Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

Morphopathological Changes of Natural Pneumonic Pasteurellosis in Calves

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ARTICLE INFO

Keywords

P. multocida infection
Calves
Fibrinous
Fibrinopurulent
OMP87

Received 09/11/2021
Accepted 04/12/2021
Available On-Line 01/01/2022

ABSTRACT

Pasteurellosis is one of the infectious diseases of calves causing huge economic losses due to high morbidity, mortality, and treatment expenses of livestock. This study was conducted to describe the clinical presentation and pulmonary lesions of pasteurellosis infected calves. 167 lung specimens were collected from the cadaver and slaughtered calves (15 days-2 years) and *Pasteurella multocida* (*P. multocida*) were identified in all the submitted samples using bacteriological examination and qRT-PCR. Our results showed upregulation of outer membrane protein (OMP) (virulent gene) of *P. multocida* in all submitted bacteriologically confirmed cases of pneumonic pasteurellosis. The clinical signs of infected calves were in form of fever, respiratory distress, frothy salivation, weakness, and inappetence. Grossly, lungs were consolidated with the presence of multiple abscesses, hemorrhages, thickened pleura, and prominent interlobular septa. Microscopically, lungs revealed fibrinous, suppurative, and fibrinopurulent bronchopneumonia with the presence of bacterial colonies, heavy infiltrates of inflammatory cells, fibrin, and RBCs inside the pulmonary alveoli and bronchioles. Multifocal necrotic areas in pulmonary parenchyma and intra-alveolar degenerated neutrophils (oat cells) were also seen in some pulmonary foci. We concluded that OMP87 overexpression reflects *Pasteurella* infection even in formalin-fixed lungs. *Pasteurella* is one of the prevalent causes of BRD and pneumonia of calves in the Kalyobiya governorate. Fibrinopurulent bronchopneumonia and multifocal pulmonary necrosis were the predominant lung alterations in calves infected with *P. multocida*.

1. INTRODUCTION

Bovine respiratory disease (BRD) is one of the serious problems affecting cattle worldwide (Kirchhoff et al. 2014). BRD causes significant financial losses among livestock due to growth retardation and increased mortality of calves (Taylor et al. 2010). *P. multocida* and *Mannheimia* (*M.*) *haemolytica* are the most common bacteria isolated from BRD infected calves (FR et al. 2019).

Pasteurella is a normal inhabitant of the upper respiratory, gastrointestinal and genital tracts of animals (Amin 2020). Many stress factors i.e. transportation and co-infection with certain respiratory viruses or bacteria could provoke *Pasteurella* to invade lower respiratory tissues and circulate to other organs (Tadesse et al. 2017, Amin 2020). *Pasteurella* infection causes many diseases in animals i.e. atrophic rhinitis in pigs, snuffles in rabbits, fowl cholera in chickens, hemorrhagic septicemia, and pneumonic pasteurellosis in ruminants (Merza 2008, Constable PD 2017).

Pneumonic pasteurellosis is a highly infectious and fatal respiratory disease-causing high mortality (30%) among cattle (Tadesse et al. 2017). The most prevalent strain of respiratory pasteurellosis isolated from diseased cattle is *P. multocida* (Bahr et al. 2021).

The most common signs in pasteurellosis infected cattle and sheep are coughing, copious nasal discharge, fever, congested mucous membranes, dyspnea, anorexia, and sudden death (Kabeta et al. 2015, Amin 2020, Bahr et al. 2021).

Prior studies have shown that *P. multocida* induces multiple pathological alterations in the lungs of infected animals. Fibrinous and suppurative bronchopneumonia were the most predominant histopathological lesions in pasteurellosis-infected sheep (Mohamed and Abdelsalam 2008, Sharma et al. 2011, Amin 2020). In pasteurellosis infected cattle, broncho-interstitial, mucopurulent, and fibrinous pneumonia were reported in many cases (Odugbo et al. 2005, Sharma et al. 2011, Praveena et al. 2014). Multifocal necrosis of pulmonary parenchyma, subpleural hemorrhage, and serofibrinous pleurisy were also recorded in other studies in infected cattle (Dowling et al. 2002, Biyashev et al. 2014). Few studies were conducted on the incidence of pasteurellosis in pneumonia calves and the pathological picture of natural pasteurellosis infection in these affected calves (FR et al. 2019, El-Seedy et al. 2020). The present study aimed to detect *P. multocida* in the pulmonary tissues of cadaver and slaughtered calves suffering from respiratory symptoms and describe the gross and histopathological changes in the lungs of pasteurellosis infected calves in the Kalyobiya governorate.

