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Original Article

Pathological and Molecular detection of *Mycoplasma ovipneumonea*e in Sheep, Basrah Province, Iraq

Waheed Khudiar Khareeb, Z[1 *], Ismail Ibrahim, Z[2], Ali Abdullah, F[3]

1. Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Basrah, Basrah, Iraq
2. Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
3. Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq

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Corresponding Author: pathovet.basra2015@gmail.com

Abstract

Mycoplasma ovipneumonea (*M. ovipneumonea*) are microorganism's causes atypical pneumonia in (sheep and goat). *Mycoplasma* is isolated frequently from pneumonic cases (lung, trachea, and nose) of sheep but can also be found in the respiratory tract of healthy sheep. This study aimed to isolate, identify, and pathological examination of *M. ovipneumonea* in sheep. Samples in the current study were collected from sheep of both sex and 6-10 months of age in Basrah slaughterhouse, suffering from respiratory signs associated with ocular, nasal discharge, and coughing. Nasal swabs were collected from the nose before slaughtering; other swabs were collected from the trachea and bifurcation of bronchus for bacterial isolation on PPLOs. Tissue specimens are frozen for DNA gene-based PCR analysis and for preparing paraffin blocks for histopathological examination. The bacterial cultures revealed isolates of *Mycoplasma* were positive on (PPLO) broth with agar from the morphological colonies of *Mycoplasmaovipeumonea* "fried egg" type colony morphology. PCR results revealed the 16S rRNA gene of *Mycoplasma sp.* The appearance revealed different stages of pulmonary changes like respiratory congestion, edema, and hemorrhagic spots on the surface of the lungs, and their air passages contained inflammatory exudate. The microscopic lesions represent acute fibrinous-suppurative broncho-interstitial pneumonia. *M. ovipneumoniae* was a prevalent respiratory infectious disease in Iraqi's sheep-Basrah province with frequent bacterial isolation, pneumonic pathological changes in animals suffer from different respiratory manifestations.

Keywords: *Mycoplasma ovipneumonia;* pathology; PCR; sheep

1. Introduction

Pneumonia is a multifactorial disease that interacts with different microbial agents. It is well documented that factors such as host defense mechanism and environmental factors, climate conditions, and transportation stress may increase the prevalence of this disease (1, 2). Pneumonia and pleura-pneumonia due to *Mycoplasma sp*. infections can cause serious health problems and economic losses in the small ruminants' industry (3, 4). This organism belongs to the Mollicutes class that has no genetic ability to produce cell walls (5). WHO recorded that the epidemiology of Mycoplasmas infection is herd problems with high morbidity. It also affects small ruminants of arid zones and addresses typical pneumonia caused by *Mycoplasma ovipneumoniae* with other microorganisms in the domestic small ruminant respiratory syndrome and infectious keratoconjunctivitis. The host, infectious agent, and environment are factors that create a complex interaction in the pathogenesis of the disease (1, 5). The variant clinical respiratory symptoms from mild to severe pneumonia and sudden death may relate to differences in strain virulence, host immune response,

and secondary pathogens. These pathogens can induce polymicrobial pneumonia, so outbreaks of severe *M. ovipneumoniae*-related pneumonia have been reported when domestic sheep were moved into higher density housing conditions or commingled. This organism could colonize and attach the ciliated respiratory epithelium resulting in decreased mucociliary apparatus function (3, 5). The pathological findings mostly showed in cranial lobes well demarcated consolidated areas affecting one or several lobules, colored red-brown to purple-grey and had a firm, meaty consistency; the cut surfaces varied from dark red and edematous to greyish and firm, occasionally with pale grey areas, multifocal abscesses were observed (6). The apoptosis induced by *Mycoplasma ovipneumoniae* in epithelial culture prepared from the bronchial epithelial cells of Ningxia Tan sheep (Ovis aries) by the production of different reactive oxygen species (ROS), methane dicarboxylic aldehyde (MDA) and anti-oxidative enzymes, as well as the mitochondrial membrane potentials, cytochrome C release, and activities of ERK and caspase signaling pathwaysi. The molecular analysis by PCR detects *M. ovipneumoniae* from animals with pneumonia by swabs from the significant bronchi and/or takes a sample of consolidated lung tissues, other swabs from the nose, middle ears, or sinuses, mainly if purulent exudate is seen. Infections related to Mycoplasma go away on their own without any medical intervention; that is when the symptoms are milder. In case of severe symptoms, a Mycoplasma infection is treated with the help of antibiotics like erythromycin, azithromycin, clarithromycin, or erythromycin (1). This study aimed to investigate the molecular prevalence of *Mycoplasma ovipneumonia*e from clinical cases in sheep.

