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Phenotypic, Genotypic Characterization and Immunological Effects of *Mycoplasma* Infection in Sheep

Sahar E. Ouda[1*], Farida H. Mohamed[2], Shereen M. Aly[2], Emad B. Ata[3], Rania Abo-Sakaya[4*] and Sabry I. Eissa[1]

[1] *Department of Mycoplasma, Animal Health Research Institute, ARC. Giza,12618, Egypt.*
[2] *Department of Immunology, Animal Health Research Institute, ARC. Giza,12618, Egypt*
[3] *Parasitology and Animal Diseases, National Research Centre, Giza 12622, Egypt*
[4] *Department of Animal Medicine, Faculty Veterinary Medicine, Benha University, Egypt.s*

Abstract

SHEEP play a vital role in the agricultural landscape of Egypt. Respiratory conditions caused by Mycoplasma are recognized as a prevalent source of economic loss within this sector. In the present study, one hundred and twenty samples were collected from sheep across three different localities from sheep farm in Giza governorate. Observations of respiratory infection symptoms were noticed, and subsequent isolation of *Mycoplasma* was carried out. *Mycoplasma arginini* was successfully isolated from two distinct localities. *Mycoplasma ovipneumoniae* was detected in the samples. Additionally, *Acholeplasma laidlawii* was isolated from all the localities under investigation. The confirmation of various strains was achieved through polymerase chain reaction (PCR) analysis and sequencing. All serum and lung tissues' samples were classified into 4 groups. Group 1 (G1) included non-infected sheep group-2 (G2) include sheep infected with *M. arginini* + *Acholeplasma*. G3: *M. ovipneumoniae* + *Acholeplasma laidlawii*. G4: sheep infected with *M. arginini* + *M. ovipneumoniae* +*Acholeplasma laidlawii*. Lysozyme activity, nitric oxide (NO), catalase enzyme and malondialdehyde (MDA) levels were measured in the serum. IL-8 and CRP mRNA were determined in lung tissues by quantitative real time-PCR (qRT-PCR). The results revealed that Mycoplasma infection significantly increased serum lysozyme activity, NO, IL-8, CRP, and MDA levels, while catalase activity was significantly reduced in infected groups (G2, G3, G4) compared with the control group (G1). Notably, multiple *Mycoplasma* infections in G4 further elevated these immunological and oxidative dysregulations. In a conclusion, infection with *Mycoplasma* sp. in sheep dysregulated innate immune response, aggravated the expression of inflammatory mediators and induced oxidative imbalance. Multiple infection with *Mycoplasma* sp. further exacerbated its detrimental effects.

Keywords: *Mycoplasma arginini*, *Mycoplasma ovipneumoniae*, *Acholeplasma laidlawii*, immune, oxidative, lysozyme, MDA.

Introduction

Farm animals are the most important source of proteins for humans [1, 2, 3]. They were subjected to multiple diseases affecting their productivity [4, 5, 6]. *Mycoplasma* sp. belongs to a group of bacteria named Mollicutes which is characterized by its minute genome size and perpetually devoid of the cell wall [7, 8]. Its infection results in great economic loss due to its varied morbidity and mortality rates in sheep and goats' populations beside its persistent infection, and endemicity worldwide [9]. Furthermore, *Mycoplasma* infection is one of the most threatens affecting development of vaccine industry [10, 11].

Mycoplasma sp. infections are associated with respiratory manifestations, including cough, nasal discharge, conjunctivitis, and fever. *M. arginini* was frequently isolated with *M. ovipneumoniae* from cases of atypical pneumonia in sheep and goats [12]. Molecular tools have helped in genotyping of many pathogens [2, 13, 14]. Molecular phylogenetic analysis had been used in the tracing, control, and prevention of diseases. In addition, molecular typing of some strains such as *M. arginini* would help in strain differentiation and determination of the suspected pathological importance of such strains [15].

*Corresponding authors: Sahar E. Ouda, E-mail: seoa200@yahoo.com, Tel.: +201100494624
Rania Abo-Sakaya, E-mail: rania.aboskayah@fvtm.bu.edu.eg, Tel.: +201068896484.
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The severity of the disease correlated with the disruption of the immune response initiated by respiratory *Mycoplasma* sp. The innate immune response represents the initial defense in limiting pathogen replication and spreading. This defense mechanism originated at the epithelial cells of respiratory mucosa [16]. These cells recognize specific pathogen-associated molecular patterns (PAMPs) of the mycoplasma and activate signaling pathways that result in the production of numerous pro-inflammatory cytokines and chemokines [17]. Among the innate immune mediators, the lysozyme and the nitric oxide (NO) play important roles in defense against infections which have been reported to significantly increase in diseased lambs with respiratory manifestation suffering from severe co-infection with *M. ovipneumoniae* and *M. arginini spp*. [18]. *Mycoplasma* sp. uses neutrophils as both a barrier and a nutrient source, thereby effectively sustain long-term survival within host tissues. These mechanisms drive continuous immune activation, oxidative stress, and tissue injury, thereby contributing to the pathogenesis and progression of many inflammatory chronic diseases [19]. Its infection has been associated with dysregulation of inflammatory cytokines and markers, including interleukin-8 (IL-8) and C-reactive protein (CRP) which play a significant role in initiating and extending mycoplasma-associated inflammatory diseases [20]. Such cytokines serve as a valuable indicator for assessing the extent of inflammatory responses [20].

Research has demonstrated that pathogen infections often cause imbalance in cells redox equilibrium and homeostasis [21]. Oxidative stress emerges because of overproduction of oxidative substances and due to the imbalance between oxidants and antioxidants [22]. Catalase enzyme is a vital antioxidant enzyme that exerts an essential role in the cellular defense against oxidative stress. Malondialdehyde (MDA) is the product of polyunsaturated fatty acid lipid peroxidation. It is the most researched biomarker for lipid peroxidation and oxidative stress in damaged tissue [23].

