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Traditional isolation and antibiotic susceptibility testing of *Mycoplasma ovipneumoniae* from respiratory-diseased sheep

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Abstract

Background: Respiratory diseases in sheep may result in sudden death or protracted illness, reducing feed efficiency, productive and reproductive performances in addition to causing slaughter condemnations. *Mycoplasma ovipneumoniae* is one of the main pathogens of sheep pneumonia, which becoming increasingly serious in sheep in Iraq.

Aims: Isolation of *M. ovipneumoniae* from the suspected pneumonic sheep, and then, molecular confirmation of study isolates using the conventional molecular assay. Also, antibiotic susceptibility testing was conducted to indicate the sensitivity of *M. ovipneumoniae* isolates to different antibiotics.

Materials and methods: A total of 37 pneumonic adult sheep were subjected to collection of nasal swabs that transported into PPLO broth, and cultured on PPLO agar. The isolates of *M. ovipneumoniae* were subjected to molecular examination by the conventional polymerase chain reaction (PCR). The Kirby-Bauer disc diffusion susceptibility test was served to determine the susceptibility patterns of *M. ovipneumoniae* isolate against 10 types of antibiotics using the Muller Hinton agar.

Results: An overall 13.51% of study sheep were positive to *M. ovipneumoniae* that forms distinctive colonies on PPLO AGAR with generally small, appearing as tiny dew drops or resembling fried eggs. Light microscopy revealed blue Dienes staining of the colonies, indicating that they were Mycoplasmas. Oil microscopy showed lavender Giemsa staining of the cell body, with a predominance of spherical and filamentous forms. Targeting the *16S rRNA* gene, molecular findings of the conventional PCR assay revealed that all study isolates were *M. ovipneumoniae* (100%). Relation to antibiotic susceptibility, significant higher rate of resistance was seen in Amoxicillin and Penicillin G (100%) while lower rate of resistance was shown in Florfenicol, Norfloxacin, and Tylosin (0%). Subsequently, the higher rate of intermediate sensitivity was recorded in Ciprofloxacin, Florfenicol, and Gentamicin (40%); whereas, the lower rate of sensitivity was detected in Amoxicillin and Penicillin G (0%). However, significant higher rate of sensitivity was observed in Norfloxacin and Tylosin (100%); while, the lowest values were recorded in Amoxicillin, Ciprofloxacin, and Penicillin G (0%).

Conclusion: *Mycoplasma ovipneumoniae* is primarily found in clinically pneumonic sheep. However, additional research into survival of individuals that have been exposed to *M. ovipneumoniae* and subsequently recovered could help elucidate the potential long-term impacts of polybacterial pneumonia.

Keywords: Mycoplasmosis, Drug sensitivity, Inhalation, Polymerase chain reaction (PCR), Iraq

INTRODUCTION

Despite the relatively long acquaintance with mycoplasmas; their natures, relationships to other organisms, and taxonomic status were for long time continuing enigma to microbiologists (Mejbel and Ibrahim, 2025). Owing to their minute size and ability to pass through filters, which blocked the passage of bacteria, the mycoplasmas were considered to be viruses for years (Ali and Abdullah, 2024; Salih; 2024; Wu *et al.*, 2024). Advancements in the 1960s and 1970s of our knowledge about ultrastructure,

cell membrane, genome, and metabolic pathways of mycoplasmas led to the recognition that *Mycoplasmas* are the smallest and simplest self-replicating organisms (Roachford *et al.*, 2019; Ali et al., 2024). Naturally, this finding has raised the intriguing question as to the place of mycoplasmas in the evolutionary scheme. The extreme simplicity and compactness of *Mycoplasma* cells led Morowitz and Wallace (1973) to propose that *Mycoplasma* is the most primitive extant organisms, representing the descendants of bacteria that existed prior to the development of a peptidoglycan cell wall.