2. Materials and Methods

2.1. Study Design

Ninety sheep 6-10 months old in a slaughterhouse in Basra city, Iraq, from (February and October/ 2020) were clinically evaluated for respiratory function; temperature, nasal discharge, cough test, and chest auscultation were also adopted (1).

2.2. Clinical Samples Collection

Ninety nasal and broncho-bifurcation swabs were collected from the diseased sheep in the slaughterhouse of Basra city. The swabs were collected in cotton swab tube PPLO broth and then transmitted in an icebox as early as possible to the Laboratory department at the University of Basra/ College of Veterinary Medicine for isolation and identification. The genetic investigation was done to identify and classify the local isolate from Mycoplasma species (2).

2.3. Characterization of *Mycoplasma ovipneumonea*

These organisms were isolated and identified as described previously (1) each nasal sample was swabbed on PPLO broth of pH 7.2 and then incubated at 37°C for two days. According to manufacture instructions for media culturing, which are specific for the growth of *Mycoplasma* in broth or agar of PPLO, then identify and characterized by molecular investigation for 16S –protein gene expression for bacterial detection from the respiratory swab samples. Then, they were closely observed for their morphology in a CO2 enriched environment in Oxoid Anaerobic Jars (Thermo Fisher Scientific, U.K.) with Oxoid CampyGen /37°C / 3-7 days, then cultured on solid PPLO media as in (1).

2.4. Molecular Detection of Isolates

For PCR amplification, 30–100 ng of template DNA and 20–40 µM primers were used.DNA was extracted from swabs using a DNA extraction kit (HiMedia, India) and stored in an elution buffer at -20˚C until use. The 16S rRNA gene was amplified and sequenced using the 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1544R (5'-AGAAAGGAGGTGATCCAGCC-3') primers (7), giving a 1536 bp product. The conditions for amplification were 95 'C / 3 min, followed by 35 cycles of 95 °C / 45 s, 55 °C / 45 s, and 72 °C / 1 min, and final elongation at 72 °C / 5 min. The Pathogen watch platform was used to confirm the identity of the *Mycoplasma*

*Ovipneumonea*isolates generated from the WGS data. The genomes of the strains were visualized using the GView Server.

The National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline

(PGAP; version 4.3) and Rapid Annotation using Subsystem Technology (RAST). The phylogenetic tree was constructed using MegAlign by the Neighbor-joining method based on ClustalW.

2.5. Histopathological Examination

After necropsy of sheep, the specimens from bronchus and lung were fixed in 10% formalin for 72 h. The histopathological preparation from processing through graduated ethyl alcohol (70%, 80%, 90%, 100% twice), then clearing twice with xylene. Blocking by liquid paraffin 56 º C then sectioning by microtome at 5µm. Then staining was performed with Hematoxylin and Eosin (1, 8).

3. Results

3.1. Bacterial Isolation

The positive results of *Mycoplasmas* from sheep (6-10 months aged) were obtained on PPLO broth supplemented with 20 ml of Horse serum, Yeast extract (25% w/v), 25 mg Thallous acetate, and Penicillin (2000 units) for two days. On PPLO agar, mycoplasmas isolated had "fried egg" type colony morphology (Figure 1). All isolates were identified by conventional PCR technique and histopathological examinations.

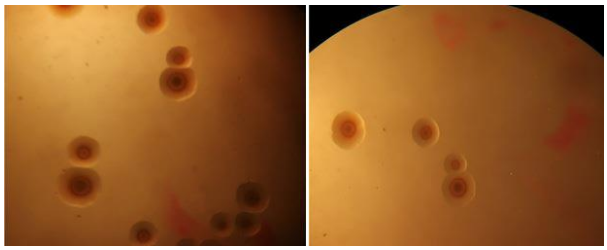

Figure 1. Fried egg colonies of *Mycoplasma ovipneumonia* on PPLOs

3.2. Molecular Detection

All growing bacterial colonies on PPLOs were positive for 16S protein, and the sequences revealed the genotyping of *Mycoplasma ovipneumonia* (Figure 2).

