# IMMUNOPATHOLOGICAL CHANGES IN SHEEP EXPERIMENTLY INFCETED WITH *MYCOPLASMA OVIPNEUMONIAE* BY INTRANASAL AND INTRATRACHEAL ROUTES

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## ABSTRACT

**Mycoplasma ovipneumoniae (M. ovipneumoniae) is a type of contagious bacteria have respiratory clinical signs; nasal mucinous discharge, sneezing, coughing, dullness, and in some cases with diarrhea and the necropsy findings represent lesions of pneumonia in different stages; pulmonary edema and hyperemia, consolidation, hemorrhage and hepatization. that causes atypical pneumonia and pleuropneumonia in small ruminants, it is belongs to a group of bacteria named Mollicutes which characterized by its minute genome size and perpetually devoid of the cell wall. The aim of studies indicates the bacterial, molecular, immunopathological and immunohistochemical investigation in sheep with two experimental routs of infection in two parts. The current isolate of Mycoplasma ovipneumoniae was obtained from respiratory tract swabs of sheep in Basrah abattoir and then cultured in PPLO broth and agar and detected genetically by 16SrRNA and GOP3/MGSO. Nine sheep were divided into G1 and G2 groups inoculated with My. ovipneumoniae 5ml (1X107 CFU/ml) intranasal and intratracheal, G3 control group. IgGs levels on day 30 were high in tow groups. Fibrino-suppurative tracheitis and interstitial bronchopneumonia were prominent in gross appearance. Microscopically: necrosis of ciliated pseudostratified epithelium with inflammatory cells. It could be concluded that My. ovipneumoniae was a primary causative agent of pleuro-bronchopneumonia in sheep.**

**Keywords: respiratory infection, gene detection, immunoglobulins, small ruminant.**



# التغيرات المناعية المرضية في الاغنام المصابة تجريبيا بجراثيم *Mycoplasma ovipneumoniae* داخل الانف والرغامي

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## المستخلص

أن البكتريا Mycoplasma ovipneumoniae نوع من أنواع البكتريا المعدية حيث أنها تسبب أعراض تنفسية سريرية مثل ارتشاحا انفية مخاطية والعطاس والكحة والخمول والإسهال في بعض الحالات المرضية وأما الملامح التشريحية المرضية أي ما بعد الموت ستظهر المراحل المختلفة من التهاب النسيج الرئوي مثل وجود السوائل وورود الدم الى منطقة الالتهاب كالتصلد النسيجي وكذلك ظهور النزف ومناطق التكبد الرئوي وهذ ا معناه ان البكتريا تسبب ذات الجنب النموذجي أوذات الرئة الجنبي التي تصاب بها فصيلة المجترات الصغيرة كالأغنام والماعز. إن بكتريا Mycoplasma ovipneumonia من عائلة Mollicutes التي تتميز بصغر الجينوم الخلوي وفقدانها للجدار الخلوي ومن هذه الأصابات تم الحصول على العزلة الحالية *Mycoplasma ovipneumoniae* من مسحات للجهاز التنفسي لأغنام مذبوحة في مسلخ البصرة والمزروعة في مرق PPLO والأكار الصلب وتم الكشف عنها بواسطة جينات SrRNA16 وGOP3 / MGSO. تجريبيا تم تقسيم تسعة أغنام إلىمجموعة الحقن الأنفي (G1) مجموعة الحقن عبر القصبة (G 2) واصابتها بـ ($1X10^7$ CFU /ml) *Mycoplasma ovipneumoniae*داخل الأنف والرغامي، G3 مجموعة سيطرة. وبعد ذلك تم قيمIgGs كانت مرتفعة في المجموعتين المصابة في اليوم 30. عيانيا لوحظ التهاب الرغامي الليفيني القيحي والالتهاب الرئوي القصبي الخلالي. مجهريا انسلاخ ونخر الظهارة العمودية المهدبة الكاذبة للجهاز التنفسي مع نضخة التهابية. يمكن الاستنتاج أن المايكوبلازما الرئوية كانت المسبب الرئيسي السائد لمرض الجنبة القصبية الرئوية في الأغنام.

