

DETECTION OF *MYCOPLASMA DISPAR* IN BOVINE RESPIRATORY DISEASE BY POLYMERASE CHAIN REACTION ASSAY IN SULAIMANIYAH CITY

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ABSTRACT

This study was aimed to identification and detection of *Mycoplasma dispar* by conventional real-time polymerase chain reaction (PCR) assays followed by routine hematoxylin & eosin technique to further confirmation of *Mycoplasma* species related to the lesions observed in diseased calves. Ten samples of infected lung tissue were gathered and fixed in 10% neutral buffered formalin and handled for making a microscopic slide after confirming *Mycoplasma dispar* infection by PCR. Histopathological examination of the lung revealed two types of pneumonia; chronic suppurative pneumonia in 60% of the cases and 40% of diffuse interstitial pneumonia. *Mycoplasma dispar* was confirmed based on genetic analysis. Slemani/2018 field isolate showed 99.84% homologies with four reference isolates of *Mycoplasma dispar* in NCBI GenBank. Phylogenetic analysis indicated a close relation to *Mycoplasma ovipneumoniae*, *Mycoplasma hyopneumoniae*, and *Mycoplasma flocculare*, often isolated from sheep and goats. Therefore, further study is crucial to identify Iraqi livestock's circulating *Mycoplasma* species related to respiratory diseases.

Keywords: , *Mycoplasma dispar*, Bovine pneumonia, Interstitial pneumonia, Suppurative pneumonia, PCR.

صالح

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الكشف عن *Mycoplasma dispar* في امراض الجهاز التنفسي البقري بواسطة فحص البوليميراز المتسلسل في مدينة السليمانية

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المستخلص

هدفت هذه الدراسة إلى تحديد واكتشاف تباين الميكوبلازما عن طريق مقاييسات تفاعل البلمرة المتسلسل التقليدي في الوقت الحقيقي (PCR) متبوعة بتقنية الهيماتوكسيلين والأبوزين الروتينية لمزيد من التأكيد على أنواع الميكوبلازما ذات الصلة بالآفات التي لوحظت في العجول المريضة. حيث تم جمع عشر عينات من أنسجة الرئة المصابة وتثبيتها في الفورمالين بنسبة 10% وتم التعامل معها لعمل شريحة مجهرية بعد التأكد من الإصابة بـ *Mycoplasma dispar* بواسطة PCR. كشف الفحص التشريحي المرضي للرئة عن نوعين من الالتهاب الرئوي. الالتهاب الرئوي القيحي المزمن في 60% من الحالات و 40% من الالتهاب الرئوي الخلالي المنتشر. على أساس التحليل الجيني تم تأكيد من الحالات بأنها مصابة بـ *Mycoplasma dispar*. حيث أظهرت العزلة الحقلية من السليمانية / 2018 تماثل 99.84% مع أربع عزلات مرجعية من *Mycoplasma dispar* في NCBI GenBank ، وأشار تحليل علم الوراثة إلى وجود علاقة وثيقة بالمفطورة الرئوية *Mycoplasma ovipneumoniae* و *Mycoplasma hyopneumoniae* و *Mycoplasma flocculare* وغالبًا ما تكون معزولة عن الأغنام والماعز. لذلك، من الضروري إجراء مزيد من الدراسة لتحديد أنواع الميكوبلازما المتداولة في الماشية العراقية والمتعلقة بأمراض الجهاز التنفسي.

الكلمات المفتاحية: *Mycoplasma dispar*، الالتهاب الرئوي البقري، الالتهاب الرئوي القيحي المزمن، الالتهاب الرئوي الخلالي المنتشر، PCR.