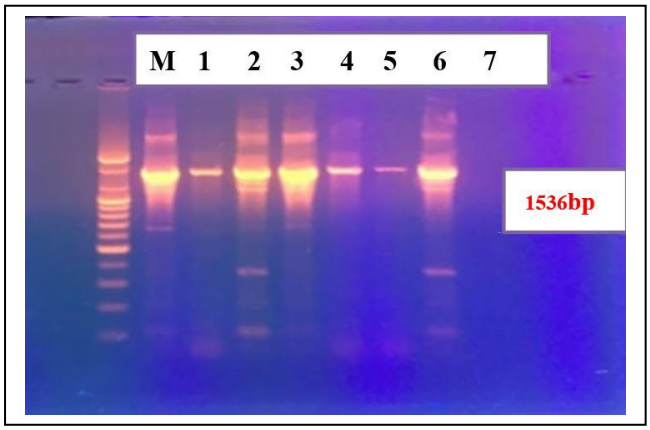

Figure 2. Ladder marker represents the amplification of 16s rRNA gene of unknown bacterial species fractionated on 1% agarose gel electrophoresis stained with Eth. Br. M: 100bp. (Lane 1 resembles 1536bp PCR products) (9)

3.3. DNA Sequencing and Sequence Alignment

M. Ovipneumonea species identification was confirmed by DNA Sequencing and Sequence Alignment. PCR implications were sent to the Macrogen company laboratory in Korea. Assembling DNA sequences in both directions using forward and reverse of the same primers used in PCR yielded a fragment containing 1536 bp. The phylogenetic tree was based on the 16s rRNA gene sequence of *M. ovipneumoniae* isolate of Iraq with standard strain and others *M. ovipneumoniae* published in GenBank (Figure 3). A network of *M. ovipneumoniae* 16S rRNA haplotypes of isolates of Iraq showed in figure 4.

3.4. Clinical and Gross Manifestation

The post-mortem examination of carcasses previously have clinical respiratory signs like fever (in some cases), cough, and nasal discharge (Figure 5), mostly appeared pneumonic lungs with different stages as colored shape (Figure 6A), with hemorrhagic areas and congested trachea (Figure 6B).

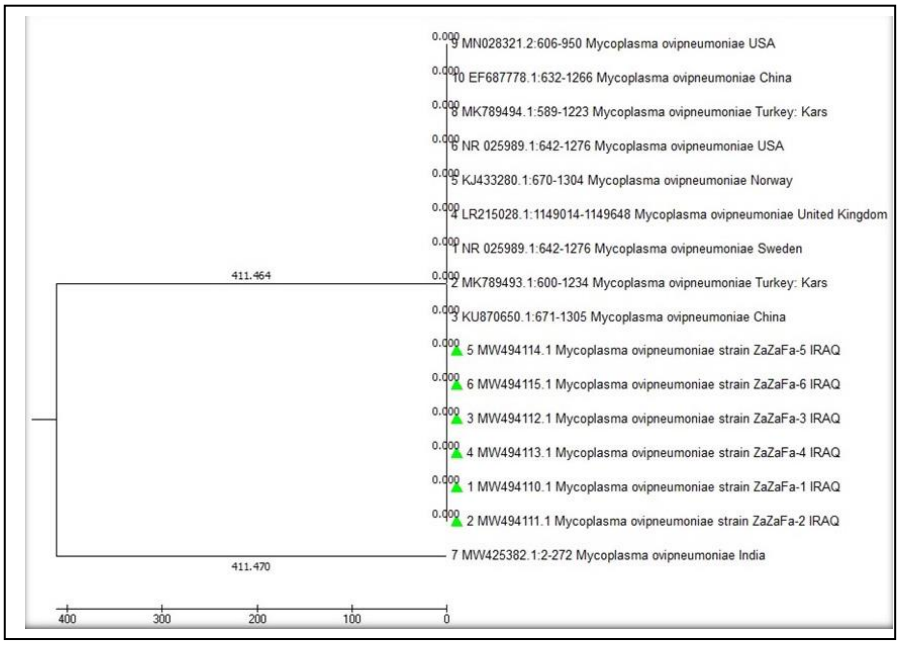

Figure 3. *Mycoplasma ovipneumoniae* strain ZaZaFa-2 16S ribosomal RNA gene, partial sequence