الكلمات المفتاحية: الاصابات التنفسية، كشف الجينات، مستوى الاضداد، المجترات الصغيرة



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## INTRODUCTION

*Mycoplasma ovipneumoniae* (*My. ovipneumoniae*) is more strongly associated with pneumonia than previously targeted pathogens (16) which it has small wall-less bacteria of the class Mollicutes and cause many diseases arranged by the World Organization for Animal Health (OIE) including atypical pneumonia, contagious agalactia (CA), chronic respiratory arthritic syndrome (CRAS), contagious caprine pleuropneumonia (CCPP) (11) and infection in chicken (4) most pathological lesions congestion and edema with red and gray hepatization, hemorrhages in the trachea and tracheal bifurcation, and pleurisy (5). Infection probably starts in lambs shortly after birth having spread from carrier ewes, from which the mycoplasma can be recovered repeatedly by swabbing the nasal mucosa (10).*My. ovipneumoniae* causes lethal pneumonia in sheep as it is the infectious agent in ovine pleuropneumonia (34) and highly contagious in almost every flock, causing major economic losses in the ovine industry worldwide (33). Compared to other pathogenic mycoplasmas, studies on *My. ovipneumoniae* are restricted by many aspects including the lack of the entire genomic sequence (8). Histopathology of M. ovipneumoniae-infected lung tissue revealed chronic bronchopneumonia known as "atypical" or chronic non-progressive pneumonia, as well as extensive hemorrhagic pneumonia with extensive alveolar hemorrhage of naturally-infected lambs' lungs, which appears greyish with red areas of collapse varying in size. The key histological hallmarks include interstitial thickness caused by septal cell proliferation, monocyte buildup in alveoli, and lymphoid hyperplasia surrounding bronchioles and arteries (15). The microscopic lesion s in the lungs were similar to those described by to (36) The principle e lesions were thickening of the alveolar septa by proliferative changes in the alveolar walls, hyperplasia of bronchiolar epithelium and alveolar atelectasis with minimal exudative changes and intraluminal aggregates of neutrophils (13). There was activation of intrapulmonary lymphoid tissue that formed peribranchial cuffs and Nasal swabs, broncho-alveolar lavages, pleural fluid, lung and mediastinal node culture yield variable results, indicating the need for new culture media (29), The study aimed to investigate the immune-pathological changes post experimental infection with *Mycoplasma ovipneumoniae* in sheep post molecular detection by conventional PCR (2).

## MATERIALS AND METHODS

### Bacterial isolation and characterization

*My. ovipneumoniae* was isolated from nasal swabs cultured on PPLO broth and agar 37°C, 5% CO2 for 5-7 days. The *16S rRNA* gene was amplified and sequenced 8F (5-AGAGTTTGATCCTGGCTCAG-3) and 1544R (5AGAAAGGAGGTGATCCAGCC-3) primers giving a1536 bp product. The PCR conditions for amplification were 95 ºC for 3 min, followed by 35 cycles of 95 ºC for 45 s, 55 ºC for 45 s and 72º C for 1 min, and final elongation at 72 °C for 5 min.

**IgG levels :** ELISA kit (www.icllab.com) was performed for quantifying IgG in infected and control groups according to the manufacturer's instructions. The optical density (OD) values were read at 450 nm using a plate reader (Wellkang Ltd., London, UK).

**Preparation of infective dose:** The bacterial inoculation prepared according to their growth in PPLO agar (23) and (26) were determined the colony forming unit per milliter in 5ml dose were inoculated Intranasaly and intratrachealy.

