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The effect of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* infection on innate immune response and hematological parameters of lambs

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Research

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Abstract

Background: Sheep are among the important livestock in agriculture in Egypt. Respiratory conditions associated with Mycoplasma infection are considered common sources of economic losses. Moreover, Mycoplasma infection in livestock can disrupt the hematological profile, innate immune response. Nonetheless, there is a scarcity of studies examining the impact of *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) and *Mycoplasma arginini* (*M. arginini*) mixed infections on the innate immune response and hemogram profile in lambs.

Objective: To explore how mixed infection with these two mycoplasma species influences both the innate immune parameters comprising lysozyme and nitric oxide and the hemogram profile in lambs.

Materials and methods: Nasal and ocular swabs were taken from apparently healthy and diseased lambs aged between 2 and 5 months for isolation of Mycoplasma species**.** Identification of the recovered isolates was carried out via polymerase chain reaction (PCR) assays. Moreover, serum and blood with anticoagulant were obtained from four healthy lambs and four co-infected ones with the two Mycoplasma species (*M. arginini* and *M. ovipneumoniae*) for nitric oxide (NO), serum lysozyme activity and hematological analysis.

Results: The isolation of *M. arginini* and *M. ovipneumoniae* from the sick lambs was validated by PCR analysis. It was determined that four lambs had a combined infection with both species. In comparison to the lambs that appeared to be healthy, the Mycoplasma-co-infected lambs had marked higher amounts of lysozyme and nitric oxide. Hematological investigations revealed alterations in the hemogram of sheep co-infected with low normal erythrocyte counts. In contrast to the seemingly healthy lambs, the leucogram of the co-infected lambs also revealed leukocytosis due to neutrophilia.

Conclusion: Co-infection with *Mycoplasma ovipneumoniae* and *M. arginini* species in lambs altered the innate immune response and the hemogram profile, which may be accountable for the disease pathogenesis and progress.

Keywords: *M. ovipneumoniae, M. arginini, innate immunity, and hemogram profile.*

Introduction

Mycoplasmas are tiny wall-less prokaryotes classified within the Mollicutes group **(Parker *et al.* 2018)**. Over 100 Mycoplasma species exist and those within the *Mycoplasma mycoides* group cause diseases in ruminants **(Weiser *et al.* 2012)**. Approximately 40 Mycoplasma species have so far been described in domestic ruminants including cattle, sheep and goats **(Chazel *et al.* 2010)**. In ruminants, clinical manifestations accompanying with *Mycoplasma* infection vary from pneumonia to conjunctivitis, arthritis and mastitis **(Kumar *et al.* 2013)**. Mycoplasma infections cause great economic losses due to the high morbidity and mortality rates they induce in sheep and goat populations in African countries including Egypt as well as in European countries and India **(Sandip *et al.* 2012)**. Different mycoplasmas have been isolated from the respiratory systems of sheep and goats. *M. ovipneumoniae* is the most common species identified in these animals. This species has been detected in both healthy and diseased sheep and goats **(Ruffin, 2001)**. Additionally, *M. arginini* has been frequently isolated from healthy and diseased sheep and goats alone or in association with other pathogenic bacteria **(Chazel *et al.* 2010)**.

Understanding how mycoplasma infections impact the immune system is vital for treatment. The primary immune response serves as the body's initial defense against these pathogens and it's a significant factor in dealing with such infections **(Kogut *et al.* 2020)**. Key elements of this response include lysozyme, an enzyme that fights microbes and nitric oxide (NO), which facilitates inflammation. Lysozyme is crucial in the host's inherent immune response. Its primary function involves breaking down the β-1,4-glycosidic bonds found in the peptidoglycan of bacterial cell walls. This action causes bacteria to burst open and perish **(Masschalck and Michiels, 2003)**. Lysozyme is principally effective against Gram-positive bacteria, which contain a thick peptidoglycan layer. It can also trigger the lysis of Gram negative bacteria when combined with other antimicrobial agents. Moreover, lysozyme acts as an immunomodulatory agent controlling both innate and adaptive immunity **(Ragland and Criss, 2017)**. Nitric oxide also plays a key role in host immune defense predominantly generated by activated macrophages and neutrophils through inducible nitric oxide synthases. It acts as a pro-inflammatory agent and influences immunological responses defending against a range of infectious pathogens **(Roberts *et al.* 2024)**. On the other hand, the excessive and long-lasting creation of NO has its down sides as it changes how host cells work causing oxidative stress, wide spread inflammation and injury to tissues **(Li *et al.* 2002)**.