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INTRODUCTION

For the first time, *Mycoplasma dispar* was isolated and identified in the calves infected with pneumonia by Gourlay and Thomas (10) in England. *Mycoplasma dispar* is a common inhabitant of healthy cattle's upper and lower respiratory tracts. *Pasteurella multocida*, *Arcanobacterium pyogenes*, or *Mannheimia haemolytica* were found in 50 percent of the herds studied in several investigations, and bacterial agents of the syndrome, such as *Pasteurella multocida*, *Arcanobacterium pyogenes*, or *Mannheimia haemolytica*, coexisted with these instances (2). *Mycoplasma dispar* is one of the pathogenic organisms of bovine respiratory disease, resulting in significant financial losses worldwide (17, 19, 24). *M. dispar* infection was found recently in bovine populations suffering from respiratory disorders in Brazil (5) and Italy (3). *M. dispar*, like other *Mycoplasma* species, belongs to the *Mycoplasma* variety in the Mollicutes class. It has no cell wall. *M. dispar* is one of the most challenging mycoplasma species to culture *in vitro*, requiring a specific medium and being one of the slowest growing mycoplasma species. *M. dispar* can cause mastitis and mild pneumonia (17). This microbe is usually isolated from pneumonic calves' lungs and nasal swabs, although it can also be obtained from healthy calves (23). Overall, *Mycoplasma pneumonia* has been portrayed as "cuffing pneumonia" because lymphoid hyperplasia shows up around the airways and expands with time (21). *Mycoplasma dispar* is frequently related to alveolitis (26), in which neutrophils, macrophages, and edema liquid aggregate in the alveolar wall and spaces. Certain authors have described a chronic lymphocytic ("cuffing") pneumonia in which hyperplasia of peribronchial and peribronchiolar lymphoid tissue causes constriction of the airways, lumina, and pressure, as well as the collapse of adjacent pulmonary parenchyma (1). *Mycoplasma dispar* is commonly found in animals suffering from respiratory diseases, although it can also be found in healthy animals. This species has been isolated in several countries and is mainly spread from infected to healthy animals through respiratory secretions. Mycoplasmas

are picky, slow-growing microbes that grow in complex environments. As a result, molecular techniques are required to diagnose them (15). Mycoplasmas can cause actual disease in cattle herds coming about in critical negative financial impacts. Fast diagnosis will help in the control and prevention of the disease. Notwithstanding disease outbreaks, there are several possibilities regarding the source and spread of the disease across the herd. Despite slow results, culture gives a definitive isolate used for DNA extraction to study the source of contamination and relatedness of isolates to different organism strains. Polymerase chain response measures give quick symptomatic outcomes (20). The genomic data of *M. dispar* is restricted, as the genome succession of *M. dispar* reference strain ATCC 27140 was delivered in the NCBI information base in 2015 without any additional examination. Besides, the virulence factors and phylogenetic relationship of *M. dispar* remain indistinct. The role of *Mycoplasma dispar* as an active agent in bovine respiratory disease cases is ineffectively explained in the Kurdistan Region of Iraq. So, standard histopathological and molecular methods for diagnosing natural bovine pneumonic cases with *Mycoplasma dispar* related lesions were implemented in this study. The results will be helpful for additional studies on the pathogenic mechanisms and genetic characterization of *M. dispar* in our region.

MATERIALS AND METHODS

DNA extraction: According to the manufacturer's instructions, a genomic DNA extraction kit (GeneNet, Korea) was used to extract DNA from affected lung tissue. The isolated DNA was then kept at -20°C until the PCR experiment was performed.

PCR amplification

Using GeneNet PCR Premix, the PCR amplification process was carried out according to the manufacturer's instructions. The PCR primers were designed to detect *Mycoplasma dispar*'s 16S rRNA gene, with the forward primer ACTCCTACGGGAGGCAGCAGTA and reverse primer TGCACCATCTGTCACTCTGTAAACCTC to amplify 710bp (27). The primers were constructed by Macrogen (Korea). The PCR

reaction was performed with 5 µl of DNA template and 1 µl of 10 pmol forward and reverse primers. The reaction was then made up to a final volume of 20 µl with DEPC-H₂O. The thermal cycler (BIO-RAD, USA) parameters were; an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec; and a final extension phase run at 72°C for five minutes.