2. MATERIAL AND METHODS

2.1. Specimen collection

A total of 167 lung specimens were collected from cadaver and slaughtered calves with a history of respiratory distress. The age of these calves varied from fifteen days to three years. All the specimens were collected from abattoirs and farms located at Kalyobiya Governorate, Egypt in the period from November 2019 till August 2021.

2.2. H&E staining and Histopathology examination

Small tissue specimens from the lungs of dead and slaughtered calves were collected and fixed in 10% neutral buffered formalin. After fixation, the tissue specimens were trimmed, washed, dehydrated, cleared, and embedded in paraffin wax. The paraffin tissue block was sectioned at 5 µm thickness and stained with hematoxylin and Eosin (H&E) as previously described (Bancroft and Gamble 2008). Images were acquired by the Nikon Eclipse E800 microscope equipped with an OMAX eyepiece camera..

2.3. Bacteriological examination

2.3.1. Sample collection

Nasal swabs from morbid calves and Lung samples from cadaver and slaughtered animals were collected under aseptic condition and submitted to Tanta Animal Health Research Institute for bacteriological examination.

2.3.2. Bacterial isolation and identification

The isolation and identification of *P. multocida* in nasal swabs and lung specimens were performed as previously described (Marru et al. 2013).

2.4. DNA Extraction and qRT-PCR

DNA was extracted from some formalin-fixed pneumonic tissues (n=23) using Gene JET genomic DNA extraction kit (Catalog # K0721, Fermentas life Sciences, European Union). The isolated DNA was amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA, # K0221) and OMP87 bovine specific primers (Table 1).

The web-based tool, Primer 3 (http://www.genome. wi.mit.edu/ cgi-bin/primer /primer3_www.cgi) was used to design these primers based on published bovine OMP87 sequences. To ensure the primer sequence is unique for the template sequence; the similarity was checked with other known sequences with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). The final reaction mixture was placed in a StepOnePlus real-time PCR system (Applied Biosystems, Life Technology, USA). The qRT-PCR condition included an initial denaturation step at 95 °C for 5 min, amplification for 40 cycles with denaturation step at 95 °C for 15s, followed by a temperature gradient of 48 °C for 30s for the annealing step, and extension at 72 °C for 30 s and the PCR program was carried out. Critical threshold (Cq) values of OMP87 gene were collected and analyzed.

Table 1 Primers used for qRT-PCR

Gene	Forward primer	Reverse primer	Size (bp)
Omp87	*AGGTGAAAGAGGTT ATG*	*TACCTAACTCAACCAAC*	200

3. RESULTS

3.1. Bacteriological results

P. multocida bacteria were isolated from all the submitted nasal swabs and lung sections. The colonies were in form of small grey colonies (1-2 mm in diameter) on blood agar. *P. multocida* appeared as gram-negative, non–spore-forming coccobacilli with bipolar staining features. All the results of biochemical tests further confirmed the identity of *P. multocida*.

3.2. Quantitative Real-Time PCR (qRT-PCR)

The mRNA level of *OMP87* of *P. multocida* was upregulated in all the submitted fixed specimens collected from bacteriologically confirmed *P. multocida* where the CT (cycle threshold) was found to be 22.2 ± 0.9. Specimens were considered as positive for pasteurellosis when the sigmoidal amplification curve was similar to the positive control before cycle 30.

