Morphopathological Changes of Natural Pneumonic Pasteurellosis in Calves

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Abstract

Pasteurellosis is one of the infectious diseases of calves causing huge economical 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 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 presence of multiple abscesses, hemorrhages, thickened pleura, and prominent interlobular septa. Microscopically, lungs revealed fibrinous, suppurative and fibrinopurulent bronchopneumonia with 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 Kalyobiya governorate. Fibrinopurulent bronchopneumonia and multifocal pulmonary necrosis were the predominant lung alterations in calves infected with *p. multocida*.

Keywords: *P. multocida* infection, Calves, Fibrinous, Fibrinopurulent, *OMP87*

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 the 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 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 pig, snuffles in rabbits, fowl cholera in chicken, hemorrhagic septicemia and pneumonic pasteurellosis in ruminants **(Merza, 2008, Constable et al., 2017).**

Pneumonic pasteurellosis is 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,Yaman et al., 2018).** 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 Kalyobiya governorate.

Materials and Methods

Specimen collection:

A total of 167 lung specimens were collected from cadaver and slaughtered calves with 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. This sudy was approved by the Ethical Committee of Faculty Of Veterinary Medicine , Benha University Egypt (BUFVTM 09 /11/2021).

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 were sectioned at 5 µm thickness and stained with hematoxylin and Eosin (H&E) as previously described **(Bancroft and Gamble, 2008)**. Images were acquired by Nikon eclipse E800 microscope equipped with OMAX eye piece camera. The histopathological lesions were calculated based on their incidence (percentage) and severity (mild to severe) in the lung tissues.

Bacteriological examination

- ***Sample collection***

Nasal swabs and Lung samples were collected under aseptic condition and submitted to Tanta Animal Health Research Institute for bacteriological examination.

- ***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)** .

DNA Extraction and qRT-PCR

DNA was extracted from formalin fixed pulmonary tissues using Gene JET genomic DNA extraction kit ***(Catalog # K0721, Fermentas life Sciences, European Union***). The isolated DNA were 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 omp87 sequences. To ensure primer sequence is unique for the template sequence; we checked similarity to other known sequences with BLAST (*www.ncbi.nlm.nih.gov/blast/Blast.cgi).* The final reaction mixture

was placed in a StepOnePlus real time thermal cycler (Applied Biosystems, Life technology, USA) 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*

Results

Bacteriological results

P. multocida bacteria were isolated from most 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, oxidase positive, non–spore-forming coccobacilli with bipolar staining features. All the results of biochemical tests proved the identity of *pasteurella multocida*.

Quantitative Real-Time PCR (qRT-PCR)

The mRNA level of OMP87 of *P. multocida* was upregulated in most fixed specimens 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.

Gross lesions

Most examined lungs had uniform or confluent purplish to red or gray consolidated lobules with occasional petechial hemorrhages (Figure 1 A). On cut surface, there was 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 with patchy or diffuse thickening of pleura (Figure 1 C-D). Abscesses (circumscribed whitish-tan nodules) were seen in some of these lungs.

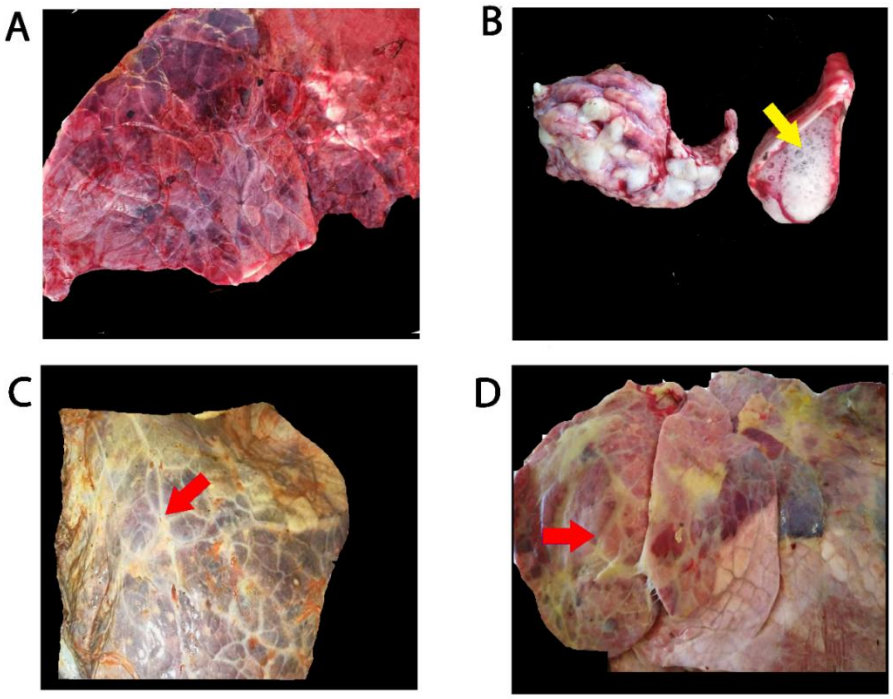

Figure 1 Gross lesions of lungs in calves naturally infected with *P. multocida*. Lung tissues displayed purplish to red or gray consolidated lobules with petechial hemorrhages (A), frothy fluid (yellow arrow) in trachea and bronchioles (B), and diffuse (C) or patchy (D) thickening of pleura and interlobular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