Electrophoresis and gel analysis

Running 5 µl of PCR product on an agarose gel (1%w/v) at 125 V for 40 minutes was used for electrophoresis. The gel was prestained with 10 µl of safe dye (0.4 g/ml) and photographed with a UV transilluminator (UVETIC, U.K). The amplicon sizes are measured using a 100-bp DNA ladder's migration pattern as a guide.

Sequencing the PCR products

Twenty µl of PCR product was sequenced from both primers (Macrogen sequencing service, Korea). Both sequences were aligned and trimmed using NCBI (bl2seq) then published in GenBank with accession number MK503977.

Sequence comparison and phylogenetic analysis: The sequence identity of *Mycoplasma dispar* strain Slemani/2018 isolates was confirmed by blast method at the NCBI Home page. The phylogenetic tree was constructed among *Mycoplasma dispar* strain Slemani/2018 isolate and all published *Mycoplasma* isolates showing significant alignments in NCBI. 1000 replicates of the original data were used to

Sample collection and histological technique

Samples from ten affected calves' lungs were taken from the farm with sterile tools and were

preserved with a 10% neutral buffered formalin. The tissues were processed by routine paraffin embedding technique. Sections of 5 µm thick sections were prepared and stained with routine hematoxylin and eosin (H&E stain) and examined under a light microscope for mycoplasma infection-associated histological changes (Olympus CX41, Japan) equipped with a digital camera (Olympus DP25, Germany).

RESULT AND DISCUSSION

PCR Result: Slemani/2018 field isolate showed 99.84% homologies with four reference isolates of *Mycoplasma dispar* in NCBI GenBank. Phylogenetic tree constructed based on 16 ribosomal RNA partial nucleotide sequence alignment of the 25 genomes (Figure 1), the isolates were distinctly divided into six groups: *Mycoplasma dispar*, *Mycoplasma ovipneumoniae*, *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, *Mycoplasma conjunctivae*, and *Mycoplasma mycoides*. The topology of the phylogenetic tree indicated that the field isolates Slemani/2018 is clustered within the group that contains *Mycoplasma dispar* reference isolates. To our knowledge, this is the first time the species *M. dispar* has been detected in Kurdistan/North Iraq. The vast majority of PCR examinations produced correct results in this study. The phylogenetic analysis based on partial length 16S rRNA sequences confirmed that *Mycoplasma dispar* is genetically related to *Mycoplasma ovipneumoniae*, *Mycoplasma hyopneumoniae*, and *Mycoplasma flocculare*. These species are often isolated from sheep and goats. This finding of genomic analysis follows a previous study that supports the result (4). The genetic relationship of *M. dispar* and *Mycoplasma*