3.3. Gross lesions

Most examined lungs had a uniform or confluent purplish to red or gray consolidated lobules with occasional petechial hemorrhages (Figure 1A). On the cut surface, there were different sized tan to greyish-white confluent areas reflecting the bronchiolar and peribronchiolar inflammation. Most trachea, bronchi, and bronchioles were packed with mucoid, purulent, or mucopurulent exudate (Figure 1B). In a large subset of examined lungs, there were thickened conspicuous interlobular septa (giving a marbling appearance to the lungs) with patchy or diffuse thickening of pleura (Figure 1C-D). Circumscribed whitish-tan abscesses were seen in some of these lungs.

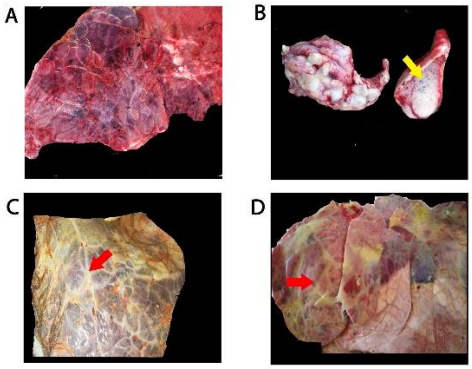

Figure 1 Gross lesions of lungs in calves naturally infected with *P. multocida*. Lung tissues displayed purplish to red or gray consolidated lobules with petechial hemorrhages (A), frothy fluid (yellow arrow) in trachea and bronchioles (B), and diffuse (C) or patchy (D) thickening of pleura and interlobular septa (red arrows) giving the marbling appearance to the lungs.

3.4.Histopathology

The histopathological examination of the lungs of calves revealed extensive damage to alveoli, bronchioles, interstitial tissue, and pleura. Most cases showed moderate to severe bronchopneumonia. The predominant two types of bronchopneumonia were suppurative necrotizing- and fibrinous-bronchopneumonia. suppurative necrotizing bronchopneumonia was characterized by multifocal to confluent, irregular necrotic areas in the pulmonary parenchyma. These necrotic areas were mostly surrounded by infiltrates of polymorphonuclear and mononuclear cells with oedematous alveoli in the vicinity.

3.4.1. Alveoli

Alveolitis was the predominant finding in most examined alveoli.

Most alveoli were expanded by an exudate composed of homogenous eosinophilic material (scanty amount) and infiltrates of alveolar macrophages, lymphocytes, live and degenerated neutrophils, and occasional syncytial and giant cells (Figure 2A-B). Multifocal areas of coagulative necrosis are surrounded by inflammatory cellular aggregations of lymphocytes, macrophages, and neutrophils in many examined lungs (Figure 2C). In some alveoli, fibrin threads intermingled with cellular infiltrates predominantly lymphocytes and macrophages or degenerated inflammatory cells were also evident (Figure 2D). In some foci, bacterial colonies were prominent in some alveoli (Figure 3A). Hyaline membranes in form of eosinophilic homogenous, thickened layer lining alveoli were seen in some cases (Figure 3B).