Histopathology

The histopathological examination of 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 pulmonary parenchyma. These necrotic areas were mostly surrounded by pus, infiltrates of PMN, mononuclear cells and histocytes with oedematous alveoli in the vicinity.

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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 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). 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 mild affected cases, there were only alveolar oedema with few leukocytic infiltrations (Figure 3F).

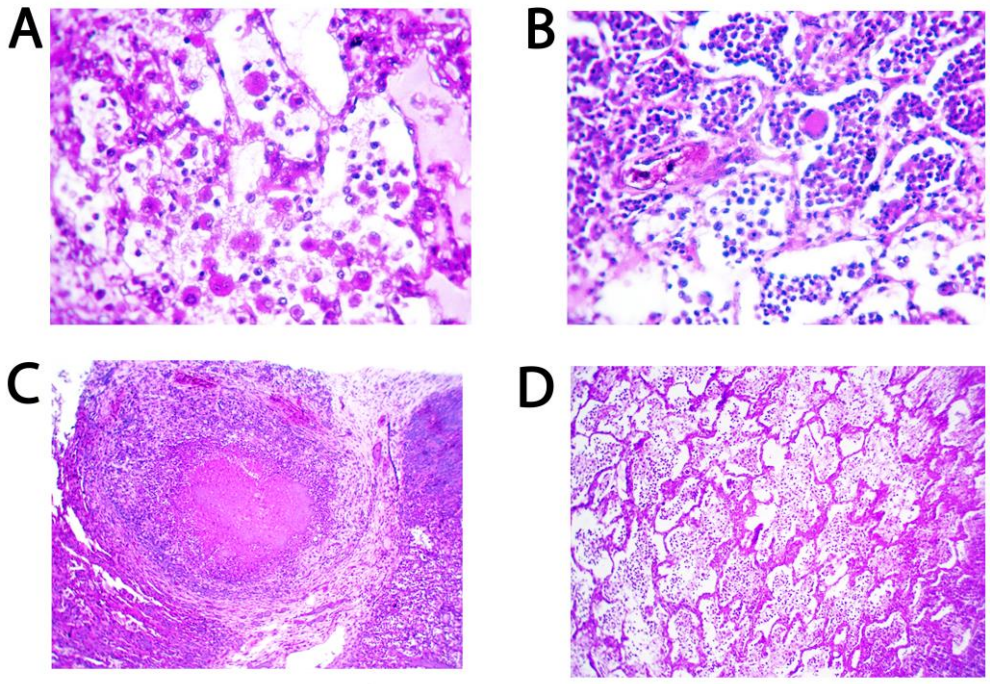

Figure 2 Pulmonary photomicrographs of calves infected with *P. multocida* in calves. Alveolar lesions included alveolar oedema infiltrated with macrophages and syncytial cells (400X) (A), severe infiltration of alveolar lumen with neutrophils, lymphocytes, macrophages, and few giant cells (400X) (B),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