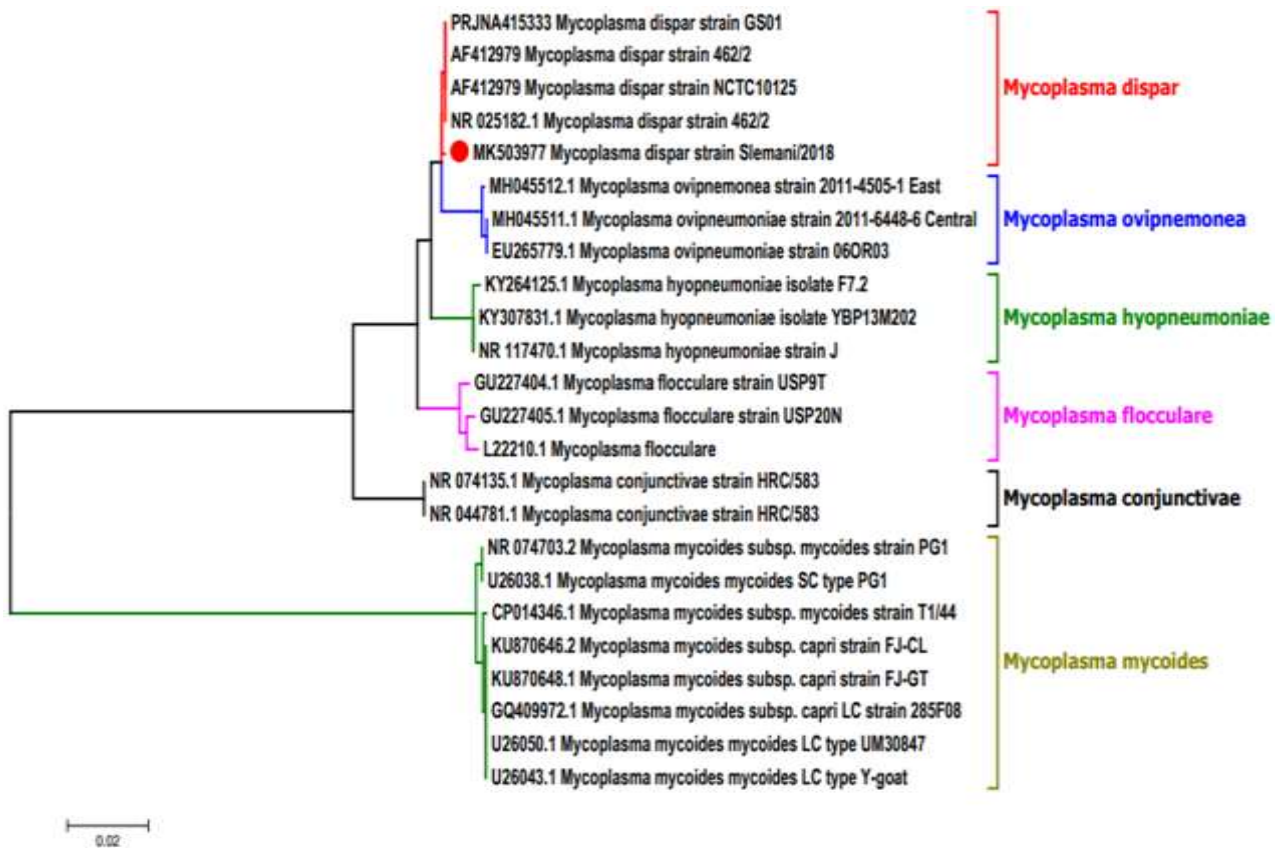


Figure 1. *M. dispar* nucleotide phylogenetic tree estimated with the Neighbor-Joining technique using MEGA version 10 based on DNA sequence of 16S rRNA gene. Bootstrap analysis with 1000 replicates confirmed the topology. The red circles represent the Sulaimani *M. dispar* sequence

mycoides is distinct. However, in this study, they had the same histopathological changes.

Histopathology Findings

Figure 2 demonstrates the histological analysis of lung lesions in calves that have been affected by *Mycoplasma dispar*. Two patterns of pneumonia were reported. Chronic suppurative bronchopneumonia was detected in 60% of affected calves. The histological features in Figure 1 shows pronounced chronic suppurative bronchopneumonia, focal bronchitis (blockage of bronchi by exudate accumulation), and bronchiectasis. The lumen of large bronchi, terminal bronchiole, and alveoli were obliterated wholly or partially by infiltration of neutrophil and mononuclear inflammatory cells, particularly macrophage and lymphocytes with massive mucopurulent exudate. Also, irregular bronchial dilation (bronchiectasis) with sloughing of lining epithelium was observed, as shows in Figures 2 a and c. Furthermore, the pulmonary vasculature displayed hyperemia filled with RBC associated with inflammatory cells; free RBCs are seen scattered through the