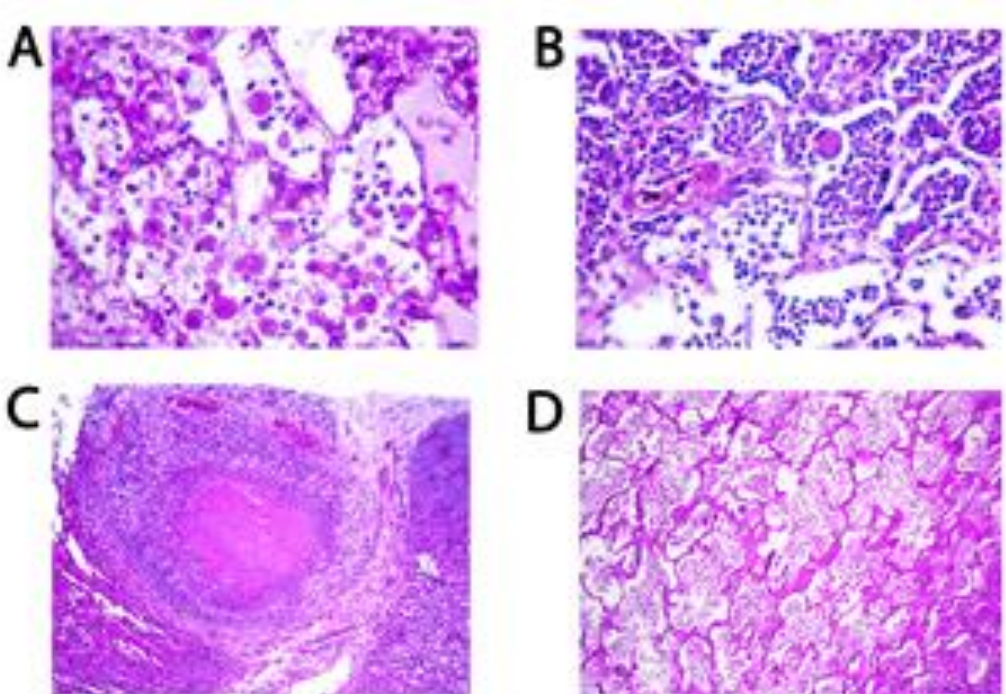

Figure 2 Pulmonary photomicrographs of calves infected with *P. multocida* in calves. Alveolar lesions included alveolar edema infiltrated with macrophages and syncytial cells (400X) (A), severe infiltration of the alveolar lumen with neutrophils, lymphocytes, macrophages, and few giant cells (400X) (B), suppurative necrotizing inflammation of pulmonary parenchyma surrounded by mono and polymorphonuclear inflammatory cells (100X) (C) and intra-alveolar fibrin threads deposition (100X) (D).

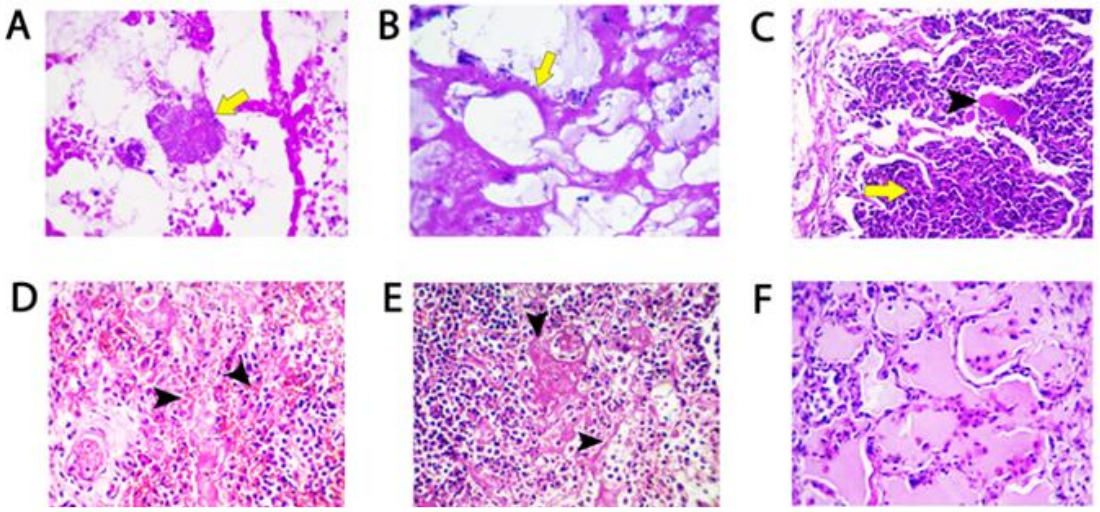

Figure 3 Lungs of calves infected by *P. multocida* showing bacterial colonies (arrow) inside the alveoli (400X) (A), hyaline membrane (arrow) lining the alveoli (400X) (B), amphophilic homogenous structureless small mass (arrowhead) among degenerated streamed inflammatory cells (oat cells) (arrow) (400X) (C), intra-alveolar hemorrhage (arrowheads) (400X) (D), necrosis of alveolar wall (arrowheads) and fibrin deposition (400X) (E) and intralveolar edema infiltrated with few leukocytes (400X).