**Experimental design:** Nine adult sheep (6-10) months old and weight 21.28 Kg divided randomly and equally into three groups: **G1** and **G2** inoculated 5ml ($7X10^7$ CFU/ml) intranasal (I/N) (28) intratracheal I/T (24) respectively. **G3** control group. Gross changes of respiratory system and preserved (nasal septum, trachea and lung) in 10% formalin then tissue sections stained with Hematoxylin and Eosin to examined under the light microscope (25).

## RESULTS AND DISCUSSION

**Culture growth and Gene expression:** The egg fried colonies were the characteristic of *Mycoplasma* (21), identification was done by specific primers evaluated by conventional PCR (24) as done with other bacterial species (14). Six samples were tested by PCR with primers 8F and 1544R, a 1536 bp region of *16S rRNA* gene (Fig. 1) and specific primers

(GPO3) F (MGSO) R a 281 bp (Fig. 2), indicate the genetic assortment of *My. ovipneumoniae* was fairly high.

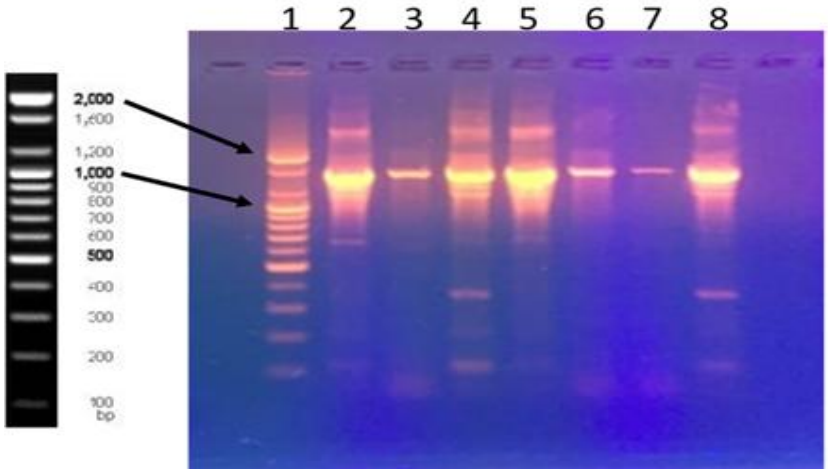


**Figure 1. Amplification of *16S rRNA* gene of *Mycoplasma ovipneumoniae* fractionated on 1% agarose gel stained with Eth.Br. Lane1: DNA marker (100bp). Lane 2-8: amplicons of seven samples of *Mycoplasma ovipneumoniae* with approximately 1536b**

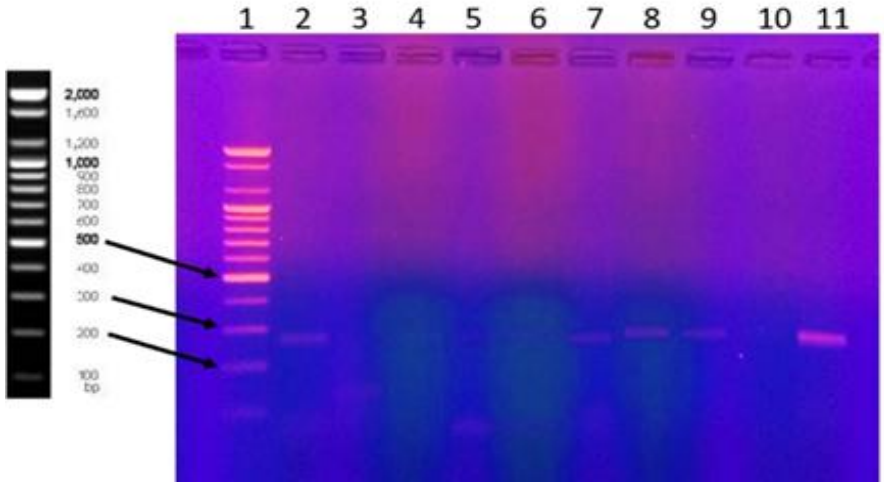


**Figure 2. Ladder marker represents the amplification of GOP3/MGSO gene of unknown bacterial species fractionated on 1% agarose gel electrophoresis stained with Eth.Br. M: 100bp. (Lane 1 resemble 281bp PCR products).**