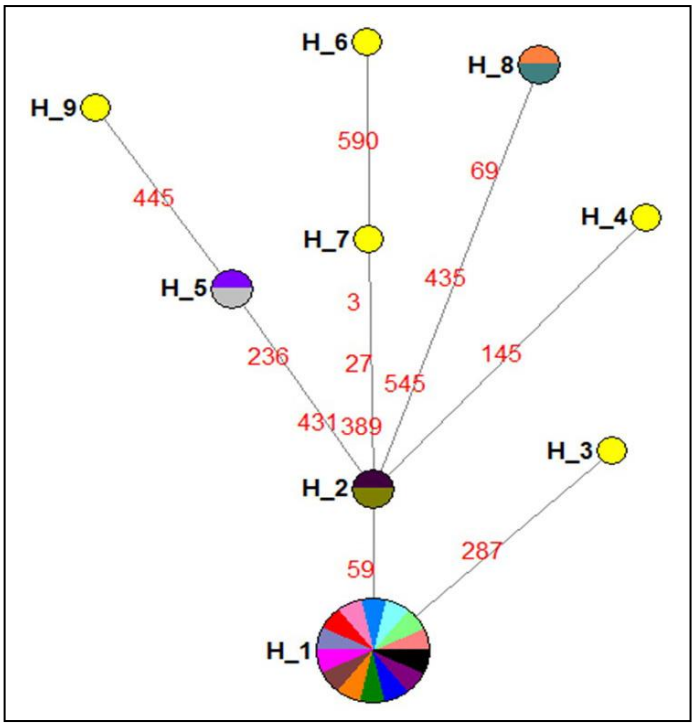

Figure 4. A network of M. ovipneumoniae 16S rRNA haplotypes. Haplotype 1 (H_1) includes M. ovipneumoniae 1 (DQ000588.1), M. ovipneumoniae 2 (NR_025989.1), M. ovipneumoniae 3 (MK789496.1), M. ovipneumoniae 4 (MK789495.1), M. ovipneumoniae 5 (MK789494.1), M. ovipneumoniae 6 (MK789492.1), M. ovipneumoniae 7 (MK789490.1), M. ovipneumoniae 8 (MK789489.1), M. ovipneumoniae 9 (MK789484.1), M. ovipneumoniae 10 (MK789483.1), M. ovipneumoniae 11 (MK789482.1), M. ovipneumoniae 12 (MK789475.1), M. ovipneumoniae 13 (KJ433280.1) and M. ovipneumoniae 14 (EF687778.1). H_2 includes M.ovipneumoniae_15 (MK789493.1) and M.ovipneumoniae_16 (KU870650.1). H_3 represents M. ovipneumoniae 17 (EU265779.1) and H_4 represents M.ovipneumoniae 18 (KU870647.1). H_5 represents M. ovipneumoniae 19 (MW494114.1) and sample 5. H_6 represents sample 1, and H_7 represents sample 2. H_8 represents sample 3 and sample 4. H_9 represents sample 6

Figure 5. Nasal discharge (arrow) from nose infected sheep with *Mycoplasma*

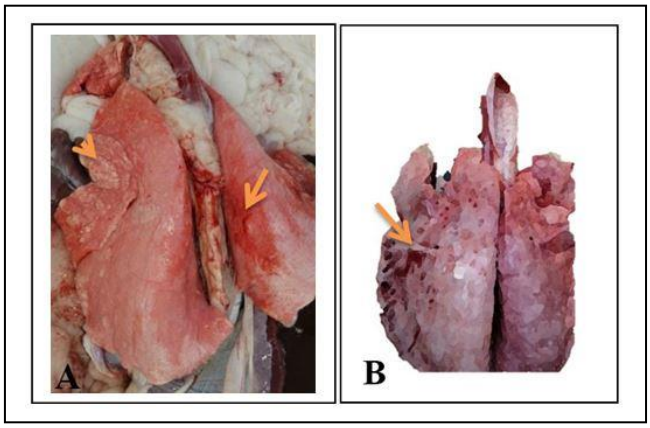

Figure 6. Lung of sheep with red consolidated areas (arrow) in both lobes accompanied with subpleural pale raised emphysematous areas (head arrow) and gray consolidated with ecchymosis hemorrhage foci (arrow)