Amphophilic homogenous structureless small masses were scattered among degenerated neutrophils and macrophages with streamed nuclei (oat cells) in the lumen of some alveoli (Figure 3C). Few alveoli had intra-alveolar extravasated RBCS (Figure 3D). Necrosis of alveolar walls with loss of alveolar architecture and fibrin thread deposition was also observed (Figure 3E). In mildly affected cases, there was only alveolar edema with few leukocytic infiltrations (Figure 3F).

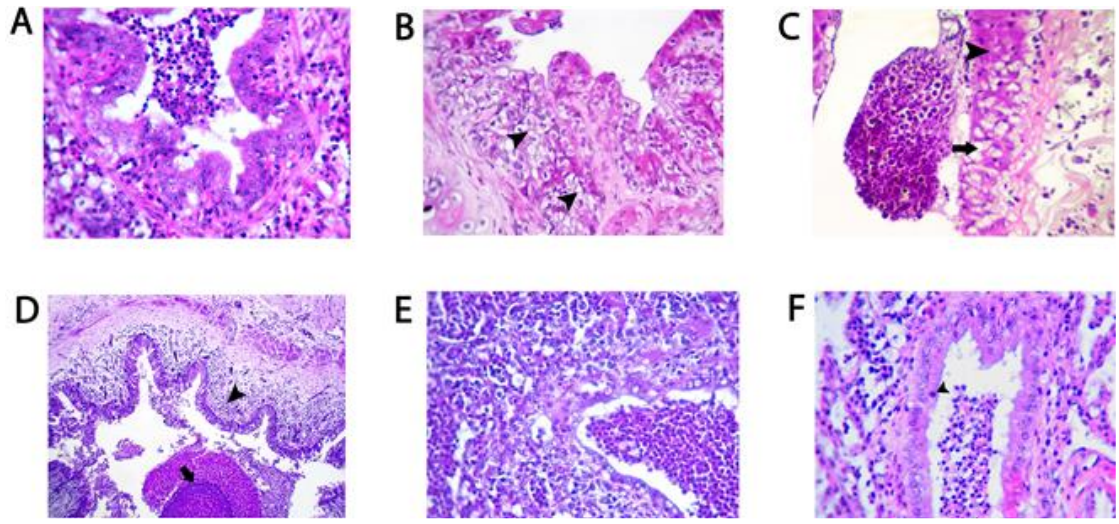

Figure 4 Bronchioles of calves infected by P. multocida showing hyperplasia of their lining epithelium (400X) (A), cytoplasmic vacuolation of their mucosal epithelia (400X) (B), degeneration and necrosis of bronchial wall with intraluminal necrotic cellular debris and inflammatory cells (400X) (C), inflammatory cellular infiltration of lamina propria of bronchiole with intraluminal purulent material (100X) (D), extensive inflammatory cellular infiltration in the wall of bronchiole, lumen and peribronchiolar space (400X) (E) and leukocytic invasion of bronchial mucosal epithelia (400X) (F).

3.4.2. Bronchioles

Suppurative bronchiolitis was the common finding in most lungs of pasteurellosis infected calves. The bronchiolar mucosal epithelium was predominantly hypertrophic and hyperplastic, often piled up to 4 layers thick (Figure 4A). Hyperplasia of goblet cells and vacuolation of bronchiolar epithelia were also observed in some of these lungs (Figure 4B). Many bronchioles showed partial to complete necrosis of their walls with denuding of their mucosal epithelia in the lumen and extensive inflammatory cellular infiltration in lumen and lamina propria mainly lymphocytes, macrophages, and neutrophils (Figure 4C-D). Similar cellular infiltrates were also evident around and in the lumen of bronchioles (Figure 4E). Few large multinucleated giant cells were also seen around some bronchioles among the cellular infiltrates. Transmigrated intraepithelial leukocytes were observed among bronchial epithelial cells in a few pulmonary tissues (Figure 4F).