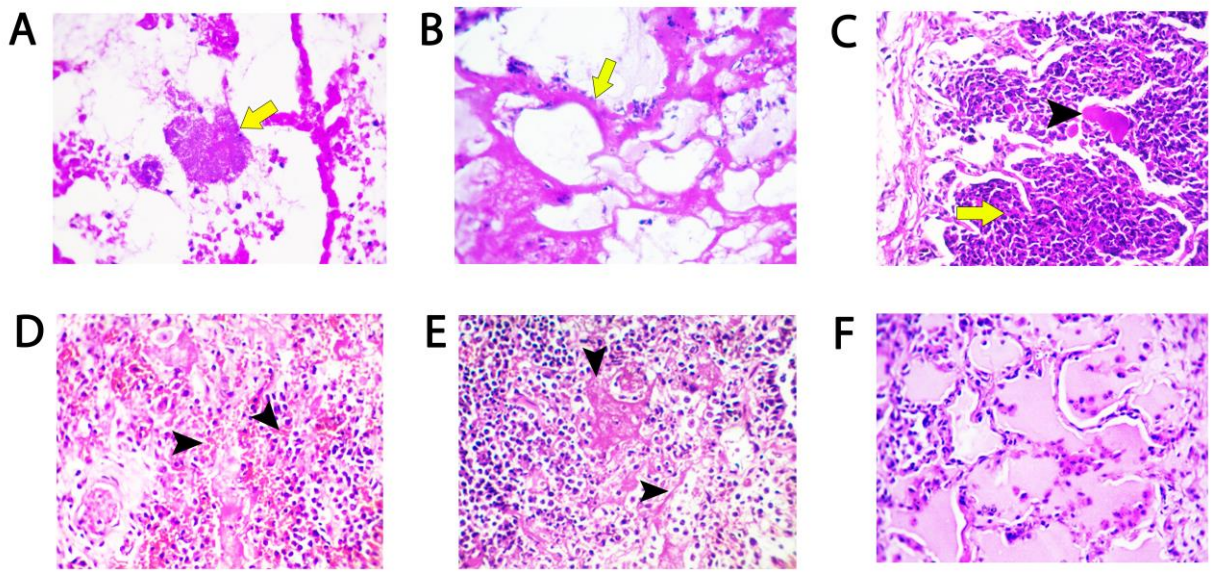

Figure 3 Lungs of calves infected by *P. multocida* showing bacterial colonies (arrow) inside the alveoli (400X) (A), hyaline membrane (arrow) lining the alveoli (400X) (B), amphophilic homogenous structureless small mass (arrowhead) among degenerated streamed inflammatory cells (oat cells) (arrow) (400X)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

Bronchioles:

Suppurative bronchiolitis was the common finding in most lungs of pasteurellosis infected calves. 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 few pulmonary tissues (Figure 4F).

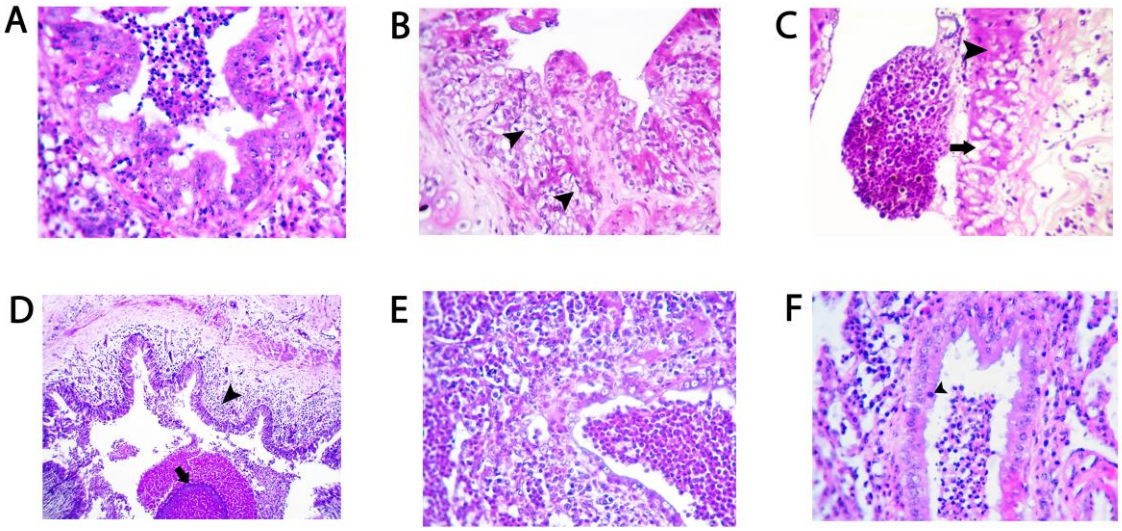

Figure 4 Bronchioles of calves infected by *P. multocida* showing hyperplasia of their lining epithelium (400X) (A), cytoplasmic vacuolation of their mucosal epithelia (400X) (B), degeneration and necrosis of bronchial wall with intraluminal necrotic cellular debris and inflammatory cells (400X) (C), 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).

Interalveolar space (Interstitial tissue) and blood vessels

Focal to diffuse thickening of interalveolar space was noticed in most pulmonary tissues due to oedema, 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 deposition of fibrinopurulent exudate (Figure 5C). Vascular changes included moderate to severe congestion of inter-alveolar and peribronchial blood vessels, hemorrhage and peri-vascular oedema. 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 oedema and hyperplasia of smooth muscles of some pulmonary blood vessels were also seen in few pulmonary tissues.

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).