pulmonary tissue. The remaining 40% of lung lesions are characterized by chronic broncho-interstitial pneumonia, with lymphocytes, macrophages, and neutrophils infiltrating not only the terminal bronchioles and blood vessels but also the bronchiole walls with peri-bronchi fibrosis, thickening of alveolar septa due to proliferation of alveolar epithelial cells and hyperemia in all the pulmonary vasculature as show in Figure 3. Pleuritis was also noted more specifically in the suppurative bronchopneumonia type due to infiltration of neutrophils in the visceral pleura—also, fluid exudate and neutrophil inflammatory cells accumulated within the alveolar lumen, as in (Figure 4). *Mycoplasma dispar*, which stands apart as a significant microorganism of BRD, delivered a significant health condition for dairy cattle worldwide. It incurs extensive economic losses for beef herds and is one of the general reasons for calves' mortality with other microbial agents (9). By itself, *M. dispar* can cause gentle respiratory infection yet is frequently isolated from pneumonic lungs with different microorganisms (3).

Histopathological findings in our study reported chronic suppurative bronchopneumonia in maximum numbers of affected calves, with few interstitial bronchopneumonia cases, following the pathological lesions reported by Gabinaitiene, Siugzdaite and Zilinskas (9). Microscopic examination in the current study showed necrotic lung without caseation that disagrees with Toutenchi Mashhour, they detected caseated lung, while inconsistent with our data

regarding interstitial pneumonia in the affected lesion (25). There are thirteen recognized mycoplasma species in cattle, with *Mycoplasma bovis* and *Mycoplasma mycoides mycoides* being the most virulent and causing significant economic losses. (18). *M. bovis* is the source of various dairy cattle disorders, including pneumonia, mastitis, joint ache, otitis, keratoconjunctivitis, endocarditis, and neurological disorders (7).