The aim of the current study was to detect and isolate as well as genotype the *Mycoplasma* spp. associated with severe respiratory manifestation, and deaths in sheep under field conditions and assessing the innate immune markers (serum lysozyme activity, and NO), the inflammatory mediators (IL-8 and CRP mRNA) in lung tissue and oxidative stress indicators (serum catalase and MDA).

Material and Methods

Ethical approval

All the investigation conducted on animal populations were in accordance with the Animal Welfare directives. The present study was ethically approved by the Ethics Committee of the Faculty of Veterinary Medicine, Benha University, Egypt, (Approval number BUFVTM01-09-25).

Sampling

During September 2025, an outbreak affected three commercial sheep farms in Giza governorates. Upon clinical investigation of the diseased animals in the different farms, fifteen animals were subjected to fresh slaughtering or emergently slaughtered by the farm owner for having lung tissues, and serum samples from each farm. Also, forty-five nasal swabs, and serum samples were collected from contact sheep. These samples were sent to microbial diagnostic laboratories in Animal Health Research Institute (Dokki, Giza) for testing, where mixed Mycoplasma infections were identified.

Mycoplasma isolation, and phenotypic characterization

The collected lung tissues, and nasal swabs were processed aseptically. Isolation of Mycoplasma spp. was done by culturing on the specific PPLO agar plates and examined daily for 21 days till the appearance of mycoplasma colonies.

Digitonin sensitivity test was done on the obtained isolates for differentiation between *Mycoplasma* and *Acholeplasma* using filter paper disc impregnated in 0.2ml of 1.5% (w/v) ethanol solution of digitonin as previously described [24]. *Mycoplasma* spp. are digitonin sensitive, while *Acholeplasma* spp. are digitonin resistant.

Biochemical characterization was carried out by glucose fermentation, arginini deamination and film

and spot tests as previously described [24].

Serological identification

Growth inhibition test was carried out using standard antisera kindly supplied by Mycoplasma Department, Animal Health Research Institute, Dokki, Giza as previously described [25].

Genotypic characterization

DNA extraction was applied directly by adding 500 µl PBS to each swab and 5mg pulverized tissue samples using a DNA extraction kit (Qiagen, Germany, GmbH) according to the manual directions. Finally, the purity and concentration of the eluted DNA was measured spectrophotometrically. The DNA samples were stored at −20 °C until amplification.

For molecular detection and genotyping, PCR was applied using generic primers common for Mollicutes and species-specific primers as shown in Table (1). PCR was carried out in (GTC96) thermocycler Cleaver scientific Ltd in total reaction volume of 50 µl.

The reaction mixture included 25 µL DreamTaq Green Master Mix (2X) (Fermentas, Waltham,

USA), 1 µL (10 pmol) of each primer (Sigma-Aldrich, St. Louis, USA), 5 µL template DNA and nuclease-free water up to 50 µl according to the manufacturer's instructions. The obtained PCR products were detected by electrophoresis on a 1.5 % agarose gel in Tris-acetate-EDTA buffer (against 100 bp DNA ladder), then visualized using ultraviolet transilluminator.

Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.08834719 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 354 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4].

Assessment of the innate immune parameters

Serum and lung tissue samples were categorized into groups according to the species of mycoplasma infection in each group. G1: apparently healthy sheep non-infected sheep), G2: sheep infected with *M. arginini* + *Acholeplasma laidlawii*. G3: *M. ovipneumoniae* + *Acholeplasma laidlawii.* G4: sheep infected with *M. arginini*+ *M. ovipneumoniae* + *Acholeplasma laidlawii*.

Measurement of lysozyme activity

This was done using the agarose gel plate lysis assay [30]. Briefly, Micrococcus lysodeikticus (50 mg) was mixed with 1% agarose in 0.06 M PBS (pH 6.3) and poured into petri dishes. Serum samples (25 µl) were added to wells, and after 18 hours the hydrolysis zones were measured to determine lysozyme concentration using a standard curve.

Determination of serum nitric oxide (NO) level

Briefly, 100 µl of serum samples were added to an equal volume of Griess reagent in a 96-well ELISA plate and incubated at 25 °C for 10 min. Absorbance was measured at 540 nm, and nitric oxide concentration was determined using a $NaNO_2$ standard curve as previously described [31].

Determination of IL-8 and CRP expression levels in lung tissues by quantitative real time-PCR (qRT-PCR)

RNA extraction and purification from lung samples were performed using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). Primers specific for IL-8 and CRP along with ß2M internal control primers are presented in (Table 2). The SYBR green qRT-PCR reaction was performed in a Stratagene MX3005P real time PCR machine and the results were analyzed based on the amplification curves and threshold cycles (CT) values (stratagene MX3005P software). To determine the fold changes' difference in the mRNA gene expression of the different samples, the CT of each sample was compared with those of the positive control group according to the "-$2^{\Delta\Delta Ct}$" method [32].

Assessment of catalase enzymatic activity

The levels of catalase enzyme were measured in serum samples by using a colorimetric method [33].

Quantification of malondialdehyde level (MDA)

MDA levels were assessed in serum samples by using colorimetric method [34].

Statistical analysis

The obtained data were statistically analyzed by one-way analysis of variance test (ANOVA). Data were presented as mean ± standard error (SE) using SPSS 2020. Statistical significance was set at $p<0.05$.

Gen Bank accession numbers

GenBank accession number of the generated nucleotide sequence of *Mycoplasma ovipneumoniae* isolate is **PX660475 and** *Mycoplasma arginini* reported in this study is **PX944911**; while *Acholeplasma laidlawii* is **PX944921.**

Results

Clinical examination

Animals were examined clinically and exhibited various signs of respiratory manifestations including nasal and ocular discharges, fever (40-42°C) with depression, cough, abnormal chest sound upon chest auscultation, persistent cough and nasal discharge varied from serous to purulent. Although some animals showed hyperthermia, others had normal range. High fatality rate was also recorded.