The clinical picture of *Mycoplasma* infections in humans and animals is more suggestive of damage due to host immune and inflammatory responses rather than to direct toxic effects by *Mycoplasma* cell components (Ali and Ali, 2019; Benedetti *et al.*, 2020). Currently, the most extensively studied attachment organelle and adhesin are *M. pneumoniae* that primarily parasitizes the human respiratory tract and is associated with primary atypical pneumonia (Wang *et al.*, 2025), and *M. genitalium* that grows extensively in the human genital tract and is associated with non-gonococcal urethritis (Hamzah and Ibrahim, 2024; Qiu *et al.*, 2025). However, the adhesion organelle of *Mycoplasma* is maintained by a complex cytoskeleton composed of three major substructures: the terminal button, rod, and wheel complex (or bowl), present in the tip, centre, and back, respectively (Martinelli *et al.*, 2016; Al-Abedi and Al-Amery, 2021). Subsequent investigations have reported a high degree of genomic heterogeneity among *M. ovipneumoniae* strains in different countries (Xu *et al.*, 2011; Kılıc *et al.*, 2013; Einarsdottir *et al.*, 2018; Jaÿ *et al.*, 2020; Ali, 2024).

Mycoplasma ovipneumoniae is an important pathogen causing respiratory disease in small ruminants worldwide. It is also a common predisposing factor for other bacterial pneumonias, particularly pasteurellosis and viral infections, which may exacerbate the pathological process (Deeney *et al.*, 2021; Al-Juwari, 2023). Nonetheless, the reason for presence of *M. ovipneumoniae* in both apparently healthy and affected animals is still unresolved but clearly indicates other factors are important in disease etiology (Mousel *et al.*, 2021; Soomro *et al.*, 2023). In view of its largely unknown and variable impact and the occurrence of other priority diseases, national control authorities are unlikely to instigate eradication measures for *M. ovipneumoniae* any time soon and further surveys are clearly necessary to determine its true prevalence and economic impact (Dawood *et al.*, 2022; Maksimović *et al.*, 2022; Gardner, 2023; Mustafa and Jawad, 2024). Therefore, this study aims to isolation of *M. ovipneumoniae* from the suspected pneumonic sheep, and then, molecular confirmation of study isolates using the conventional PCR assay. Also, this study conducts the antibiotic susceptibility testing for study isolates to indicate its sensitivity to different antibiotics.

MATERIALS AND METHODS

Ethical approval

The current study was licensed by the Scientific Committee of the Department of Pathology and Poultry Diseases in the College of Veterinary Medicine (University of Baghdad).

Samples and M. ovipneumoniae isolation

A total of 37 pneumonic sheep of various ages and sexes, were subjected to collection of nasal swabs under aseptic conditions, and transported into PPLO broth (Oxoid, England) to the laboratory. For bacterial isolation, PPLO agar was prepared following the manufacturer instruction (Oxoid, England). Briefly, samples were serially diluted in PPLO broth without crystal violet (21g/l), 20% de-complemented horse serum, 10% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate, 0.04% penicillin). This was followed by incubation of broths at 37°C in 5% CO_2 for 7-14 days. Broths were checked daily for *Mycoplasma* growth, which was indicated by turbidity and the appearance of the floccule's materials at the bottom of the culture tube. All the tubes with former appearance were filtered using 0.45µm and 0.2µm syringe filters to remove debris and the fast-growing microbes. The filtrates were cultured onto PPLO agar and incubated at 37°C in 55% CO_2 for 7-14 days. The plates were checked three days after inoculation for the appearance of *Mycoplasma* colonies indicated by fried egg observations under the light microscope. The suspected colonies were continued to be sub-cultured to obtain a single colony pure culture. Giemsa staining (Solarbio, China) was used to permissively identify

the isolates according to the morphology of their colonies (Hadi et al., 2020; Chen et al., 2024; Mustafa and Jawad, 2024; Salih, 2024).