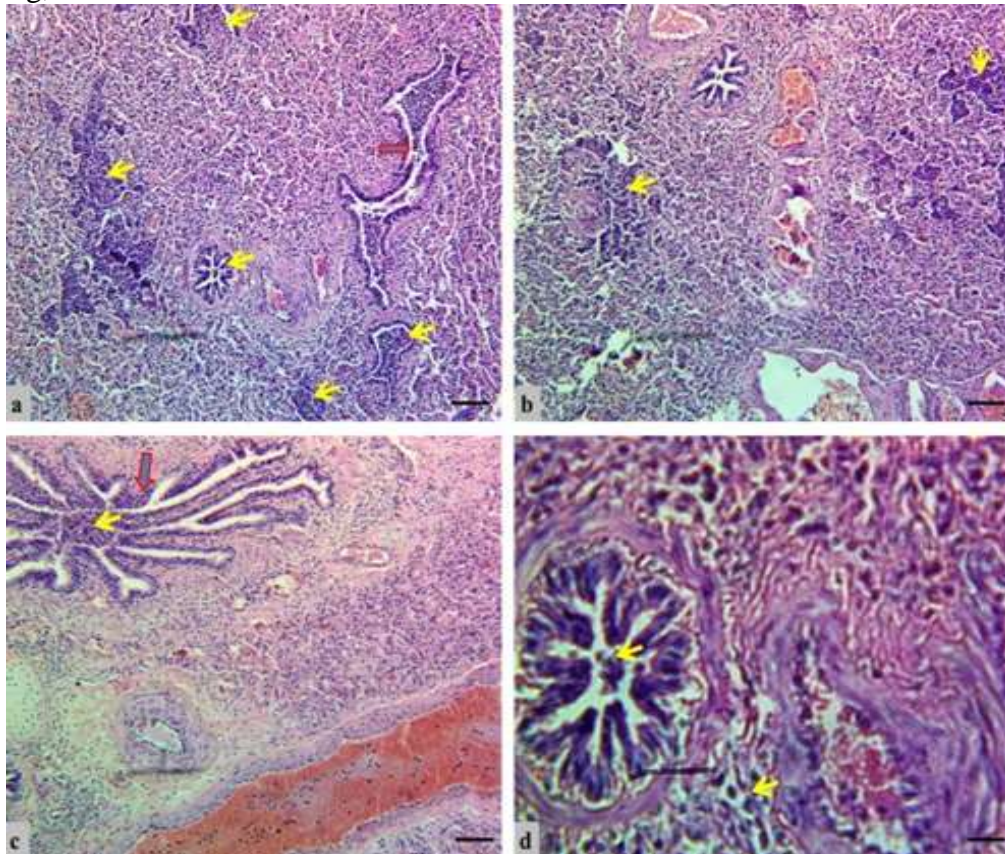


Figure 2. Microscopic section of the lung tissue affected by *Mycoplasma dispar*. a-d: Massive amounts of mucopurulent exudate and necrotic debris within a lumen of a large bronchus associated with marked epithelial sloughing and typical atelectasis (blue arrows). Present of suppurative exudate in bronchiole, bronchiole terminals, and lumina alveolar (yellow arrows). Hyperemia of pulmonary vasculature and scattering of free RBCs through the pulmonary tissue (H&E stain, a-c 50µm, and d 20 µm).

The lung sample revealed an exudative catarrhal-purulent inflammation of the bronchi/bronchiole with lymphohistiocytic cell proliferation associated with the bronchial wall. The histopathological findings followed two earlier studies (8, 12) while disagreeing with other reports stating that *Mycoplasma bovis* infection in cattle is related to extensive consolidation of caseous necrosis foci (11, 16). Occasionally, the lesions are accompanied by multiple necrotic foci, abscesses, and necrosis in some infected calves (6, 13) that agree with

our findings in *Mycoplasma dispar* infection. During our histological study, about 40% of the lungs section showed diffuse chronic bronchointerstitial pneumonia, which disagrees with a former report in which 72.7% of *Mycoplasma bovis* cases showed foci of chronic bronchointerstitial pneumonia (22). Furthermore, a study by Khodakaram-Tafti and Lopez (14) showed necrosis and suppurative lesions and areas of coagulative necrosis.