Previous researches concerning *Mycoplasma* infections in farm animals have suggested that *Mycoplasma* species could alter the innate immune responses. Previous studies have stated that *Mycoplasma bovis* infection resulted in an increase in lysozyme activity and NO production in cattle suffering from abortion and mastitis **(Ramadan *et al.* 2009** and **Ahmed et al. 2019)**. Likewise, experimental infection with *Mycoplasma bovis* in albino mice led to a significant elevation of lysozyme levels **(Fathi, 2022)** indicating a prospective role of *Mycoplasma* infection in altering host immune responses. Also, an earlier research showed that lipoprotein from *M. arginini* altered the function of the human monocyte and promoted the inflammatory response **(Herbelin *et al.* 1994)**. Moreover, the mycoplasma biofilm was found to stimulate the phagocytic cells to release the lysosomal enzymes and reactive oxygen and nitrogen species **(McAuliffe *et al.* 2006)**.

However, limited researches display the effect of *M. ovipneumoniae* and *M. arginini* mixed infection on lambs innate immune response and hemogram profile; therefore, the current study investigated the effect of these two *Mycoplasma* species on the innate immune markers (lysozyme and NO) and hemogram profile of infected lambs.

Materials and Methods

Animals and sampling

Twenty diseased sheep suffering from recurrent outbreak infection with pneumonia in Menofia Governorate were investigated. They were treated with antibiotics till the disappearance of respiratory symptoms, then a new pneumonia outbreak associated with lameness appeared in lambs aged between 2 to 5 months in the same farm and so, further clinical examination were done on these lambs. Nasal and ocular swabs were taken from lambs either ap-

parently healthy (group 1, G1) or diseased (group 2, G2), 20 each and subjected for mycoplasma isolation and identification. Moreover, serum and anti-coagulated blood samples were taken from the investigated lambs to evaluate the serum lysozyme activity and NO levels and hematological analysis, respectively. The anti-coagulated blood samples were collected on dipotassium ethylenediaminetetraacetic acid (EDTA) as an anticoagulant.

***Mycoplasma* isolation and characterization**

Mycoplasma isolation and propagation on liquid and solid pleuropneumonia-like organisms (PPLO) media was done as described previously **(Sabry and Ahmed, 1975)**. Moreover, sensitivity of the recovered isolates to digitonin was done to differentiate between Mycoplasma and Acholeplasma species **(Sabry and Ahmed, 1975)**. Characterization of the isolates was further carried out using film and spot formation assay as mentioned elsewhere **(Fabricant and Freundt, 1967)** and biochemically via fermentation of glucose and deamination of arginine as described earlier **(Erno and Stipkovits, 1973)**.

Molecular identification of *Mycoplasma* species

DNA extraction

To perform polymerase chain reaction (PCR), DNA was extracted according to the methods described previously **(Yleana *et al.* 1995)**. Briefly, five milliliters of 24 h broth cultures of the recovered isolates were centrifuged at 12,000 rpm for ten minutes. The pellets were suspended in 50 µL of phosphate-buffered saline (PBS) after being rinsed in 1 mL of PBS (pH 7.2). After 10 minutes of direct heating at 100°C in a water bath to rupture the cell wall, the cell suspension was allowed to cool on ice for five minutes. Following five minutes centrifugation of the cell solution, DNA in the supernatant was collected and kept at - 20°C until used.

PCR amplification and cycling protocols

A total reaction volume of 50 µL consisting of 25 µL of master mix (2X), 1 µL of 10 pmoL of each primer (Sigma-Aldrich, USA, Table 1), 5 µL of extracted DNA and nuclease free water up to 50 µL was prepared. In thermo cycler (GTC96) Cleaver scientific Ltd., PCR reactions were carried out with the following amplification programs for molecular identification of *M. arginine*, initial denaturation at 94°C for 5 minutes, then 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 10 minutes. Moreover, the cycling conditions for *M. ovipneumoniae* identification were 94°C for 5 minutes, then 35 cycle comprising 94°C 30 seconds, 58°C 45 seconds and 72°C 45 seconds), followed by a final extension cycle at 72° C for 10 minutes.