Molecular testing

DNA Extraction

Based on the manufacturer's instruction of the G-Spin™ Total DNA Extraction Kit (Intron Biotechnology, South Korea), protocol (F) was followed to extract the DNAs from the freshly prepared colonies of *M. ovipneumoniae*. Briefly, 1 ml of cultured bacteria cell was transferred into 2 ml tube, centrifuged for 1 min at 13,000 rpm with discarding of supernatant. The pellet bacterium was re-suspended completely with remnant supernatant vigorously by vortex. A total of 200µl Buffer CL, 20µl Proteinase K and 5µl RNase A Solution were added into sample tube and mixed by vortex vigorously. The lysate was incubated at 56°C using the water bath for 10min; and when the lysis was completed, 200µl of Buffer BL was added into the upper sample tube and mixed thoroughly. Then, the mixture was incubated at 70°C for 5min, centrifuged at 13,000rpm for 5 min, and a total 350µl of supernatant was transferred into a new 1.5ml tube and centrifuged for 1.5ml. Then, a 200µl of absolute ethanol was added into the lysate, and mixed well by vortex. After centrifugation at 13,000rpm for 5 min, the filtrate was discarded, and the Spin Column was transferred into a new Collection Tube. After centrifugation at 13,000rpm for 1 min, the filtrate was discarded and the Spin Column was transferred into a new 2ml Collection Tube. A 700µl of Buffer WA was added to the Spin Column, centrifuged for 1min at 13,000rpm, and the flow-through was discarded. Then, a 700µl of Buffer WB was added to the Spin Column, centrifuged for 1min at 13,000rpm, and the flow-through was discarded. Then, the Column was placed into a new 2ml Collection Tube to be centrifuged additionally for 1min, and the flow-through was discarded. After placing the Spin Column into a new 1.5 ml tube, 30µl of Buffer CE was added directly to be incubated for 1min at room temperature; and then, centrifuge for 1min at 13,000rpm to elute. Finally, the concentration and purity of each obtained DNA sample was quantified using the Nanodrop Spectrophotometer (Thermo Scientific, UK) at an absorbance of A260/A280nm.

MasterMix prepration

Targeting the *16S rRNA* gene, one set of primers (JB1:5′-TGG TGG CAA AAG TCA CTA GC-3′ and JB2:5′-AGC CAT TGT AGC ACG TGT GT-3′) was designated based on the NCBI-GenBank database *M. ovipneumoniae* isolate (ID: MW494115.1) and provided by the Bioneer Company (South Korea). Following the manufacturer instructions of the ready to use PCR-PreMix Kit (Bioneer, South Korea), the MasterMix tubes were prepared at a final volume of 20µl (5µl DNA Template, 1µl for each F and R primers, and 13µl Free-Nuclease Water). Then, the MasterMix tubes were subjected to the PCR thermocycler (Bio-Rad, USA) conditions that involving 1 cycle for initial denaturation (95°C/5min); 35 cycles for denaturation (95°C/30sec), annealing (58°C/30sec), and extension (72°C/30sec); and 1 cycle for final extension (72°C/7min).

Agarose-gel electrophoresis

The PCR products were analyzed by electrophoresis using 1.5% agarose gel stained with Ethidium Bromide and TAE-1X buffer at 80mA and 100Volt, and for 90min. The PCR products were visualized under the ultraviolet Transilluminator (Biobase, China) and the positive samples were identified at 400bp.

Antibiotic susceptibility testing

The Kirby-Bauer disc diffusion susceptibility test was served to determine the susceptibility patterns [sensitive (S), intermediate (I), or resistance (R)] of *M. ovipneumoniae* isolate against various antimicrobial compounds on the Muller Hinton agar (Oxoid, UK). Ready to use antibiotic discs (Liofilchem, Italy) were used in the current study including Amoxicillin (10µg), Azithromycin (15µg), Ciprofloxacin (5µg), Erythromycin (15µg), Florfenicol (30µg), Gentamicin (10µg), Norfloxacin (30µg), Penicillin G (10µg), Tetracycline (30µg) and Tylosin (30µg).

Statistical analysis

The obtained results were analyzed by GraphPad Prism version 8.0.2 (*GraphPad Software, USA*). One-Way ANOVA and the *t*-test were applied to detect significant variation between values of study at

$p<0.05$ (*). Values were represented as either mean ± standard errors (M±SE) or as number (percentage) [No. (%)] (Gharban and Yousif, 2020).

RESULTS

In current study, nasal swabs of totally 37 pneumonic sheep, cultivated on PPLO agar, revealed that 5 (13.51%) were positive to M. *ovipneumoniae* that forms distinctive colonies with generally small, appearing as tiny dew drops or resembling fried eggs (Figures 1, 2). Light microscopy revealed blue Dienes staining of the colonies, indicating that they were Mycoplasmas. Oil microscopy showed lavender Giemsa staining of the cell body, with a predominance of spherical and filamentous forms (Figure 3).