Postmortem examination (PM)

The PM examination of the lungs and tracheas revealed a variety of pathological lesions with different degrees of pleurisy including congestion and edema. The lungs exhibited areas of red and gray hepatization while haemorrhage was observed in the trachea and tracheal bifurcation (Fig. 1).

Isolation and biochemical identification of Mycoplasma

Isolation from nasal swabs and lung tissues produced colonies with a characteristic fried egg shape appearance under dissecting microscope, when grown on PPLO agar media for at least 7 days (Figs 2 A and B).

Out of 60 samples (nasal swabs: n= 45 and lung: n= 15), 28 samples were positive for mollicutes with total incidence 46.7%. The recovery rate from nasal swabs was 35.5% whereas from lung tissues was 66.7% Table (3), Fig (2A).

The biochemical characterization of twenty-eight mollicutes isolates depended on digitonin sensitivity (Fig. 2B) glucose fermentation, arginini deamination test and; film and spot formation as shown in Table (4).

Serological identification

Growth inhibition test was employed for identification of isolates from sheep; *Mycoplasma arginini, Mycoplasma ovipneumoniae and Acholeplasma laidlawii* were identified serologically with percentages of 13.3%, 11.7% and 18.3% respectively using standard specific antisera Table (4). Two un-typeable Mycoplasma isolates with a percentage of 3.3% were detected in nasal swabs also. *Acholeplasma laidlawii* was more prevalent. In lung tissues, mixed infection with mycoplasma was detected.

Genotypic characterization of the isolated mycoplasma

No Mycoplasma agalactiae was detected in all examined DNA samples. *Mycoplasma ovipneumoniae, Mycoplasma arginini* were assayed positive for amplification in the examined samples either alone or mixed infection in lung tissues. Both *Mycoplasma ovipneumoniae*, and *Mycoplasma arginini* were detected in pulmonary lesions of sheep alone or as mixed infection. The use of genus-specific primers for mollicutes helped in the detection of other species in samples negative for Mycoplasma *ovipneumoniae* and *Mycoplasma arginini*.

Sequence analysis of the 16SrRNA for the isolated mycoplasma strains

The genetic relatedness between the isolated *Mycoplasma* with those reported in Egypt revealed genetic similarity of 98.49% & 99.53% between the isolated *Mycoplasma ovipneumoniae* isolate **PX660475** & *Mycoplasma arginini* isolate **PX944911** and the corresponding isolate strains which circulate in Egypt respectively as well as 94.55-94.84% & 98.60-99.07% homology with those reported in different countries worldwide respectively (Figs. 3 & 4). In addition, phylogenesis showed 97.11% homology between *Acholeplasma laidlawii* isolate PX944921 and other selected *Acholeplasma laidlawii* circulating in Egypt and other countries worldwide (Fig. 5).

Serum lysozyme and NO assays

The results of serum lysozyme are presented in Figure 6 (A). Sheep in G4 which were infected with three *Mycoplasma* species exhibited the highest mean value of lysozyme activity (65.40 ± 11.39), with a significant increase ($P < 0.05$) compared to those in G1 and G2. Furthermore, G2 (*M. arginini* + *Acholeplasma laidlawii* and G3 (*M. ovipneumoniae* + *Acholeplasma laidlawii* infected animals) showed significantly higher lysozyme activities (35.70 ± 1.15 and 51.54 ± 6.66; respectively) in relation to the apparently healthy group (G1) (16.56 ± 0.63). On the other hand, no significant difference was observed between G2 and G3 (Fig. 6A).

Nitric oxide (NO) assay results are shown in Figure 6 (B). Mixed *Mycoplasma* infection (G4) resulted in significantly elevated NO levels (9.51 ± 0.17) ($P < 0.05$) compared to the other groups. Likewise, G2 and G3 exhibited significantly higher NO levels (6.03 ± 0.11 and 6.16 ± 0.23; respectively) than the apparently healthy group (G1) (4.78 ± 0.09). However, no significant difference was observed between G2 and G3 (Fig. 6B).

IL-8 and CRP mRNA gene expression in sheep lung tissues

The relative fold change of IL-8 and CRP mRNA expression based on threshold values are presented in Table 5. All infected groups (G2, G3 and G4) induced significant higher fold change of both IL-8 and CRP mRNA compared to the non-infected group (G1). The highest significant values for both genes were in G4. Moreover, a significant difference in IL-8 expression was observed between G2 and G3; in contrast, no significant difference was found between these two groups regarding CRP mRNA gene expression.

Serum catalase enzyme and malondialdehyde (MDA) levels in different groups

Figure 7(A) shows that all infected groups (G2, G3 and G4) induced significantly ($P < 0.05$) lower catalase enzyme values (14.56 ± 0.21, 14.32 ± 0.18 and 13.82 ± 0.30; respectively) compared to that of the non-infected group (G1) (23.44 ± 0.55). However, no significant difference was observed between all infected groups. G4 showed the lowest catalase value.

Results of MDA Figure 7(B) revealed that G4 had the highest significant value (2.20 ± 0.17) among all groups. Although there is no significant difference in MDA values between G2 and G3 (1.61 ± 0.05 and 1.74 ± 0.06), both were significantly higher than the non-infected group (G1) (1.07 ± 0.08).

Discussion

In the respiratory system of small ruminants, the most commonly detected Mycoplasma species are *M. arginini, and M. ovipneumoniae* that are associated with the so called atypical non progressive pneumonia [38]. The identification of mycoplasma can be accomplished by conventional methods including culture examination, biochemical testing,

serological tests as growth inhibition and molecular tests such as PCR [39]. An early study showed that nasal and lung isolates represent similar population, although, nasal strains may differ in their ability to colonize the lungs [40].