Table (1). Primer sequences used for the PCR analysis

Specificity	Primer sequence (٥'-٣')	Amplicon size (bp)	Reference
M. arginini	MAGF: 5'-GCATGGAATCGCATGATTCCT − 3' GP4R: 5'- GGTGTTCTTCCTTA-TATCTACGC-3	(bp)	**Timenetsky *et al.* (2006)**
M. ovipneumoniae	F: GGAACACCTC CTTTCTACGG R: CCAAGGCATC CACCAAATAC	402	**Besser *et al.* (2012b)**

Electrophoresis
Using agarose gel (1.5%) electrophoresis in tris -acetate-EDTA buffer, PCR products were visualized under UV light using 100 bp DNA ladder as a size marker.

Evaluation of serum lysozyme activity
The activity of lysozyme in serum samples of lambs from G1 and G2 was evaluated by agarose gel plate lyses assay as described previously (**Schultz, 1987)**. Briefly, fifty milligrams of lyophilized *Micrococcus lysodeikticus* bacteria were dispersed in 100 mL of 1% agarose in 0.06 m PBS solution at pH 6.3. The agarose was then poured into petri dishes, where 25 µL of serum samples and standard lysozyme were tested in individual wells. After 18 hours, the diameter of the zones of clear hydrolysis was measured. The concentration of lysozyme was determined using a logarithmic curve created from various concentrations of standard lysozyme solutions.

Evaluation of serum nitric oxide levels
It was carried out on serum samples of lambs from G1 and G2 according to the method detailed previously **(Yang *et al.* 2010)**. A total of 100 µL of serum samples that had been deproteinized was mixed with an equal volume of Griess reagent in a flat bottom 96-well ELISA plate at 25º C for 10 minutes. The absorbance was recorded at 540 nm with an ELISA reader and the nitric oxide concentration was determined from a standard curve using sodium nitrite ($NaNO_2$).

Evaluation of the hematological profile
Hematological analysis was carried out on the anti-coagulated blood samples from lambs in G1 as well as G2 by calculating the total erythrocytes (RBCs) counts x 10^6 / µL and the differential leucocytes counts x 10^3 / µL.

Statistical analysis
Statistical analysis of the obtained results was carried on using IBM SPSS software version 23.0 (IBM SPSS Statistics for Mac OS, Armonk, NY, USA). Expression of results was done as mean values ± standard error (SE).

Results
Clinical examination
The infected lambs displayed nasal and eye discharges along with inflamed corneas and conjunctiva, coughing, lameness and rise of body temperature.

Results of mycoplasma isolation and identification
Seventeen Mycoplasma isolates (42.5%) were obtained from nasal and ocular swabs collected from examined lambs (Table 2). The isolates were further identified through conventional identification tests and then categorized using PCR assays with specific primers (Table 3). Colonies of Mycoplasma isolates resembled the appearance of fried egg under stereomicroscope (Figure 1). Nine of these isolates were identified as *M. arginini*, four were recognized as *M. ovipneumoniae*, while four isolates were classified as undifferentiated Mycoplasma species (Table 3 and Figures 1 and 2). Co-infection with both *M. arginini* and *M. ovipneumoniae* was identified among four infected lambs in G2.

Table (2). Isolation percentages of Mycoplasma and Acholeplasma species from examined lambs

Digitonin test result (Identified species)	No. of recovered isolates from lambs (%)		
	Apparent Healthy (20)	Diseased (20)	Total (40)
Resistant (*Acholeplasma*)	3 (15)	5 (25)	8 (20)
Sensitive (*Mycoplasma*)	4 (20)	13 (65)	17 (42.5)

Table (3). Isolation percentages of Mycoplasma species from collected swabs from lambs along with their biochemical identification and PCR results.