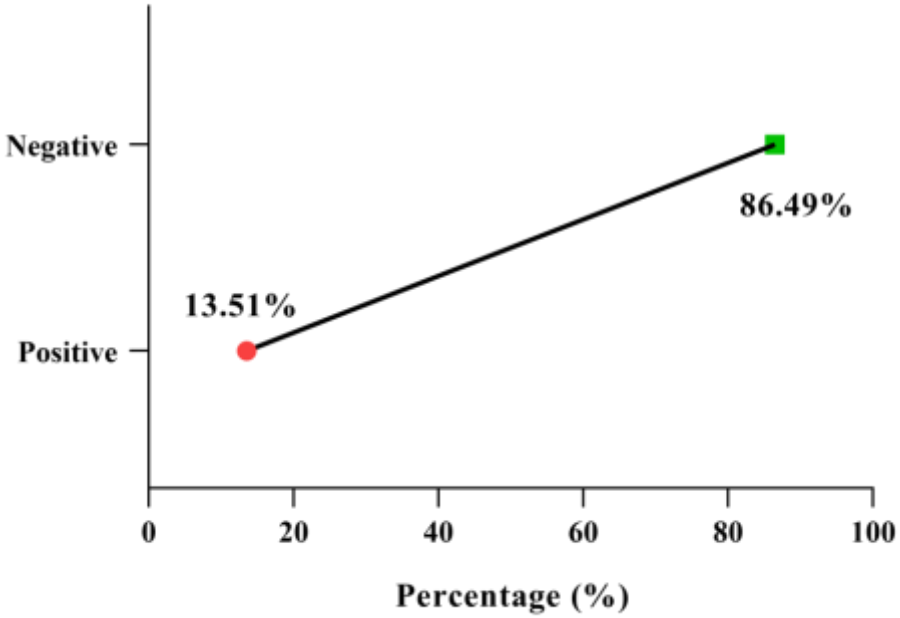

Figure (1): Total results for cultivation the nasal swabs of totally 37 pneumonic sheep

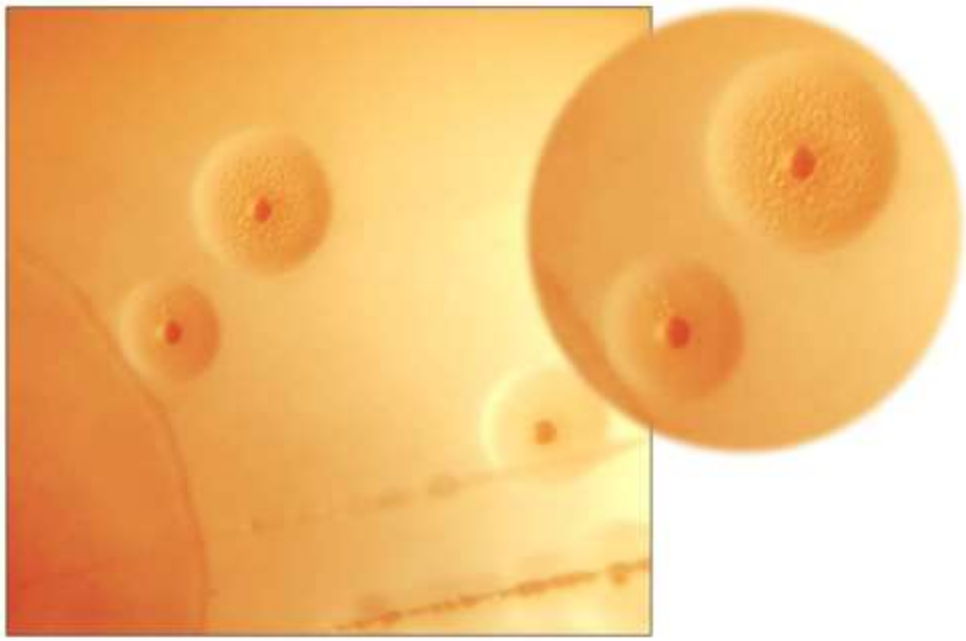

Figure (2): Colonies of *M. ovipneumoniae* on PPLO agar characterized as round without a central umbilicus (10X)