*Mycoplasma* species not enough by PPLO growth culture they selected their isolates by detection 16SrRNA gene (14) due to wide spreading of these pathogens in world including Iraq regions the current result was the first in Basra province, Iraq to isolate *Mycoplasma* from animals with respiratory distress as occurred with other bacterial species. The experimental infected sheep with *My. ovipneumoniae* represents respiratory signs agreed with observations of (37), (38) and (17) their bacterial isolates on PPLO give *Mycoplasma* growth from swab samples in both groups and identified as *Mycoplasam ovipneumoiae* by molecular analysis of their genome as the researchers recommended polymerase chain PCR.

**IgG titers:** Serum antibody ELISA performed before intranasal and intratracheal inoculation found immunoglobulin levels of 31.64±4.83 and 6.26± 4.01ng/ml, respectively. On day 30, immunoglobulin levels were significantly higher in intranasal and intratracheal inoculation at 61.73±18.41 (t-statistic: 2.738; Standard error: 10.989; 95% CI: -0.4196 to 60.5996; DF: 4; P = 0.05) and 137.82±64.15 ng/ml (t-statistic: 3.542; Standard error: 37.138; 95% CI: 28.4480 to 234.6720; DF: 4; P = 0.02) respectively (Tab.1). The difference between intranasal and intratracheal inoculation routes concerning IgG concentration was considered to be statistically non-significant (t-statistic: 1.97; Standard error: 38.56; 95% CI: -30.97 to 183.145; DF: 4; P = 0.12) (Fig. 3).

**Table 1. IgG levels before and after infection with *Mycoplasma ovipneumoniae* intranasal and intratracheal in sheep**

| Type of inoculation | | IgG (ng/ml) Mean ±SD | t- test P value | SE | 95% CI |
|---|---|---|---|---|---|
| Intranasal I/N | Before | 31.64 ± 4.83 | 0.05 | 10.9897 | 0.4196 to 60.5996 |
| | After | 61.73±18.41 | | | |
| Intra tracheal I/T | Before | 6.26 ±4.01 | 0.02 | 37.138 | 28.4480 to 234.6720 |
| | After | 137.82±64.15 | | | |

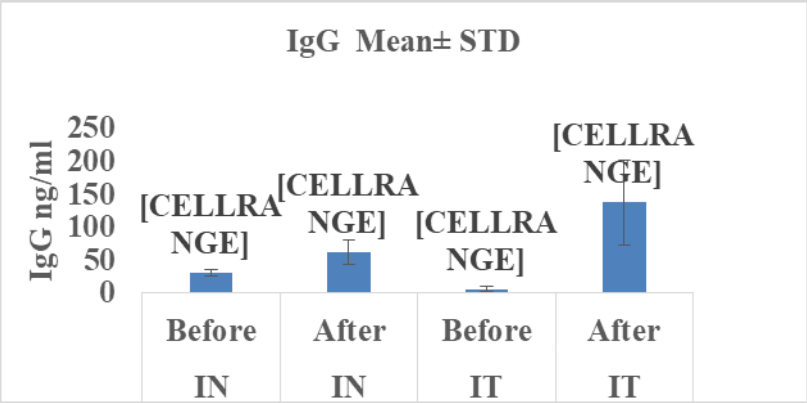


**Figure 3. IgG levels before and after infection with *Mycoplasma ovipneumoniae* intranasal and intratracheal in sheep**

**Pathological examination**

**Gross findings:** The paranasal sinuses of experimental infection after 4 weeks (trachea and lungs) showed different lesions including congestion of mucus membrane lined nose with fibrinous material (Fig. 4) and other findings were tracheal congestion with frothy material and ecchymotic hemorrhage with gray consolidation areas mainly at antero-ventral portion of lung (Fig. 5).