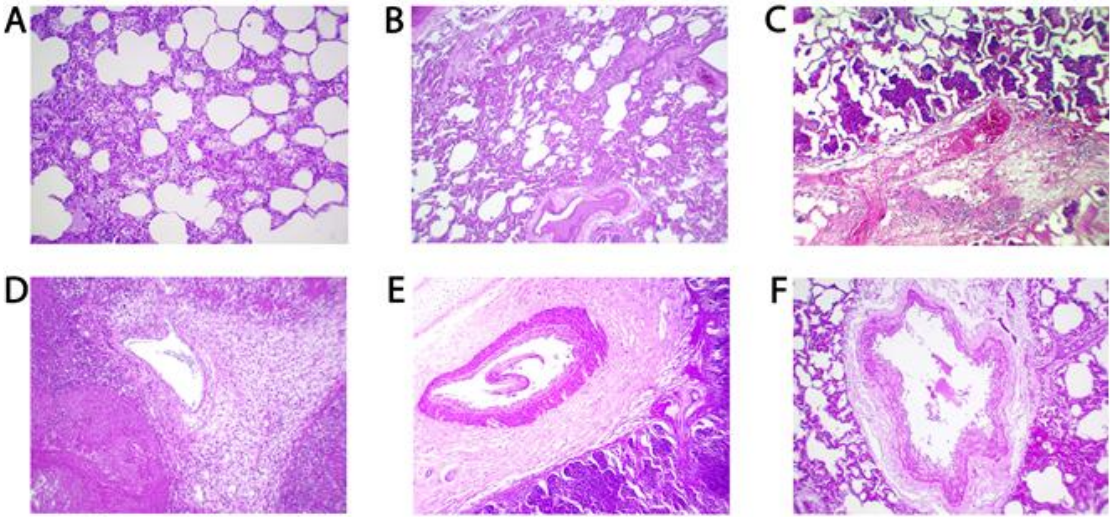

Figure 5 Lungs of calves infected by *P. multocida* Showing inflammatory cellular infiltration in the interstitial tissues mainly lymphocytes and histocytes (100X) (A), Atelectasis of pulmonary alveoli due to interstitial pneumonia (100X) (B), interlobular septal thickening due to fibrinopurulent inflammation (100X) (C), perivascular fibroplasia (400X) (D) and sloughing of endothelial cells of the large pulmonary blood vessel with degeneration of their walls (400X) (E-F).

3.4.3. Interalveolar space (Interstitial tissue) and blood vessels

Focal to diffuse thickening of interalveolar space was noticed in most pulmonary tissues due to edema, reactive pneumocyte hyperplasia, fibrous CT proliferation, and inflammatory cellular infiltration (Figure 5 A). In some areas where extensive thickening of interalveolar space was seen, atelectasis of alveoli was also prominent (Figure 5B). In addition, there was multifocal interlobular septal thickening due to the deposition of fibrinopurulent exudate (Figure 5C). Vascular changes included moderate to severe congestion of inter-alveolar and peribronchial blood vessels, hemorrhage, and perivascular edema. Perivascular fibroplasia and inflammatory cellular infiltration were also prominent in some pulmonary sections (Figure 5D). Some pulmonary blood vessels were denuded of endothelial cells and filled with necrotic debris (Figure 5E-F). Pyemic emboli and vascular thrombi were seen in many pulmonary tissues (Figure 6A-B). Fibrinoid necrosis was also reported in the wall of some large pulmonary blood vessels (Figure 6B). Disruption and vacuolation of tunica media of some pulmonary blood vessels were among the rare lesions seen in examined cases (Figure 6C). Perivascular edema and hyperplasia of smooth muscles of some pulmonary blood vessels were also seen in a few pulmonary tissues.