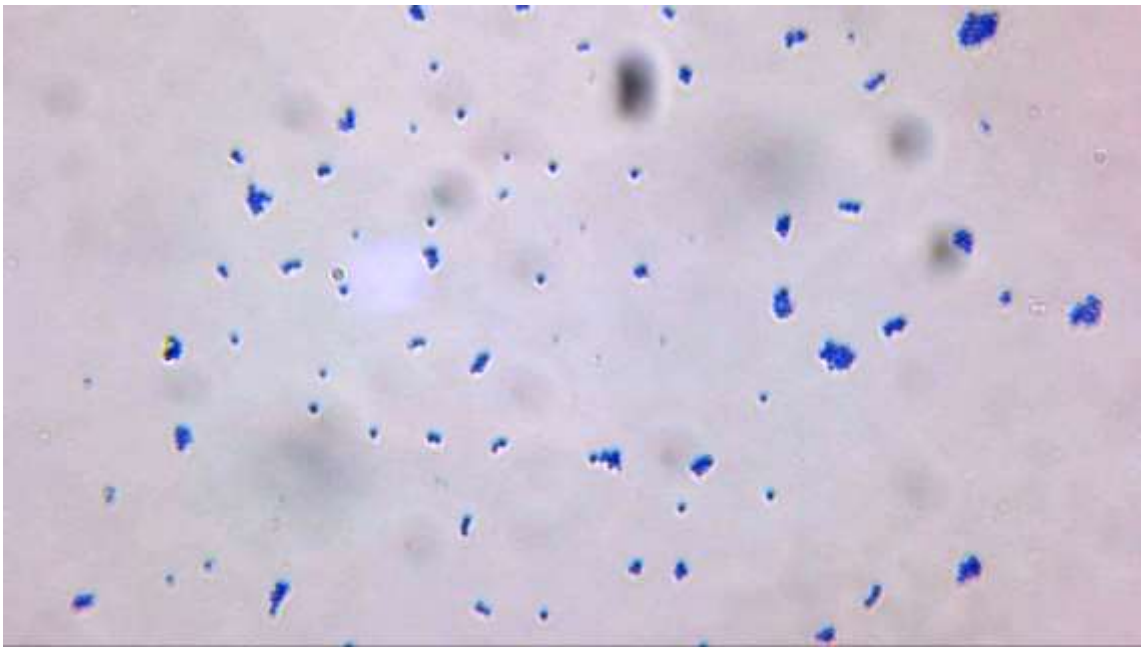

Figure (3): Giemsa staining of purified colonies (400X)

Targeting the *16S rRNA* gene, molecular findings of the conventional PCR assay revealed that all study isolates were *M. ovipneumoniae* [100% (5/5), (Figures 4).

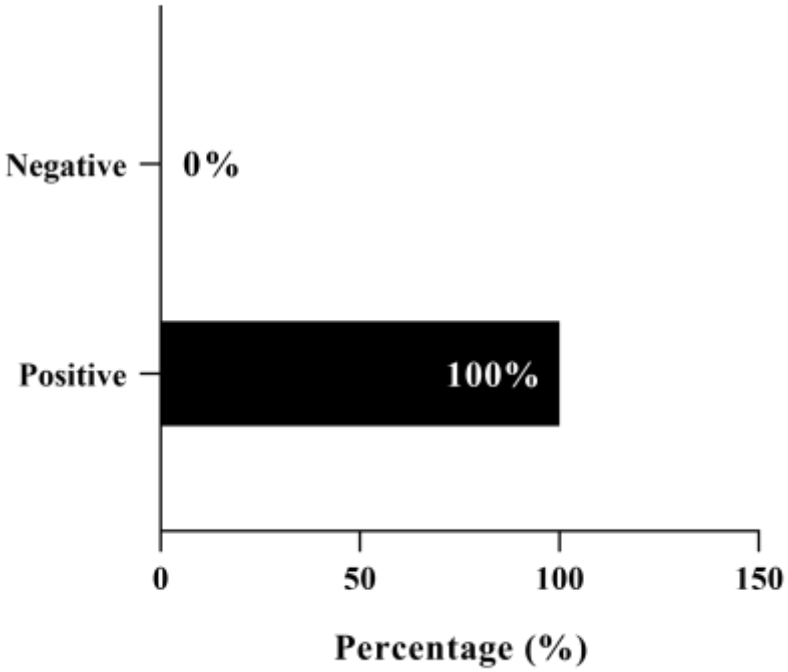

Figure (4): Total results for molecular PCR testing of *M. ovipneumoniae* isolates

Relation to antibiotic susceptibility, significant higher rate of resistance was seen in Amoxicillin and Penicillin G (100%) while lower rate of resistance was shown in Florfenicol, Norfloxacin, and Tylosin (0%). Subsequently, the higher rate of intermediate sensitivity was recorded in Ciprofloxacin, Florfenicol, and Gentamicin (40%); whereas, the lower rate of sensitivity was detected in Amoxicillin and Penicillin G (0%). However, significant higher rate of sensitivity was observed in Norfloxacin and

Tylosin (100%); while, the lowest values were recorded in Amoxicillin, Ciprofloxacin, and Penicillin G (0%), (Table 1).