Culture and PCR tests produced similar results for M. arginini, but for *M. ovipneumoniae* culture alone was less accurate than PCR as mentioned by [28]. While standard PCR is useful for identification, it often fails to identify mixed cultures, which are commonly found together in *M. ovipneumoniae* pneumonia cases to overcome this limitation partial sequencing of the 16S-23SrRNA of *Mycoplasma ovipneumoniae* confirmed the molecular typing by PCR as the generated phylogenetic tree showed homology with (98.49%) between our *Mycoplasma ovipneumoniae* isolate PX660475 and *Mycoplasma ovipneumoniae* isolate (KY562849) detected in Delta region 2017 in Egypt as mentioned by [41]. and (94.84%-94.55%) homology with strains in different countries worldwide as USA during a comparative genomic analysis study that identifies potential adaptive variation in *Mycoplasma ovipneumoniae* isolated from nasal swabs of Bighorn sheep [42] and *M. ovipneumoniae* strain 150; which was isolated from a nasal swab of a sheep with respiratory disease in Central Bosnia Canton in 2009 [43].

Mycoplasma arginini is a type of bacterium that is commonly found in the respiratory tracts of sheep and often acts alongside *Mycoplasma ovipneumoniae* as a co-causative agent in atypical pneumonia occurrences. While it is present in both healthy and ill animals, it is associated with respiratory diseases that can result in lung damage and reduced performance. For both *Mycoplasma arginini* and *Acholeplasma laidlawii*, we conducted sequencing on the PCR products using common generic primers. The phylogenetic analysis of the *Mycoplasma arginini* isolate (PX944911) examined in this research demonstrated a 99.53% similarity with other *Mycoplasma arginini* isolates found in Egypt, specially the KP972458 strain obtained from sheep in Dakahlia in 2016 with higher rate(15.38%) [44] and from sheep in the Giza governorat with lower rate (2.98%) [45]. Additionally, it exhibited a homology of 99.07%-98.60% with strains from various countries around the world, including New York USA [46] whose findings from the genome analysis validated that the *M. arginini* strain from the housefly was very closely related to the isolates obtained from milk. The closest match was found with the M. arginini extracted from the milk on the dairy farm where the housefly was collected. This supports the idea that *M. arginini* might make animals more susceptible to exhibiting more serious symptoms of mastitis when other udder-related factors are present. So, the presence of M. *arginini* with other mycoplasma in case of mixed infection in sheep lungs may support its pathogenic role as mentioned by [45]. Concerning *Acholeplasma laidlawii* the phylogenetic tree showed homology (97.11%) with different percentages between *Acholeplasma laidlawii* isolate PX944921 and other selected Acholeplasma laidlawii isolated in Egypt from tissue culture [47] and two isolates from cases of ulcerative balanitis and vulvovaginitis isolated from sheep in South Africa [48].The phylogenetic analysis of the PCR products of common generic primers for *Mycoplasma arginine and Acholeplasma laidlawii* isolated from lungs showed very high sequence identity between different strains regardless their geographical distribution.

Previous studies have demonstrated that infection with *Mycoplasma* spp. can dysregulate the host immune response, particularly at the level of innate immunity [18, 41]. In the current study, sheep naturally infected with mixed *Mycoplasma* spp. under field condition exhibited significant increase in lysozyme and NO levels in G2, G3 and G4 whereas the highest levels of both mediators were in G4 infected with the three *Mycoplasma* spp. These results suggest that mycoplasma infection in sheep contributes significantly to the dysregulation of the host innate immune markers whereas the mixed infection with multiple spp. (G4) exacerbate its deleterious effects. These finding agree with previous studies that recorded increased level of serum lysozyme and NO production in lambs infected with *M. ovipneumoniae* and *M. arginini* [18]**,** as well as in cattle with *M. bovis*-induced mastitis and in experimentally infected mice [41, 42]. Lysozyme and NO are key components of the antimicrobial responses during the early phases of infection however, excessive production of NO, may potentially contribute to inflammation and tissue damage rather than effective pathogen clearance [43].

Regarding the gene expression of pro-inflammatory markers (IL-8 and CRP) in lung tissue, our results declared that mycoplasma infection in G2, G3, and G4 induced a marked elevation of both markers compared with the normal group (G1), with the highest expression observed in G4. These results suggest that mycoplasma infection induced inflammatory reaction in the lung tissue, whereas mixed infection with multiple spp. (G4) further exacerbates its inflammatory effects. IL-8 expression has been reported to increase in sheep bronchial epithelial cells against capsular polysaccharide (CPS) of *M. ovipneumoniae* [44]. Likewise, elevated IL-8 expression has been reported in *M. pneumoniae* infection in human airway epithelial cells and alveolar macrophages, which is associated with lung tissue damages [45, 46]. Additionally, the significantly elevated CRP levels in infected groups suggest a robust acute phase response to mycoplasma infection. This result agrees with [20, 47] who

reported upregulation of CRP due to *Mycoplasma* infection. The marked increase of both IL-8 and CRP in infected groups may also reflect the interplay between these two markers. CRP promotes IL-8 production via activation of ERK, p38 MAPK, and JNK pathways as stated by [48]. Conversely, IL-8 stimulates the CRP production, providing a potential feedback loop [49]. A baseline expression of these mediators protects host against pathogen invasion. However, their intense production during mycoplasma infection may subsequently damage the immune cells and trigger the systemic inflammatory response [50].