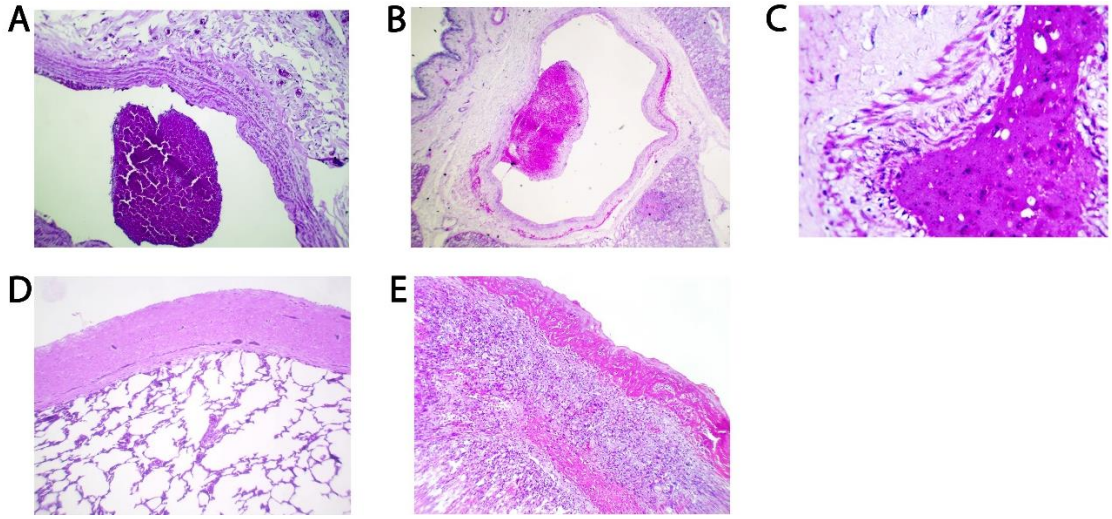

Figure 6 Vascular and pleural photomicrographs of calves infected with *P. multocida*. Lungs displayed intravascular pyemic emboli (400X) (A), thrombus (100X) (B), and degeneration of T. media and endothelial cells of large pulmonary blood vessels (400X) (C). Pleura were severely thickened (100X) (D) and occasionally hyalinized (100X) (E) in some pulmonary tissues.

3.4.4 .Pleura

Pleura was expanded in many pulmonary tissues due to mononuclear inflammatory cellular infiltration, ectatic lymphatics, edema, and fibrous C.T proliferation (Figure 6D). Hyalinization of pleural C.T was also noticed in some cases (Figure 6E).

4. DISCUSSION

BRD is a multi-factorial disease that has a catastrophic effect on the global economy. BRD causes 75% morbidity and 50% to 70% mortality among feedlot cattle (Edwards 1996, Loneragan et al. 2001). The most prevalent bacteria recovered from bovine respiratory disease (BRD) or shipping fever in cattle are *P. multocida* and *M. haemolytica* (El-Seedy et al. 2020). BRD classic presentation in beef cattle is pneumonia that mainly affects calves 3 days to 3 weeks after shipment to their feedlot (Caswell 2016). Pneumonia caused by *P. multocida* infection often occurs when the normal defenses of animals are impaired (Boyce and Adler 2006, Merza 2008, Constable PD 2017, Amin 2020) The pathological picture of pneumonic pasteurellosis in calves have not been completely elucidated and is considered a subject of argument due to the complex nature of the disease and the lack of correspondence of the results obtained by an experimental approach. In this study, we described the pulmonary alterations caused by natural pasteurellosis infection in calves in Kalyobiya Governorate, Egypt.

Since the pathological picture of pneumonia caused by *M. haemolytica*-infected lungs of cattle is similar to those produced by *P. multocida*, we used qRT-PCR in the identification of *P. multocida* in the infected lungs by measuring the expression of *OMP87* gene. All submitted pneumonic specimens (bacteriologically confirmed p. multocida cases) showed upregulation of this gene thus confirming *P. multocida* infection and reflecting the role of OMP in the pathogenesis of pasteurellosis pneumonia. It has been shown that OMPs play an important role in the virulence, colonization, invasion, and pathogenesis of *P. multocida* infection (Srivastava 1998, Lin et al. 2002, Boyce et al. 2006). In this investigation, *P. multocida* was isolated from pneumonic calves in Kalyobiya farms. Consistent with our results, the prevalence of *P. multocida* was found to be higher (2.17-fold) than *M. haemolytica* in calves suffering from respiratory diseases in upper and middle Egyptian governorates (El-Seedy et al. 2020). In another study, 88 *P. multocida* isolates were recovered from 256 nasopharyngeal swabs and lung specimens collected from emergency slaughtered calves in some Egyptian Governorates emphasizing the higher incidence of *P. multocida* (El-Jakee et al. 2016).