Table (1): Antibiotic susceptibility testing of *M. ovipneumoniae* isolates (total number = 5) against the applied antibiotics (total number = 10)

Antibiotic	Standard zones of inhibition		Susceptibility pattern		
	R	S	R	I	S
Amoxicillin	≤ 15	≥ 20	5 (100%) Aa	0 (0%) Cb	0 (0%) Db
Azithromycin	≤ 12	≥ 15	1 (20%) Db	1 (20%) Bb	3 (60%) Ba
Ciprofloxacin	≤ 21	≥ 26	3 (60%) Ba	2 (40%) Ab	0 (0%) Dc
Erythromycin	≤ 13	≥ 19	2 (20%) Db	1 (20%) Bb	2 (60%) Ba
Florfenicol	≤ 12	≥ 16	0 (0%) Ec	2 (40%) Ab	3 (60%) Ba
Gentamicin	≤ 11	≥ 15	2 (40%) Ca	2 (40%) Aa	1 (20%) Cb
Norfloxacin	≤ 17	≥ 22	0 (0%) Ec	2 (20%) Bb	3 (80%) Aa
Penicillin G	≤ 10	≥ 14	5 (100%) Aa	0 (0%) Cb	0 (0%) Db
Tetracycline	≤ 17	≥ 25	1 (20%) Db	1 (20%) Bb	3 (60%) Ba
Tylosin	≤ 14	≥ 18	0 (0%) Ec	1 (20%) Bb	4 (80%) Aa
p-value			0.0011	0.0163 S	0.0031
95% confidence interval			8.356 to 63.64	11.44 to 32.56	18.20 to 65.80
R squared			0.4909	0.7118	0.6391

Differences between large vertical and small horizontal letters refer to significant variation (S) at $p<0.05$

DISCUSSION

Mycoplasma ovipneumoniae infection in small ruminants is a serious problem in herds of small ruminants around the world as it is responsible for high economic losses and decreased animal productivity. This study found that the prevalence rate of *M. ovipneumoniae* in sheep using of culture was 13.51%. In comparison to other studies, our findings were in agreement with Mostafa (2003) who reported that the prevalence rates of *Mycoplasma* in apparently healthy sheep was 14.67% but lower than observed by Abdou (2002) who reported a 42.5% prevalence rate of *Mycoplasma*; Rania (2006) who recorded the prevalence rates of *Mycoplasma* were 40% in apparently healthy sheep, and Mousa *et al.* (2021) who recorded 46.2% in sheep suggesting that the higher prevalence rates of *Mycoplasma* in our survey may be due to the bad hygienic measures applied in animal management and husbandry practices. In Brazil, the culture of tracheobronchial lavage has been resulted in glucose-fermenting fried egg colonies in 59.28%, which were identified as *M. ovipneumoniae* (Gaeta *et al.*, 2022). In Italy, Pavone *et al.* (2023) found that

47.5% out of the 202 tested sheep were positive to Mycoplasmal infections that involved M. *ovipneumoniae* (21%), M. *arginine* (19%), and M. *ovipneumoniae* + M. *arginini* (57%). In China, the overall positivity rate of M. *ovipneumoniae* was 27.50%. *Mycoplasmas* were obtained from nasal swabs (20.0%), pleural fluid samples (10.5%), and lung samples (12.5%), (Chen *et al.*, 2024).
In the present study, the application of conventional PCR assay confirmed that all study isolate obtained by culture were M. *ovipneumoniae*. PCR is a commonly used detection method in laboratories. McAuliffe et al. (2003) designed a pair of specific primers to amplify M. *ovipneumoniae 16S rRNA* gene sequences and they are often used by Friis et al. (1976). The universal primers designed by Van Kuppeveld et al. (1994) can be used to detect the *Mycoplasma* genus and are widely used in the identification of cell contamination. Because of its unique growth characteristics, it is difficult to isolate and culture *Mycoplasma*, but it is still the gold standard method for diagnosing of disease caused by these bacteria. Showing a difference from other *Mycoplasma* colonies, M. *ovipneumoniae* colonies isolated using modified Hayflick's medium are transparent, round, with the center deeply invaginated and have no central umbilical (Zhao et al., 2021).
Our findings of antibiotic susceptibility testing shown that M. *ovipneumoniae* isolates were highly resistant to Amoxicillin and Penicillin G, intermediately to Ciprofloxacin, Florfenicol, and Gentamicin but highly sensitive to Norfloxacin and Tylosin. In comparison to other studies,

