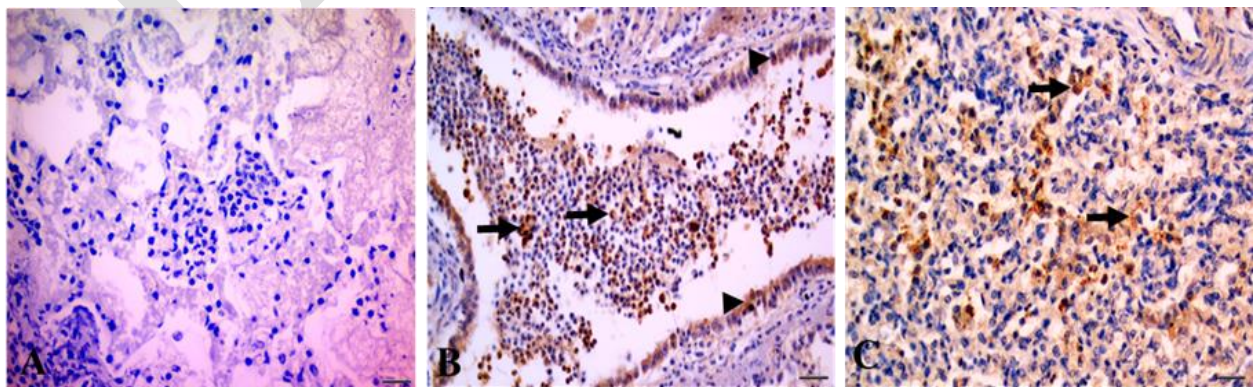


Fig. 3: Immunohistochemical staining for the diagnosis of *P. multocida* and *M. haemolytica*, DAB, A. Negative control, bar; 50 μ m, B. Immunopositive staining with *M. haemolytica* antibodies in bronchiolar epithelium (arrowheads) and inflammatory cells in their lumen (arrows), bar; 100 μ m, C. Immunopositive staining with *P. multocida* antibodies in alveolar epithelia and inflammatory cells (arrows) in the lumens of the alveoli, bar; 50 μ m.