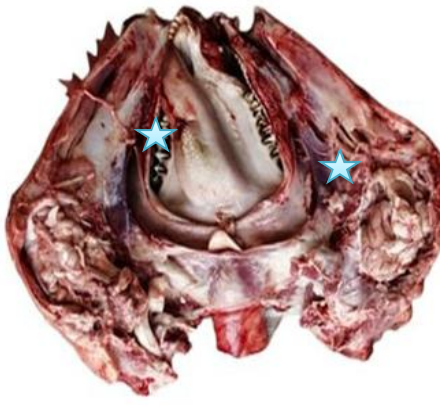

**Figure 4. The nasal cavity in G1 group shows presence of congestion with fibrinous material in both sided of intranasal mucosa (star).**

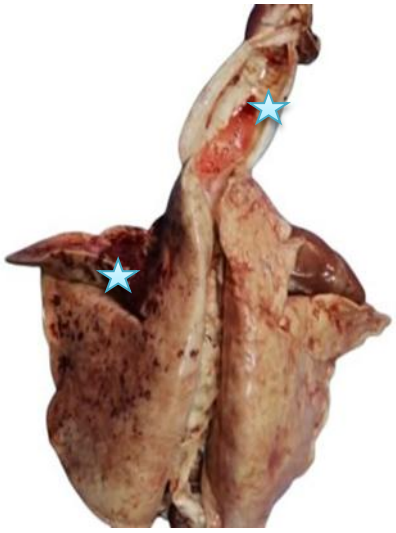

**Figure 5. The trachea and Lung from G1 group shows congestion of trachea with frothy fluids (star) and consolidation in right lung marked ecchymosis (star).**

**Histopathology findings**

**Intranasal infection:** The paranasal sinuses showed thickening of inflamed mucosa, clumping and lost cilia of pseudostratified columnar epithelium, desquamation and sloughing of epithelial cells (Fig. 6). Hyperplasia of goblet cells and mucus glands in submucosa surrounded by neutrophils and mononuclear cells (lymphocytes and macrophages, plasma cells) also perivascular cuffing. Bronchitis and bronchiolitis represented by mucopurulent exudate in their

lumen neighboring alveoli were collapsed and atelectasis (Fig. 7). Interstitial lesion from fibroplasia of pleura and thickening of inter-alveolar septa, emphysema and focal peribronchiolar aggregation of inflammatory cells (Figs. 8, 9).

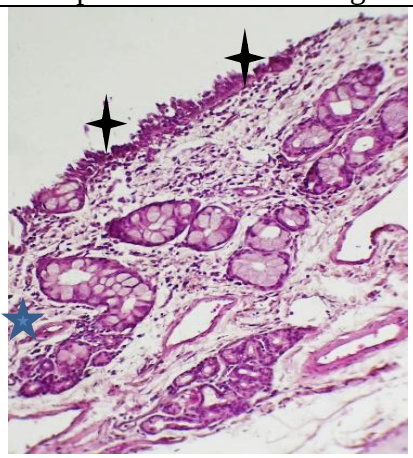

**Figure 6. Paranasal sinus membranes section showing multifocal epithelial sloughing (stars), loss of cilia and Hyperplasia (star) of mucus glands. (H&E stain, 100X).**

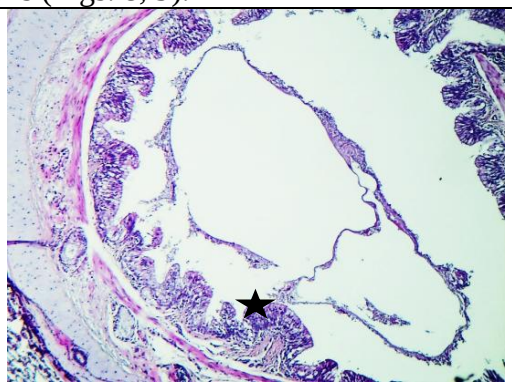

**Figure 7. Tracheal section showing moderate epithelial hyperplasia (star) of mucosa with mucinous exudate in lumen (star). (H&E stain, X100).**