A number of studies found that fluoroquinolones, tilmicosin, tulathromycin, chlortetracycline, enrofloxacin, doxycycline, and oxytetracycline are effective against *Mycoplasma*, which are the main causative agents of respiratory infections in lambs (Morgan et al., 2005; Ambroset et al., 2017; Tortorelli et al., 2017). The concomitant use of non-steroidal anti-inflammatory drugs is also recommended. All lambs with clinical signs should receive the full course of treatment. Politis et al. (2019) found that the potential value of metaphylactic treatment of clinically healthy lambs in affected herds should be assessed on a case-by-case basis. Disease management protocols should always include changes in herd management to eliminate factors contributing to the development of the disease. In comparison to the findings of two studies, Gautier-Bouchardon (2018) recorded that the overall susceptibility of M. *ovipneumoniae* isolates contrasts with the status of other *Mycoplasma* species such as M. *bovis* that has become resistant to several antimicrobial classes over the last 20 years but is comparable to that of M. *agalactiae*, which has acquired moderate antimicrobial resistance over time, with a dominant low-MIC population (Poumarat et al., 2016). Jaÿ et al. (2020) evidenced homogenous minimum inhibitory concentration (MIC) distributions with low values for all the antimicrobials tested. MICs remained dominantly below the intermediate breakpoint, except for florfenicol. Murray et al. (2020) mentioned that the efficacy of bacteriostatic agents such as oxytetracycline, florfenicol, and lincomycin against *Mycoplasma* species could depend on the ability of the antimicrobial agent to suppress the bacteria long enough for the host immune system to respond to the pathogen, and for the host to recognize and respond to the presence of a pathogen. An *in vitro* study evaluating susceptibility of M. *ovipneumoniae* to various antibiotics, Maksimović et al. (2020) found that while M. *ovipneumoniae* was susceptible to florfenicol and oxytetracycline, florfenicol had the highest MIC50 value and oxytetracycline the highest MIC90 value, indicating that high concentrations of each of these antibiotics was needed for an inhibitory effect on M. *ovipneumoniae*. Agoltsov et al. (2022) detected that *Mycoplasma* isolates with increased resistance to tilmicosin are also resistant to tylosin and lincomycin; and that treatment of respiratory *Mycoplasma* infections in lambs has been successful with the use of fluoroquinolones, tilmicosin, tulathromycin, chlortetracycline, enrofloxacin, doxycycline, and oxytetracycline. Christensen (2022) demonstrated that daily administration of oxytetracycline injections resulted in significant changes in infected animals when compared to those of control group. A single-dose therapy of antibiotic likely did not achieve therapeutic levels of the antibiotic for a length of time to allow the host's immune system to mount a response to the presence of the bacteria.

CONCLUSION

Mycoplasma ovipneumoniae is primarily found in clinically pneumonic sheep. However, additional research into survival of individuals that have been exposed to M. *ovipneumoniae* and subsequently recovered could help elucidate the potential long-term impacts of polybacterial pneumonia. Continued monitoring of the spread and evolution of M. *ovipneumoniae* isolates in terms of diversity and susceptibility is needed in the years to come. Including wildlife isolates would be useful as these might play a role in the population dynamics. This follow-up will rely on improved, harmonized diagnosis with notably the potential determination of epidemiological cut-off values to interpret the MICs

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Authors' contributions
AHM: Collection of nasal swabs and blood, measurement of IL-10, and statistical analysis of results. ZII: Isolation of M. *ovipneumoniae*, preparation of bacterial infectious inoculum and preparation of vaccine.

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