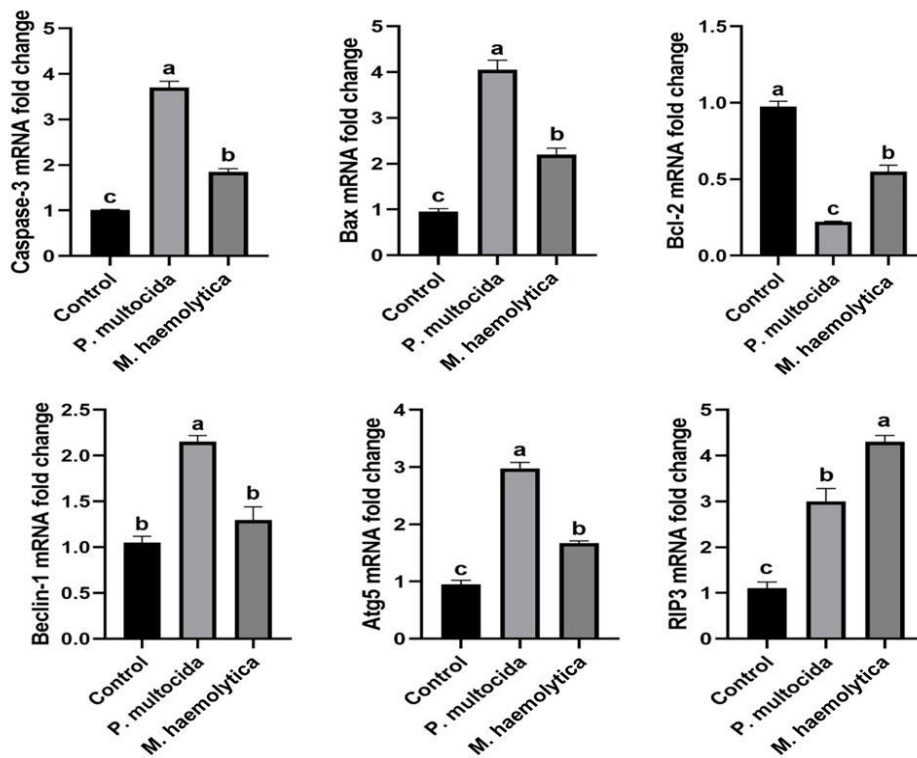


Fig. 4: Relative mRNA expression levels in *P. multocida* (n:23) and *M. haemolytica* (n:17) pneumonia compared to the control group (n:10). The data obtained were offered as Mean±SD. ^{a-c}Means within different superscripts are statistically significant ($p < 0.001$). (Bax; Bcl-2-associated protein X, Bcl-2; B-cell lymphoma 2, Atg5; Autophagy related-5; RIP3; Receptor Interacting Protein-3).

Real-time PCR (qPCR) results: The relative mRNA expressions of Bax, Bcl-2, Caspase-3, Beclin-1, Atg5 and RIP3 genes examined in the study are given in Fig. 4. Bax expression levels measured in PP lung tissues examined in the study were found to increase 4.2-fold in *P. multocida* and 2.3-fold in *M. haemolytica*, relative to the control group ($p < 0.001$). Bcl-2 expression levels were found to decrease 0.22-fold in *P. multocida* and 0.52-fold in *M. haemolytica* relative to the control group ($p < 0.001$). Caspase-3 expression levels were increased 3.8-fold in *P. multocida* and 1.9-fold in *M. haemolytica* relative to the control group ($p < 0.001$). It was observed that there was a 2.9-fold increase in Atg5 expression levels in *P. multocida* and a 1.7-fold increase in *M. haemolytica* relative to the control group ($p < 0.001$). A 2.8-fold increase in RIP3 expression levels was detected in *P. multocida* and a 4.2-fold increase in *M. haemolytica* relative to the control group ($p < 0.001$). A 2.1-fold increase in Beclin-1 expression levels was detected in *P. multocida* and a 1.2-fold increase in *M. haemolytica* relative to the control group ($p < 0.001$). The control group and the *M. haemolytica* group were statistically similar regarding Beclin-1 expression level ($p > 0.05$).

DISCUSSION

Today, cattle respiratory system diseases are one of the most important causes of death affecting beef cattle breeding in general, and they cause serious losses in the national economy due to both these deaths and high treatment costs. PP is known as one of the pivotal infectious diseases caused by *P. multocida* and *M. haemolytica* (Abed *et al.*, 2020; Tuzcu *et al.*, 2020). There is not enough information in the literature about the type and mechanisms of cell death in cases of PP caused by *P. multocida* and *M. haemolytica* in cattle; therefore, new

studies are needed within the scope of relevant processes. This study mainly aimed to reveal the effects of cell death mechanisms such as apoptosis, autophagy and necroptosis on the pathogenesis of pneumonia caused by relevant agents in cases of fibrinous pneumonia/pleuropneumonia caused by *P. multocida* and *M. haemolytica* in cattle. To our knowledge, this was the first study to evaluate the agents-related processes of apoptosis, autophagy and necroptosis in naturally infected PP.

Yaman *et al.* (2018), in the pneumonic lung study conducted on cattle for the immunohistochemical detection of *P. multocida* and *M. haemolytica*, found 23 (23%) and 17 (17%) positive, respectively. In a different study on pneumonia in calves, it was reported that *P. multocida* was detected in 8% of the lungs of calves with pneumonia, *M. haemolytica* in 42%, both *P. multocida* and *Haemophilus somnus* in 7%, and *P. multocida* and *M. haemolytica* in 2% (Hazirolu *et al.*, 1997). In a study on pneumonia conducted by Kilic and Muz (2004), *P. multocida* was detected in 6% and *M. haemolytica* in 1.8%. In the current study, 23 (15.3%) *P. multocida* and 17 (11.3%) *M. haemolytica* were detected in 150 fibrinous pneumonia/pleuropneumonia lung tissues. Additionally, 150 (10%) cases of fibrinous pneumonia/pleuropneumonia were detected in 1430 animals. Current findings showed lower rates than those obtained by Yaman *et al.* (2018), but higher than the results from the study of Kilic and Muz (2004). It was interpreted that this situation may be due to the difference in the material and methodology of the study. Additionally, in the current study, both *P. multocida* and *M. haemolytica* positivity was detected in 5 cases.