Infection with *Mycoplasma* spp. has been reported to induce oxidative stress in various host spp. In the current research, this was obvious in the infected groups particularly in G4, by a significant reduction in catalase and a significant increase in MDA. These finding reflect the occurrence of oxidative imbalance, in agreement with previous studies [51, 52]. These elevated immune and inflammatory parameters as well as the disruption in the oxidative status in the infected groups may be attributed to the pathogenic effect of specific mycoplasma components, particularly lipid-associated membrane proteins (LAMPs), CPS, variable surface proteins (Vsps), and lipoproteins. Whereas, LAMPs from *M. ovipneumoniae* are potent activators of inflammatory cytokine production by macrophages and monocytes, primarily via Toll-like receptor 2 (TLR2) – mediated NF-κB and MAPK signalling cascades [14, 53, 54]. Similarly, CPS of *M. ovipneumoniae* contributes to inflammation by triggering TLR4-MyD88-NFκB and TLR4-TRIF-IRF3 pathways thereby promoting IL-8 release in sheep bronchial epithelial cells [44]. Also, lipoproteins from *M. arginini* such as TUH-14, activate IL-1, IL-6, and TNF-α production in monocytes [55]. Moreover, Vsps and toxic metabolic byproducts produced by *Mycoplasma* spp. likely contribute to the induced- cellular oxidative damage and immune dysregulation [51, 56].

Conclusion

In conclusion, findings of the current study provide evidence that mixed infection with *Mycoplasma* spp. in sheep dysregulates the innate immune parameters and inflammatory mediators' expression as well as inducing oxidative imbalance. The infection with multiple *Mycoplasma* spp. further amplifies its pathological effects. These immunological and oxidative alterations are likely key factors contributing to the progression and severity of respiratory disease in infected animals.

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Funding statement

This study didn't receive any funding support.

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

The present study was ethically approved by the Ethics Committee of the Faculty of Veterinary Medicine, Benha University, Egypt, (Approval numbers BUFVTM01-09-25).

TABLE 1. Primers used for PCR amplification of *Mycoplasma sp.*

Primer Designation	Primer Name	Sequence (5'-3')	Annealing Temp.	Amplified Product Size	Ref.
Generic primers for Mollicutes	MGSO	-TGC ACC ATC TGT CAC TCT GTT ACC CTC-	55°C	280 bp	[26]
	GPO-3	-GGG AGC AAA CAG GAT TAG ATA CCC T-			
Primers for *M. agalactiae*	MagF	-CCT TTT AGA TTG GGA TAG CGG ATG-	60°C	360 bp	[27]
	MagR	- CCA CTT GTG CGG GTC CCC GTC-			
Primers for *M ovipneumoniae*	MoIGSF	-GGA ACA CCT CCT TTC TAC GG-	58°C	402 bp	[28]
	MoIGSR	-CCA AGG CAT CCA CCA AAT C-			
Primers for *M arginini*	MAGF	- GCA TGG AAT CGC ATG ATT CCT-	55°C	545 bp	[29]
	GP4R -	GGT GTT CTT CCT TAT ATC TAC GC-			

TABLE 2. Target genes, primers sequences and cycling conditions for quantitative real time-PCR.

Target gene	Primers sequences	RT	PD	Amplification (40 cycles)			Dissociation curve (1 cycle)			Ref.
				SD	AnO	Ex	SD	An	FD	
ß2M	5'-AGACACCCACCA GAAGATGG-3' 5'-TCCCCATTCTTC AGCAAATC-3'	50˚C 30 min.	94˚C 5 min.	94˚C 15 sec.	60˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	60˚C 1 min.	94˚C 1 min.	[35]
IL-8	5'-ATGAGTACAGAA CTTCGA-3' 5'-TCATGGATCTTG CTTCTC-3'	50˚C 30 min.	94˚C 5 min.	94˚C 15 sec.	60˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	60˚C 1 min.	94˚C 1 min.	[36]
CRP	5'-CTG GCT TGG GAG ATTG-3' 5'-AGT GAG GGT AAG GGATT-3'	50˚C 30 min.	94˚C 5 min.	94˚C 15 sec.	65˚C 30 sec	72˚C 30 sec.	94˚C 1 min.	65˚C 1 min.	94˚C 1 min.	[37]

ß2M * (ß2-Microglobulin) served as housekeeping gene (internal control). RT: Reverse transcription. PD: Primary denaturation. SD: Secondary denaturation. AnO: Annealing (Optics on). Ex:. Extension. An: Annealing. FD: Final denaturation.

TABLE 3. Total Incidence of *Mycoplasma* isolates in the examined ovine samples and digitonin sensitivity test results.

Total	Recovery Rate		Digitonin sensitivity test		*Mycoplasma* positive samples for digitonin
	Nasal swabs	Lung tissues	Nasal swabs	Lung tissues	
28/60 (46.7%)	18/45 (35.5%)	10/15 (66.7%)	8/45 (17.8%)	9/15 (60%)	17/60 (28.3%)

TABLE 4. Biochemical characterization and serological identification of the obtained isolates.

No. Of Examined mycoplasma isolates (n=28)	Biochemical Tests				Serological identification
	D	G	A	F&S	
8	**+ve**	**-ve**	**+ve**	**-ve**	*M. arginini*
7	**+ve**	**+ve**	**-ve**	**-ve**	*M. ovipneumoniae*
2	**+ve**	**-ve**	**-ve**	**+ve**	*Untyped mycoplasma*
11	**-ve**	**+ve**	**-ve**	**-ve**	*Acholeplasma laidlawii.*

D= Digitonin sensitivity; G = glucose fermentation; A= arginini hydrolysis; F&S= film and spot formation

TABLE 5. The results of IL-8 and CRP mRNA gene expression in lung tissues by quantitative real time-PCR (qRT-PCR).

Groups	IL-8	C-reactive protein (CRP)
G1	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
G2	6.45 ± 0.27^{b}	5.20 ± 0.26^{b}
G3	8.02 ± 0.10^{c}	5.03 ± 0.04^{b}
G4	9.86 ± 0.07^{d}	7.86 ± 0.05^{c}

The relative fold change of IL-8 and CRP mRNA expression was calculated by $2^{-\Delta\Delta Ct}$ method. The CT of each sample was compared with that of G1 as a normal control. G2: sheep infected with *M. arginini* + *Acholeplasma laidlawii* G3: *M. ovipneumoniae* + *Acholeplasma laidlawii.* G4: sheep infected with *M. arginini* + *M. ovipneumoniae* + *Acholeplasma laidlawii*. Data are displayed as mean ±SE at $p < 0.05$ using one-way ANOVA test. Similar small litter in the same column indicates no significant difference between groups.