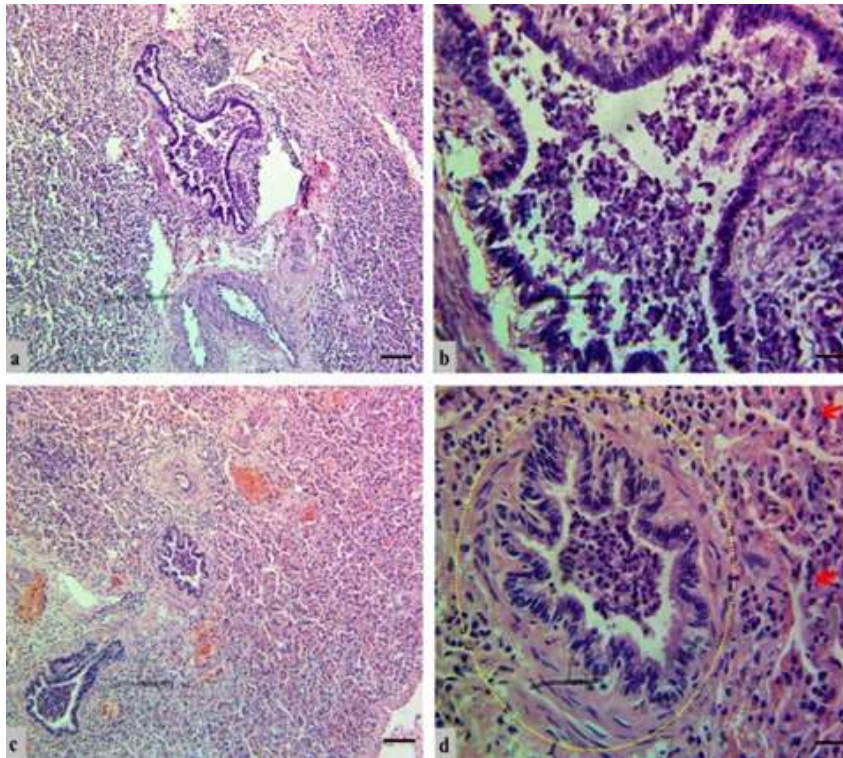


Figure 3. Microscopic section of the lung tissue affected by *Mycoplasma dispar*. a-d: Marked epithelial sloughing of a large bronchus lining epithelium associated with large quantities of mucopurulent exudate and necrotic debris in their lumen. Peri-bronchiolar infiltration of inflammatory mononuclear cells and fibrosis with mucus exudate in their lumen (yellow ring). The section shows thickening of alveolar septa due to alveolar epithelial cell proliferation (red arrows) and pulmonary hyperemia (H&E stain, a-c 50 μ m, and d 20 μ m).

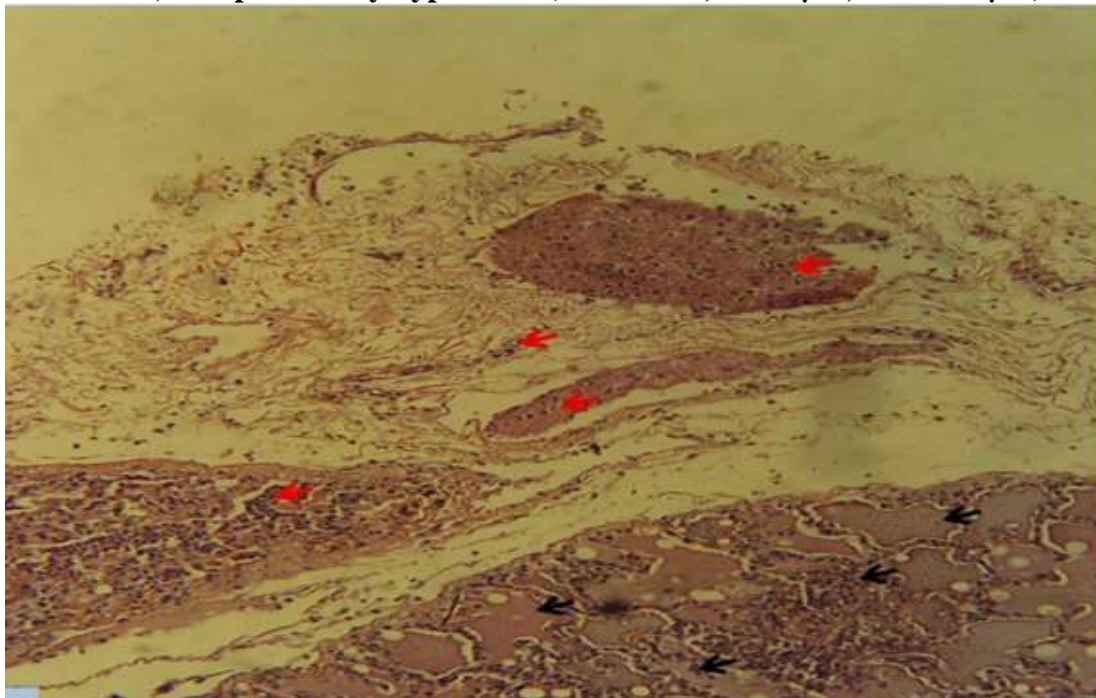


Figure 4. Microscopic lung tissue section that has been affected by *Mycoplasma dispar*. mild neutrophil infiltration in the visceral pleural and presence of fluid exudate with neutrophil infiltration in the alveolar lumen as indicated by black arrows (H&E stain, 50 μ m).

CONCLUSION

In the cattle population of Iraq's Kurdistan area, *M. dispar* causes pulmonary infections. According to the phylogenetic study,

Mycoplasma ovipneumoniae, *Mycoplasma hyopneumoniae*, and *Mycoplasma flocculare* are all isolated from sheep and goats.

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