Praveena *et al.* (2010) stated that *P. multocida* causes apoptosis in lung and reported that the necrotic changes seen in the respiratory tract epithelium may be due to caspase activity. Periasamy *et al.* (2018) suggested that *P. multocida* lipopolysaccharides induce apoptotic changes

in macrophages, lymphocytes and neutrophils. Yavuz and Dinçel (2020a) reported that Caspase-3 and 9 expression in calves with PP was triggered by inflammatory cells in the alveoli, bronchi and bronchiole epithelial cells and their lumens. Present study, it was determined that PP, induced by *P. multocida* and *M. haemolytica*, caused an increase in Bax (4.2 and 2.3 fold, respectively) and Caspase-3 (3.8 and 1.9-fold, respectively), and a decrease in Bcl-2 (0.22 and 0.52-fold, respectively), relative to the control group. Our findings were parallel to the findings of previous studies. Moreover, the present findings show that apoptosis is more effectively induced in cases of fibrinous pneumonia/pleurapneumonia caused by *P. multocida* than by *M. haemolytica*.

In general, many pathogens antagonize the initiation and maturation of autophagic processes and use them to their advantage to facilitate intracellular survival or replication. In this context, the identification of mechanisms and/or virulence factors that utilize autophagy may provide a new strategy for therapeutic intervention in infections (Mizushima and Komatsu, 2011; Zhu *et al.*, 2018; D'Arcy, 2019;). Recently, studies on the evaluation of apoptosis and autophagic processes have come to the fore in infection and cancer studies. Studies have stated that autophagy plays a pivotal role in the response to bacterial and viral infections (Ogawa *et al.*, 2005; Colombo, 2007; Rioux *et al.*, 2007). The outcome of autophagy may be pathogen-specific. In the experimental study conducted by Yuan *et al.* (2012), it was stated that *Pseudomonas aeruginosa* induced autophagy. It has been stated that this function occurs via Beclin-1 and Atg5. They also stated that autophagy induced by *P. aeruginosa*, an extracellular bacterium, represents a host protective mechanism. Zhu *et al.* (2018) reported that in cases of pneumonia caused by *Staphylococcus aureus*, inhibition of autophagy is a good strategy to prevent and/or treat the infection. Current findings revealed an increase in Atg5 (2.9 and 1.7 fold, respectively) and Beclin-1 (2.1 and 1.2-fold, respectively) in *P. multocida* and *M. haemolytica* compared to the control group. However, Beclin-1 expression in *M. haemolytica* was found to be statistically similar to the control group. Additionally, our findings regarding *P. multocida* were parallel to Yuan *et al.* (2012). Current findings revealed that autophagic changes are more prominent in pneumonia caused by *P. multocida* and provided a new perspective on the pathogenesis of the relevant disease.

It has been stated that necroptosis plays a pivotal role in the pathogenesis of many diseases occurring in the cardiovascular, neurological, pulmonary and gastrointestinal systems (Khoury *et al.*, 2020). Necroptosis is a controlled cell death characterized by necrosis morphology. RIPK3 is known as an important transducer in the necroptosis process (Nailwal and Chan, 2019; Khoury *et al.*, 2020). RIP3 expression levels were increased 2.8 and 4.2-fold in *P. multocida* and *M. haemolytica* respectively relative to the control group. Present findings revealed that necroptosis was induced by both agents, but *M. haemolytica* played a more active role in inducing the necroptosis process. We can interpret the findings as necrotic changes being more prominent in pneumonia caused by *M. haemolytica*. These results also support our findings that the necrotic changes detected in

histopathological examinations of *M. haemolytica* pneumonia are more severe.