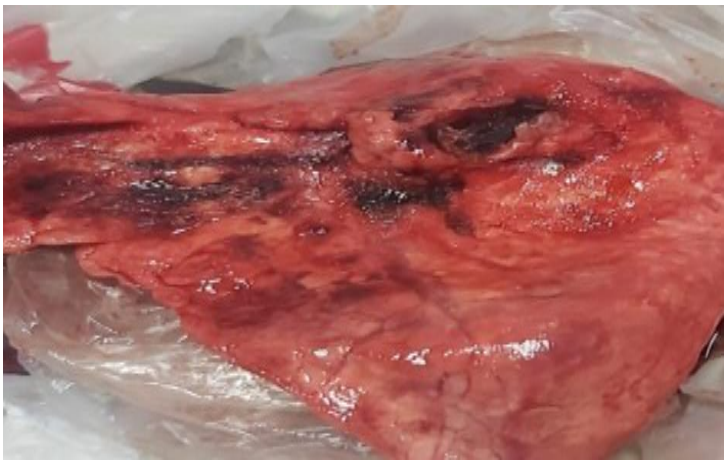

Fig.1. Lung from *Mycoplasma* sp. infected sheep with pneumonia shows congestion and edema with reddish and gray hepatization.

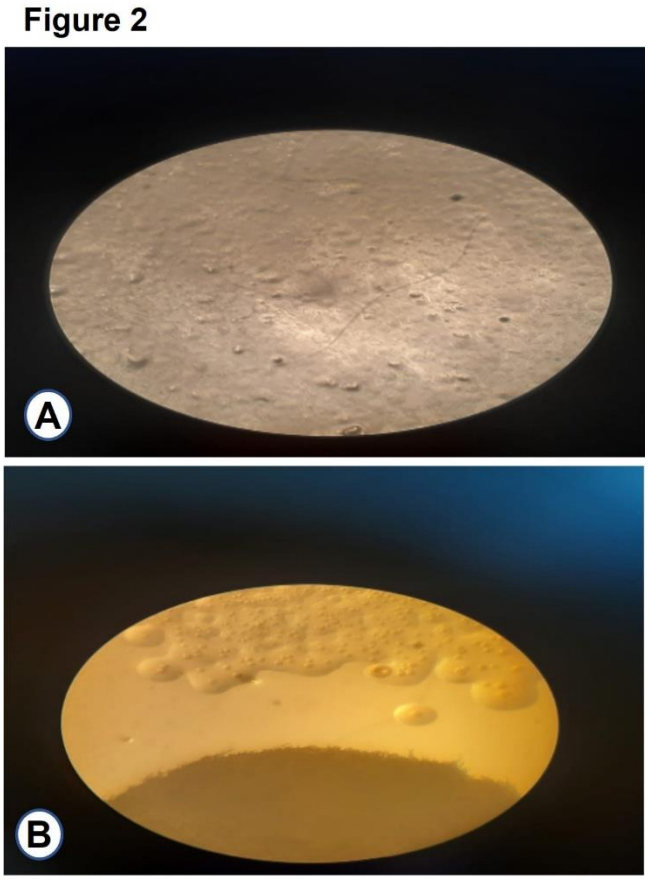

Fig. 2. Isolation of *Mycoplasma* colonies, (A): Heavy condensed *Mycoplasma* colonies from primary isolation from sheep lung, (B): *Mycoplasma* colonies with fried egg appearance under dissecting microscope showing inhibition zoon with digitonin disk.

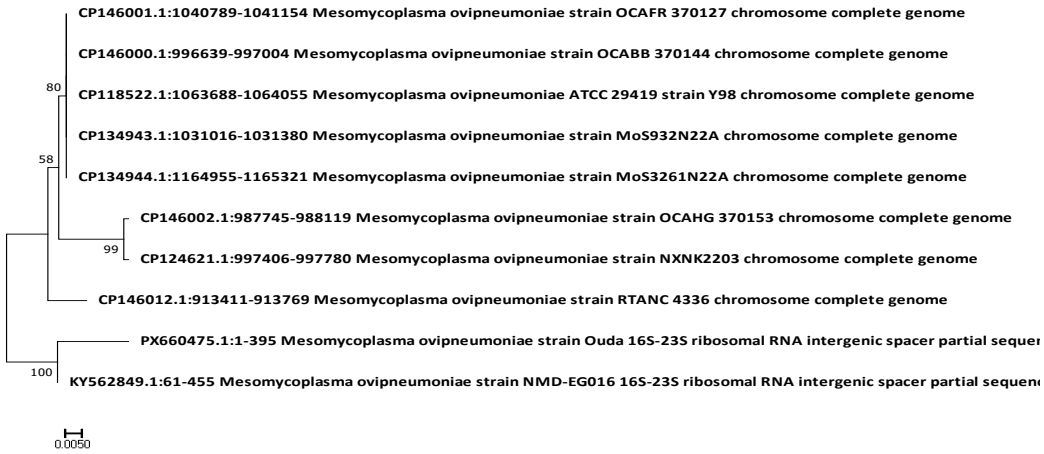

Fig. 3. Phylogenetic tree of *M. ovipneumoniae*; isolated in this study from pneumonic lung of sheep showing genetic relatedness with different strains recorded on GenBank.