3.5. Histopathological Examination

The Lung sections revealed suppurative bronchopneumonia; where there was severe damage to pulmonary tissues from alveoli, bronchi, and bronchioles due to infiltration of PMNs as suppurative exudate in the lumen of bronchi and bronchioles (Figures 7A, 7B and 8A), the mucosa shows degeneration of stratified epithelium, lost their cilia and hypertrophy with hyperplasia of goblet cells impacted with mucin (PAS) (Figure 8B). The pulmonary tissues from interstitial, heavily infiltrated with inflammatory exudate caused marked interstitial-alveolar thickening (interstitial pneumonia) may be due to injury and inflammatory process in any of the three layers of the alveolar walls (endothelium, basement membrane, or alveolar epithelium). Most branches of bronchi appeared with microscopic changes; loss of cilia, desquamation of respiratory epithelium, hypertrophy, and sometimes hyperplasia of bronchus epithelium, which lead to the presence of mucinous exudate into the lumen. There was marked thickening of pleura noted and emphysematous lesions (Figures 9A and 9B).

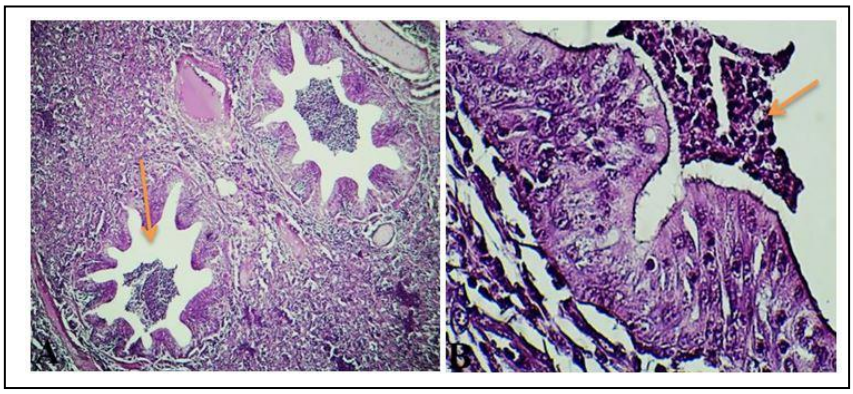

Figure 7. Histopathological sections from sheep infected with *Mycoplasma ovipneumonia* show suppurative bronchitis, bronchiolitis (arrow), and interstitial pneumonitis. (H&E stain, 100&400×)

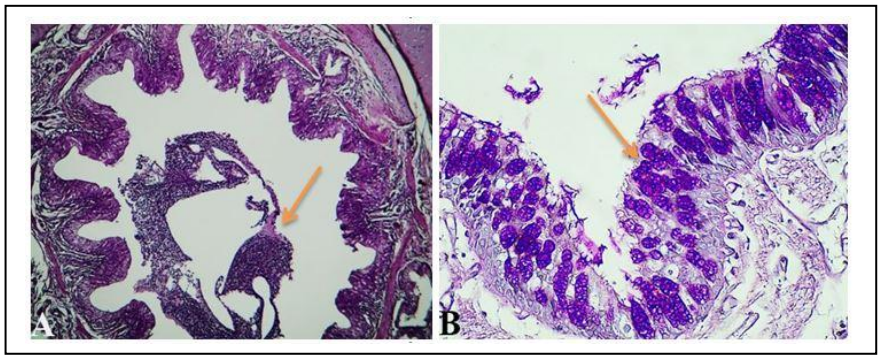

Figure 8. Histopathological sections in the bronchus show fibrino-suppurative bronchitis (arrow) and goblet cells hyperplasia (arrow) with PAS-positive mucin substance. (H&E stain, 200X, PAS stain, 400×)