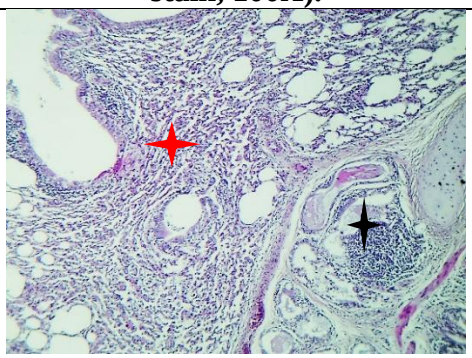

**Figure 8. Histopathological section of Lung; shows focal MNCs and PMNs (red star) in submucosa of bronchus and neighboring atelectasis alveoli (star). (H&E stain, 100X).**

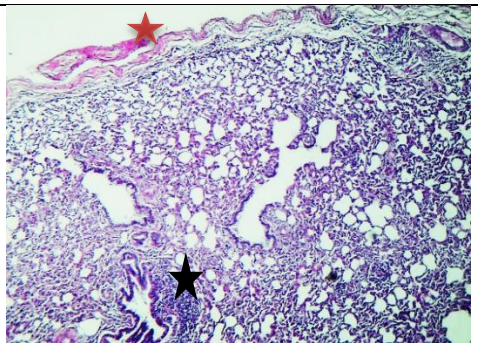

**Figure 9. Histopathological section of Lung shows; peribronchiolar lymphocytic aggregation (red star) associated with fibrosis of the visceral pleura (star). (H&E stain, X40).**

**Intratracheal infection**

Trachietis was the predominant inflammatory changes; sloughing epithelium, lost cilia with the necrotic-hyperchromatic epithelial cells and infiltration of mononuclear cells (MNCs) with few polymorphoneutrophils (PMNs) extended to subepithelial and submucosa with evidence of inflammatory edema (Fig.10). The bronchi and bronchioles contained mucinous and fibrinosuppurative exudate (Fig. 11), in lung infiltration **of** inflammatory cells (Fig. 12). Focal aggregation and diffuse inflammatory cells perivascular and peribronchiolar (Fig. 13). The studies identified that *My. ovipneumoniae* is a common disease of sheep in all major sheep-producing countries, with inflammatory changes as in microscopic features; peribronchiolar lymphocytic infiltrations are observed with diffused nonsuppurative pleuritis (26), (1) and (20) compatible with present histopathology in second group presence of muco and fibrino-suppuartive exudate in the lumen of bronchi and bronchioles attached to superficial necrotic mucosa (38). *My. ovipneumoniae* showed that this bacterium caused direct cell death via the reactive oxygen species (ROS) and the mitogen-activated protein kinase (MAPK) signaling–mediated mitochondrial apoptotic pathway (35), (3), (22) and (30). Moreover, the capsular polysaccharide caused the apoptosis of sheep airway epithelial cells via the c-Jun N-terminal kinase (JNK)/P38 MAPK