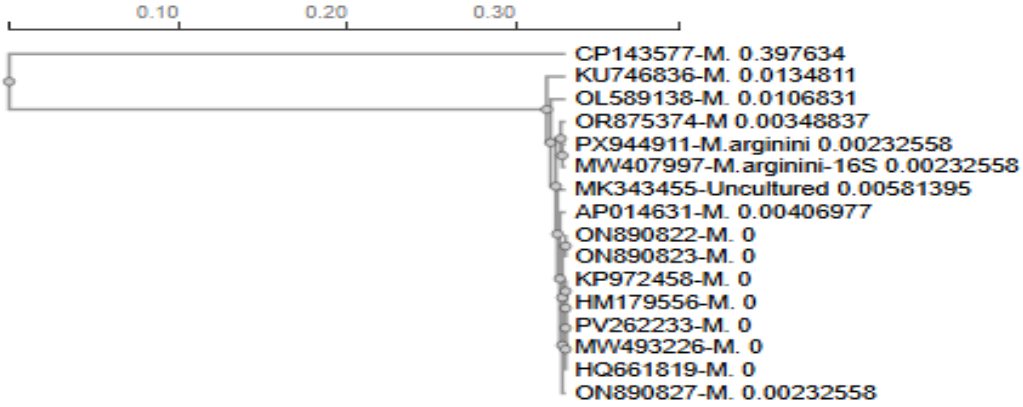

Fig. 4. Phylogenetic tree of *M. arginini;* isolated in this study from pneumonic lung of sheep showing genetic relatedness with different strains recorded on GenBank.

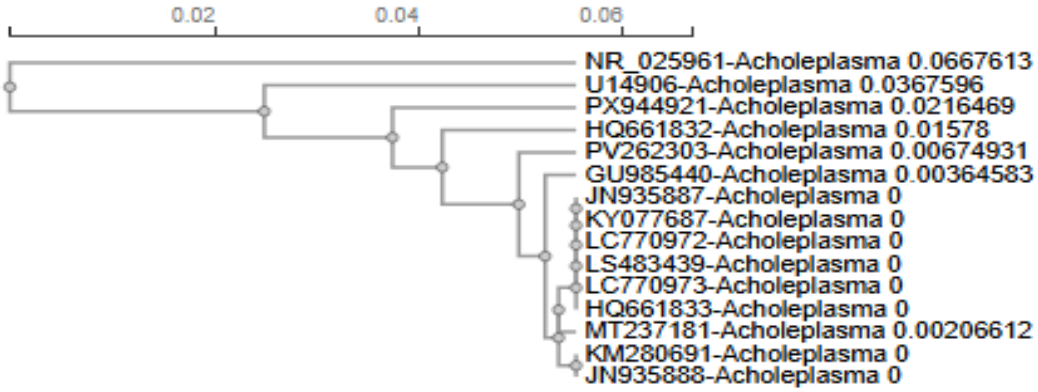

Fig. 5. Phylogenetic tree of *Acholeplasma laidlawii*; isolated in this study from pneumonic lungs of sheep showing genetic relatedness with different strains recorded on GenBank.

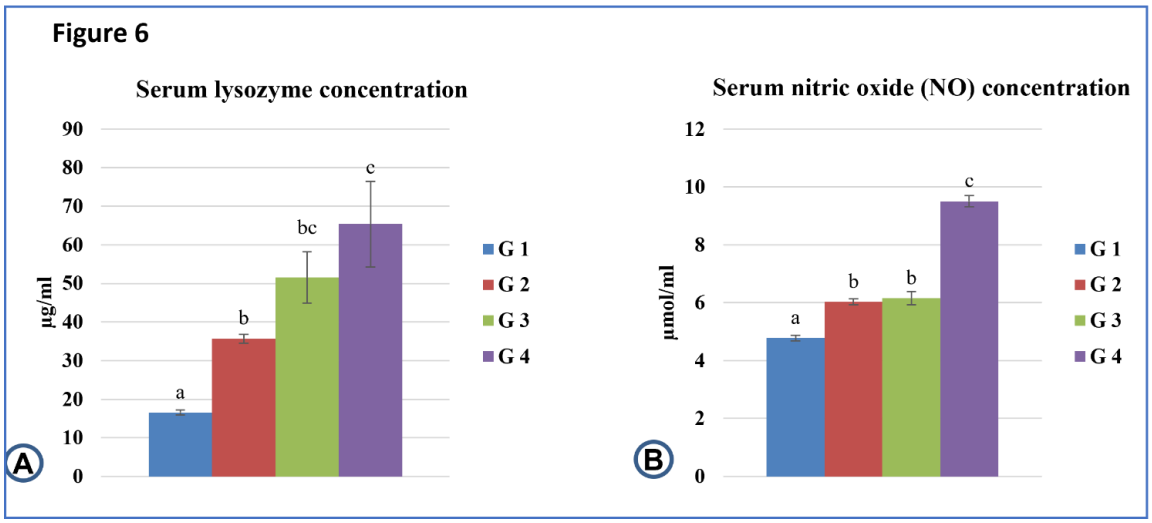

Fig. 6. The results of serum lysozyme activity assay (A) and nitric oxide (NO) assay (B) in all sheep groups. G1: non infected sheep. G2: sheep infected with *M. arginini* + *Acholeplasma laidlawii* G3: *M. ovipneumoniae* + *Acholeplasma laidlawii.* G4: sheep infected with *M. arginini* + *M. ovipneumoniae* + *Acholeplasma laidlawii*. Data are displayed as mean ±SE at $p < 0.05$ using one-way ANOVA test. Similar small litter in the same column indicates no significant difference between groups.