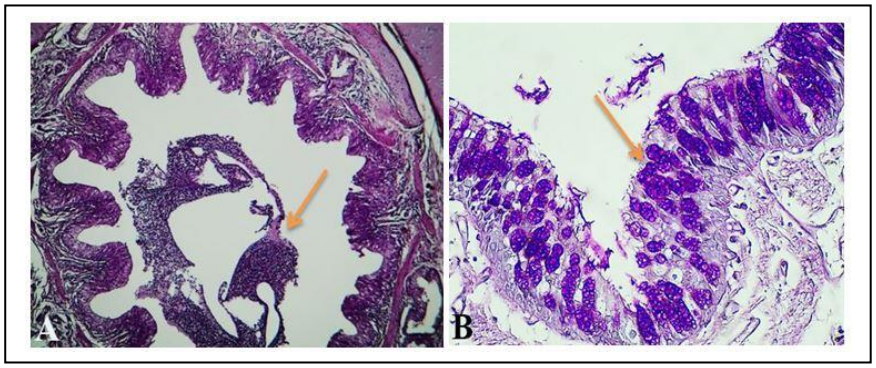

Figure 9. Histopathological sections show fibrous thickening of pleura and subpleural vascularity (arrow).Peribrochiolitis, perivascular and peribronchiolar mononuclear cells infiltration (arrow) (H&E stain, 100×

4. Discussion

Mycoplasma ovipneumonia infection was reported by (6), Einarsdottir, Gunnarsson (9), (10); the findings indicated that pathogen transmission was from ewes to lambs shortly after birth, and lambs have succumbed *to M. ovipneumoniae* as young as 6 weeks old in Iceland, although these early deaths are uncommon. It has been suggested that *M. ovipneumoniae* prevents regular ciliary activity in the host, which facilitates the invasion of the lower respiratory tract by other organisms, such as *Mannheimia haemolytica* (11), that symptoms are mostly seen during autumn and winter. Mixed strains of *M. ovipneumoniae* appear to be more pathogenic, as documented by Azizi, Tajbakhsh (12). The results reported in the current study confirmed for the first time in Basrah province- Iraq, that *Mycoplasma ovipeumonea* infection was revealed in sacrificed female and male sheep in the slaughterhouse. The initial suggestive diagnosis involves clinical signs from nasal discharges, coughing, and dullness of animal; these observations confirmed by post-mortem examination show the signs of inflammatory reaction from congestion collection of edematous fluid in pulmonary chest and exudate in trachea besides hemorrhagic types on the surfaces of lungs, these findings agreed with (3) Lungs showed pneumonic appearance with abdomen-caudal gray hepatization, severe pulmonary edema evident by tense pulmonary capsule and separated lobules with frothy white edematous fluid oozing from bronchi at cut section and prominence interlobular septa. Very clear hydropericardium was recognized with a tan color fluid filling the pericardial sac and petechial to ecchymotic hemorrhages on the heart's coronary fat, followed by

bacterial isolation and biochemical identification then confirmed by PCR of etiological agents as has been done in other studies (5). *Mycoplasma ovipneumoniae* causes subtle and unnoticed clinical signs, and infected animals have good appetites until severe damage associated with secondary bacterial infections (13, 14); bronchopneumonia was the most frequently found due to *M. ovipneumoniae*, as the causative agent of bronchopneumonia and hyperplasia of bronchus epithelium cells (15). Pathogenesis of *M. ovipneumoniae* infection in sheep and other small ruminants occurred as a result of important factors like altering macrophage activity, adhering to the ruminants' ciliated epithelium via its polysaccharide capsule, inducing the production of autoantibodies to ciliary antigens, and suppressive activity on lymphocytes, furthermore the ability of bacteria to doing as a predisposing factor for other bacterial and viral infections (16-18). In the current study, ninety swabs were collected from the nose, tracheal bifurcation, and bronchus collected from sheep in Iraq, Basrah province, subjected to bacterial identification and genetic analysis using PCR as Mycoplasma using Mycoplasma genus-specific primers (19). A positive culture was seen in 24 samples; ten confirmed *M.arginini* and four *M.ovipneumoniae* by PCR. The results showed that PCR was more successful than the culture in detecting *Mycoplasma sp.*, and it agreed with other researchers' results (20).

The prevalence of respiratory disease occurred in Iraqi's sheep-Basrah province with frequent isolation of *Mycoplasma ovipneumoniae*, which reflects respiratory manifestations and pneumonic pathological changes.

Authors' Contribution

Study concept and design: Z. W. K. K.
Acquisition of data: Z. W. K. K. and Z. I. I.
Analysis and interpretation of data:
Drafting of the manuscript: F. A. A.
Critical revision of the manuscript for important intellectual content: Z. W. K. K.
Statistical analysis: Z. I. I.
Administrative, technical, and material support: Z. I. I.

Ethics

Approval for the research study was obtained from the University of Basrah, Basrah, Iraq ethics board.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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