signaling pathway, that may explain the present results from fast and easily infection with *My. ovipneumoniae* faster than other organisms, suggesting might play an immunosuppressive role during infection in addition to causing airway cell apoptosis (9) and (12). Moreover, the infection with these pathogens promoted both transcription and translation of proinflammatory cytokine genes including interleukin, which reflected the pathology thought mainly from damage to the host immune response caused by mycoplasma infection (19), (7) and (20). Other studies have shown that a series of inflammatory reactions are caused by their lipid-associated membrane proteins (LAMPs) may facilitate adhesion to the host cell surface, thus facilitating subsequent host cell entry, leading to the host cell damage and death. (31) and (32), suggested *My.ovipneumoniae* which are etiologic factor in inducing chronic respiratory infections and asymptomatic disease in animals were suffering from the abdominal and thoracic fibrous adhesions in post mortem examination with presence of bloody fluids and inflammatory exudate, these infections rise the immune resistance and predisposed the mortal etiologic factor-like *Mannheimia hemolytica* to attach alveolar cells, proliferate, and secrete endotoxin, leukotoxin and capsular polysaccharide resulting in fibrin deposition in lungs and pleural cavity, peribronchiolar lymphocytic infiltrations are observed with diffused non-suppurative pleuritis (6)

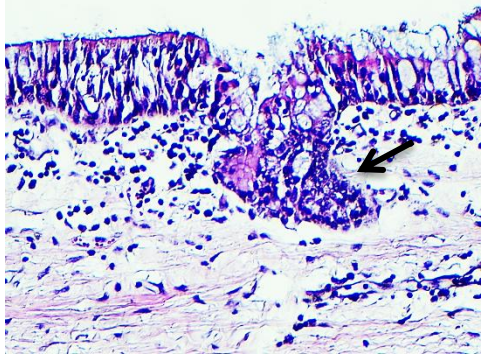

**Figure 10. Tracheal section showing thickening of the submucosal layer due to accumulation of edematous fluid and infiltration of MNCs. (H&E stain, X200).**

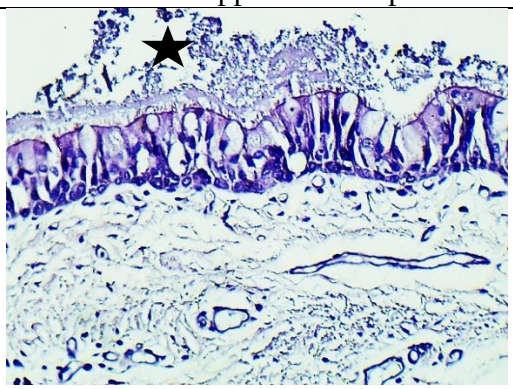

**Figure 11. Tracheal section showing focal loss of cilia and mucopurulent exudate inthe lumen (star). (H&E stain, X400).**

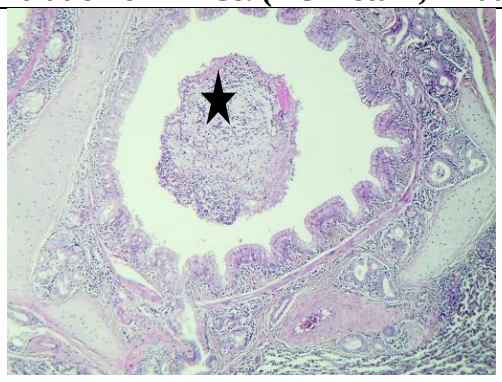

**Figure 12. Lung section showing a fibrinopurulent exudate (star) in the bronchial lumen. (H&E stain, X100).**

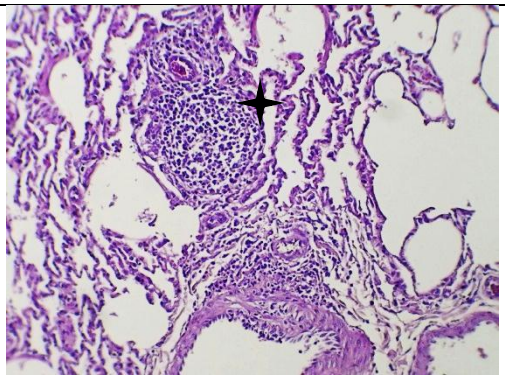

**Figure 13.Lung section showing emphysema and perivascular lymphocytic cuffing. (H&E stain, X200)**

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## DECLARATION OF FUND

The authors declare that they have not received a fund.

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