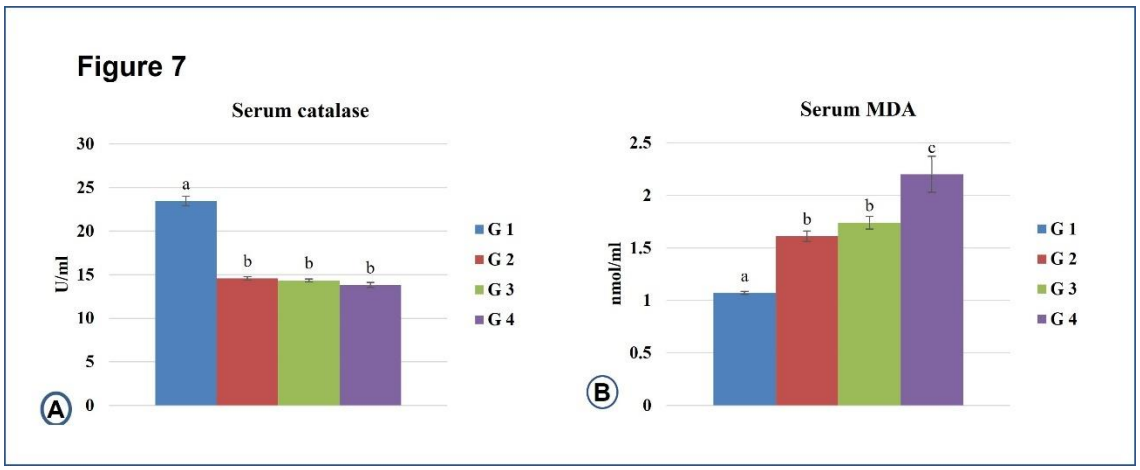

Fig. 7. The results of serum catalase enzyme assay (A) and MDA assay (B) in all sheep groups G1: non infected sheep. G2: sheep infected with *M. arginini* + *Acholeplasma laidlawii* G3: *M. ovipneumoniae* + *Acholeplasma laidlawii.* G4: sheep infected with *M. arginini* + *M. ovipneumoniae* + *Acholeplasma laidlawii*. Data are displayed as mean ±SE at $p < 0.05$ using one-way ANOVA test. Similar small litter in the same column indicates no significant difference between groups.

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التوصيف الظاهري والجيني والتأثيرات المناعية لعدوى الميكوبلازما في الأغنام

سحرالسيد عوده[1]* ، فريدة حسن محمد [2] ، شيرين محمدعلي[2] ، عماد بشير عطا[3] ، رانيا أبوسقاية[4]

[*]و صبرى اسماعيل عيسي [1]

[1] قسم الميكوبلازما، معهد بحوث صحة الحيوان، مركز البحوث الزراعية، الجيزة ، مصر.

[2] قسم المناعة، معهد بحوث صحة الحيوان، مركز البحوث الزراعية، الجيزة ، مصر.

[3] قسم علم الطفيليات وأمراض الحيوان، المركز القومي للبحوث، الجيزة ، مصر.

[4] قسم طب الحيوان، كلية الطب البيطري، جامعة بنها، مصر.

الملخص

تلعب الأغنام دورًا حيويًا في القطاع الزراعي بمصر. وتُعدّ أمراض الجهاز التنفسي الناجمة عن الميكوبلازما مصدرًا رئيسيًا للخسائر الاقتصادية في هذا القطاع. في هذه الدراسة، جُمعت مئة وعشرون عينة من الأغنام من ثلاث مناطق مختلفة في مزرعة أغنام بمحافظة الجيزة. رُصدت أعراض عدوى الجهاز التنفسي، ثم جرى عزل الميكوبلازما. وقد تم عزل الميكوبلازما أرجينيني بنجاح من منطقتين مختلفتين. كما تم الكشف عن الميكوبلازما الرئوية للأغنام في العينات. بالإضافة إلى ذلك، عُزلت الأكولبلازما لايدلاوي من جميع المناطق قيد الدراسة. تم تأكيد السلالات المختلفة من خلال تحليل تفاعل البوليميراز المتسلسل (PCR) والتسلسل الجيني. صُنفت جميع عينات المصل وأنسجة الرئة إلى أربع مجموعات. المجموعة الأولى (G1) ضمت الأغنام غير المصابة، والمجموعة الثانية (G2) ضمت الأغنام المصابة بالميكوبلازما أرجينيني والأكولبلازما، والمجموعة الثالثة (G3) ضمت الأغنام المصابة بالميكوبلازما الرئوية للأغنام والأكولبلازما لايدلاوي. المجموعة الرابعة (G4): أغنام مصابة ببكتيريا الميكوبلازما أرجينيني، والميكوبلازما الرئوية، والأكولبيلازما لايدلاوي. تم قياس نشاط الليزوزيم، وأكسيد النيتريك (NO)، وإنزيم الكاتالاز، ومستويات مالونديالدهيد (MDA) في مصل الدم. كما تم تحديد مستوى الحمض النووي الريبوزي المرسال (mRNA) للإنترلوكين-8 (IL-8) والبروتين التفاعلي C (CRP) في أنسجة الرئة باستخدام تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي (qRT-PCR). أظهرت النتائج أن عدوى الميكوبلازما زادت بشكل ملحوظ من نشاط الليزوزيم، وأكسيد النيتريك، والإنترلوكين-8، والبروتين التفاعلي C، ومستويات مالونديالدهيد في مصل الدم، بينما انخفض نشاط الكاتالاز بشكل ملحوظ في المجموعات المصابة (G2، G3، G4) مقارنةً بالمجموعة الضابطة (G1). والجدير بالذكر أن العدوى المتعددة بالميكوبلازما في المجموعة الرابعة (G4) زادت من حدة هذه الاضطرابات المناعية والتأكسدية. في الختام، أدت العدوى ببكتيريا الميكوبلازما في الأغنام إلى اضطراب الاستجابة المناعية الفطرية، وتفاقم التعبير عن الوسائط الالتهابية، وإحداث خلل في التوازن التأكسدي. وقد أدى ذلك إلى تفاقم آثاره الضارة.

الكلمات الدالة: الميكوبلازما أرجينيني، الميكوبلازما الرئوية، أكولبلازما لايدلاوي، المناعة، الأكسدة، الليزوزيم، مالونديالدهيد.

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