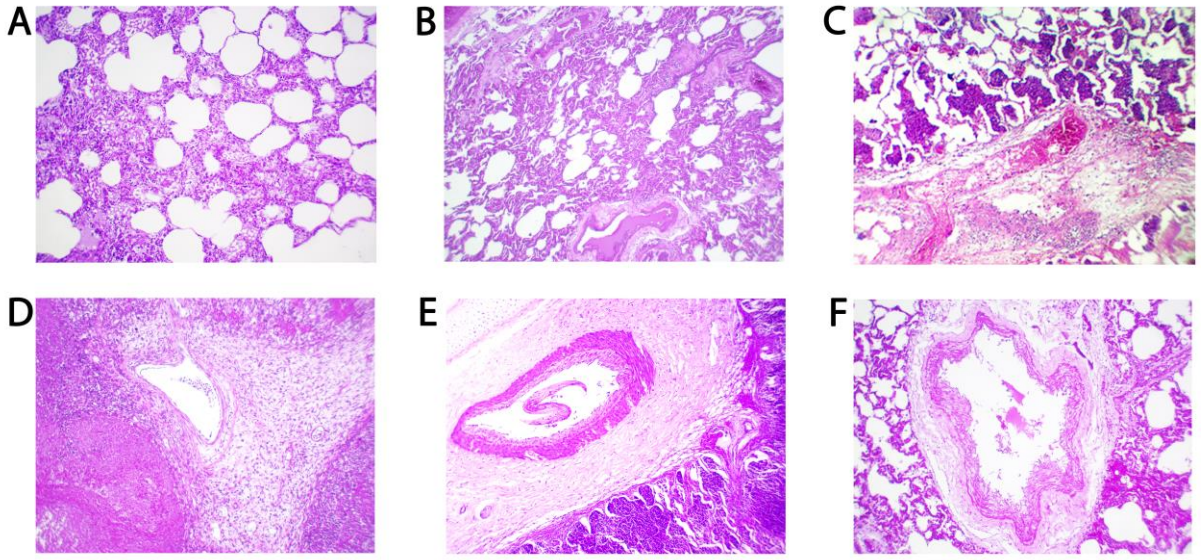

Figure 5 Lungs of calves infected by *P. multocida* Showing inflammatory cellular infiltration in the interstitial tissues mainly lymphocytes and histocytes (100X) (A), Atelectasis of pulmonary alveoli due to the interstitial pneumonia (100X) (B), interlobular septal thickening due to fibrinopurulent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

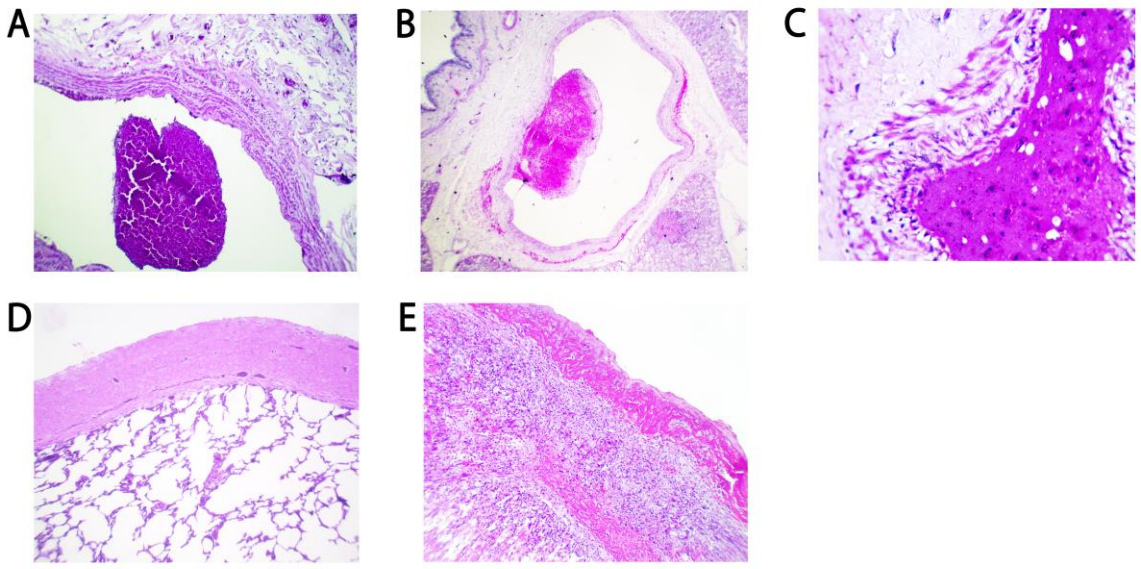

Figure 6 Vascular and pleural photomicrographs of calves infected with *P. multocida*. Lungs displayed intravascular pyemic emboli (400X) (A), thrombus (100X) (B), and degeneration of T. media and endothelial cells of large pulmonary blood vessels (400X) (C). Pleura were severely thickened (100X) (D)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

Discussion

BRD is a multi-factorial disease that has a catastrophic effect on 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 affect 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 et al., 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 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 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 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 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 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, Yaman et al., 2018).**

Microscopically, 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 et al., 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, 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., 1997a).**

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).**

Conclusions

P. *multocida* is one of the main causes of pneumonia and BRD in calves in 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 identification of pasteurella infection in pneumonic lungs. Due 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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