Wang *et al.* (2016) reported that survival increased and inflammation decreased in RIPK3-deficient animals after intratracheal injection of LPS in mice to induce respiratory distress syndrome. Pan *et al.* (2016) detected in a similar study in mice induced with oleic acid, a decrease in inflammation in the lung parenchyma with the use of Necrostatin-1 (Nec-1), an inhibitory agent. Studies show that necroptosis and the inflammatory process are closely related. Although inflammation is essential for host defense mechanisms due to its many important functions in the immune system, excessive and/or unlimited continuation can cause tissue immunopathology (Fontes *et al.*, 2015). Current findings suggest that in pneumonia caused by *M. haemolytica*, necroptosis process is more active, the response of the organism to the agent may be different, and the inflammatory process may be more severe. Some researchers report that microbial infection can cause necroptosis in host cells. It is also suggested that microbial proliferation occurs by inhibiting programmed cell death (Cho, 2018; Bedoui *et al.*, 2020).

It has been reported that necroptosis will be inhibited by Caspase-8/FADD-mediated apoptosis (Newton and Manning, 2016). Caspase-8 and Caspase-9, known as initiator caspases, exhibit very pivotal roles in the induction of apoptosis (D'Arcy, 2019). Current findings show that apoptosis is induced more in *P. multocida* pneumonias than in *M. haemolytica* pneumonias. However, it is not possible to say the same for necroptosis. The present findings revealed that *M. haemolytica* triggered necroptosis process more effectively; this suggests that the effect of Caspase-8 may also play a role, and further studies are needed in the future.

Clinical diagnosis of PP is difficult. It is mostly done with cultural and serological methods, and both methods are time consuming and laborious (Abera and Mossie, 2023; Robi *et al.*, 2024). Nowadays, more confirmatory diagnostic analyzes such as immunohistochemistry, PCR, real-time PCR, RFLP, gene sequencing and phylogenetic analyzes are used (Yaman *et al.*, 2018; Abera and Mossie, 2023). In the present study, the relevant agents in PP were confirmed by immunohistochemistry. Afterwards, apoptosis, autophagy and necroptosis gene expressions for the agents were investigated by real-time PCR. Thus, the results obtained in the study revealed the importance of necroptosis, apoptosis and autophagy processes in PP pathogenesis. Additionally, the findings from this study contributed to the literature on the differential diagnosis and treatment of pneumonia caused by *P. multocida* and *M. haemolytica*.

Conclusion: As a result, with this study, we determined with the real-time PCR method the levels of Bax, Bcl-2, Caspase-3, Atg5, Beclin-1 and RIP3 in pneumonic pasteurellosis caused by *P. multocida* and *M. haemolytica* in our country and examined the effects of cell death mechanisms such as apoptosis, autophagy and necroptosis on the pathogenesis of the agents. Our results show that the processes of apoptosis, autophagy and necroptosis play a pivotal role in the pathogenesis of pneumonic pasteurellosis. In addition, it has revealed that the processes of apoptosis and autophagy play a more active role in PP caused by *P. multocida*, and the process of necroptosis plays a more active role in PP caused by *M.*

haemolytica. Present results may provide a new perspective on the pathogenesis of pneumonic pasteurellosis.

Author's contribution: GA and MT designed the study. GA collected lung tissue samples. OK performed histopathological and immunohistochemical examinations. NT performed the qPCR examinations. All authors read and approved the final version of the manuscript.

Ethics approval statement: This study was approved by Sivas Cumhuriyet University Animal Experiments local ethics committee (dated 12.01.2022, decision no:500).

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Conflict of interest: The authors declare no conflict of interest.

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