Respiratory distress, pyrexia, anorexia, and loss of body weight were the most observed clinical signs of pneumonic pasteurellosis in this study. Similar clinical signs were also recorded by prior studies (Dowling et al. 2002, Mohamed and Abdelsalam 2008, Tadesse et al. 2017, Amin 2020). Our necropsy examination showed a variable degree of pneumonic lungs and pleurisy in calves. The pattern of lung consolidation ranged from small multifocal areas to patchy consolidation with occasional multiple abscesses or necrotic foci in some pulmonary tissues. The complex nature of infection along with *Pasteurella* may account for the variability in the pattern, severity, and distribution of lesions. Consistent with our gross lesions, previous studies have reported the cranioventral firm to hard consolidated lung tissues, areas of coagulative necrosis, interlobular edema (marbling), and pleuritis in lungs infected with pasteurellosis (DeRosa et al. 2000, Reinhold et al. 2002, Sharma et al. 2011, Praveena et al. 2014).

Microscopically, a variable degree of bronchopneumonia was observed in most examined lungs in our investigation. The most prevalent types of bronchopneumonia in pneumonic calves were purulent, fibrinous, and fibrinopurulent. These types were also reported in pasteurellosis infected lungs by other studies (Dungwonh 1985, Abubakar and Zamri-Saad 2011). *P. multocida* infection has been shown to induce neutrophilic bronchopneumonia, edema, and hemorrhage (Caswell JL 2007). Such changes were also documented in our study. Although degenerated neutrophils (oat cells) were recorded mainly in *M. Haemolytica* and *Histophilus somni* infected calves (Caswell 2016), some *Pasteurella* infected lungs showed similar cells in the alveoli in the current study.

Prior studies have correlated the presence of coagulative necrosis in the lungs as a characteristic lesion of pneumonia induced *by M. haemolytica* (Haritani 1995, Gershwin et al. 2015). However, a variable degree of coagulative necrosis was also reported in our study. It has been shown that *Pasteurella* endotoxins induce intravascular thrombosis of pulmonary veins, capillaries, and lymphatics, resulting in localized ischemic necrosis of the pulmonary parenchyma and severe intra-alveolar inflammatory reaction (mainly fibrinous exudate) (Slocombe et al. 1984, Jones et al. 1997).

Interestingly, most pulmonary compartments of examined pneumonic calves were flooded with neutrophils and chronic inflammatory cells i.e., lymphocytes, macrophages, and occasional giant cells. This finding could be attributed to the pro-inflammatory cytokines such as TNF-α, IL-β, and IL-8 released as a reaction to bacterial cell wall components in alveolar air space which promote leukocytic cellular infiltrations at the infection site (Locksley et al. 2001). Moreover, infiltration of macrophages with neutrophils was found to enhance phagocytosis and cytokine production (Prame Kumar et al. 2018). Meanwhile, the presence of inflammatory exudates in the bronchioles may reflect secondary bacterial dissemination throughout the respiratory tracts (Praveena et al. 2010).

5. CONCLUSION

P. multocida is one of the main causes of pneumonia and BRD in calves in the Kalyobiya governorate in Egypt. Upregulation of *OMP87* along with conventional methods i.e., gross, and histopathological examination of pneumonic lungs could be a reliable method in the identification of *Pasteurella* infection in pneumonic lungs. Due to the

complex nature of infection associated with *Pasteurella*; a variety of lesions was seen in pneumonic calves. Fibrinous to fibrinopurulent bronchopneumonia, vascular thrombosis, and multifocal coagulation necrosis were the predominant microscopic findings in the lungs infected by *P. multocida* in our study.

CONFLICT OF INTEREST

All authors declare no competing interest related to the content of this work.

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