Swab type (No.), biochemical identification tests and PCR results	**No. of recovered *Mycoplasma* species from lambs (%)**					
	Apparent Healthy (20)			**Diseased (20)**		
Nasal (20)	3 (15)	0	0	3 (15)	8 (40)	0
Ocular (20)	1 (5)	0	0	1 (5)	1 (5)	0
Total (40)	4 (10)	0	0	4 (10)	9 (22.5)	0
Glucose fermentation	+ ve	- ve	- ve	+ ve	- ve	- ve
Arginine deamination	- ve	+ ve	- ve	- ve	+ ve	- ve
Film and spot	- ve	- ve	+ ve	- ve	- ve	+ ve
PCR-identified *Mycoplasma* species (No.)	Undifferentiated *Mycoplasma* species (4)			Both *Mycoplasma ovipneumoniae and Mycoplasma arginine* (4) *Mycoplasma arginine* (5)		

+ ve: positive; - ve: negative

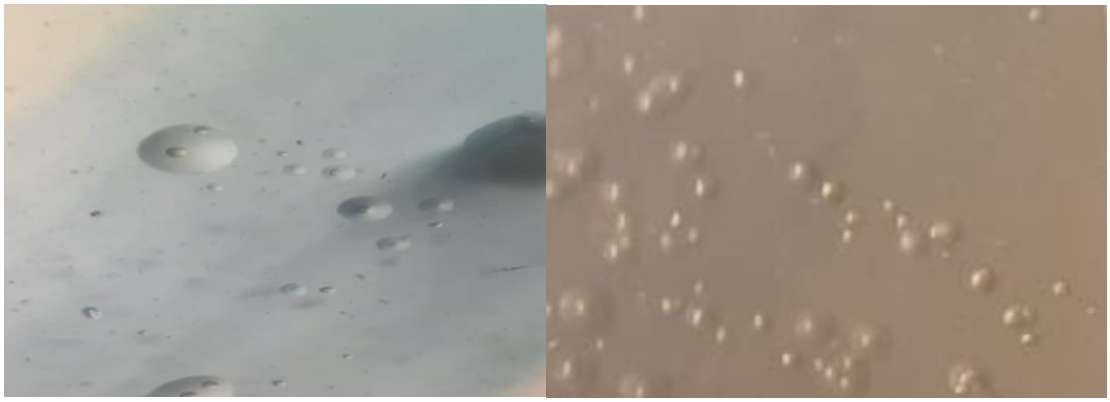

Figure (1). Fried egg shaped Mycoplasma colonies under stereomicroscope.

Figure (2). Agarose gel electrophoresis of PCR amplified products using *M. arginini* PCR primer sets. Lane1: 00 bp DNA marker, lane 2: control positive *M. arginini* strain, lanes 3-: *M. arginini* recovered isolate at (545 bp).

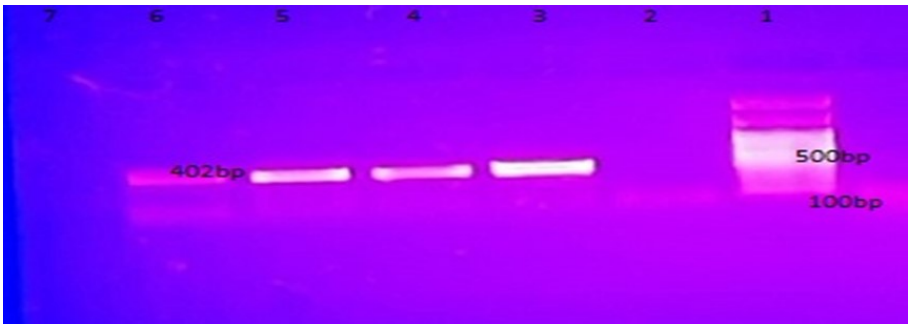

Figure (3). Agarose gel electrophoresis of PCR amplified products using *M. ovipneumoniae* PCR primer sets. Lane1: 100 bp DNA ladder, lane 2: control negative, lane 3: control positive *M. ovipneumoniae* strain, lanes 4-6: *M. ovipneumoniae* recovered isolates (402 bp).

Serum lysozyme activity and NO assays results

The serum lysozyme activity assay results shown in Figure 4 clarified that lambs co-infected with Mycoplasma species in G2 displayed a marked higher mean value of lysozyme activity (42.3± 6.9) compared to the apparently healthy control lambs in G1(15.6 ± 2.6). Similarly, the results of NO assay shown in Figure 5 demonstrated that Mycoplasma co-infected group (G2) showed a noticeable higher mean value of NO (7.01 ± 0.15) than the apparently healthy control lambs in G1 (4.53 ± 0.12).

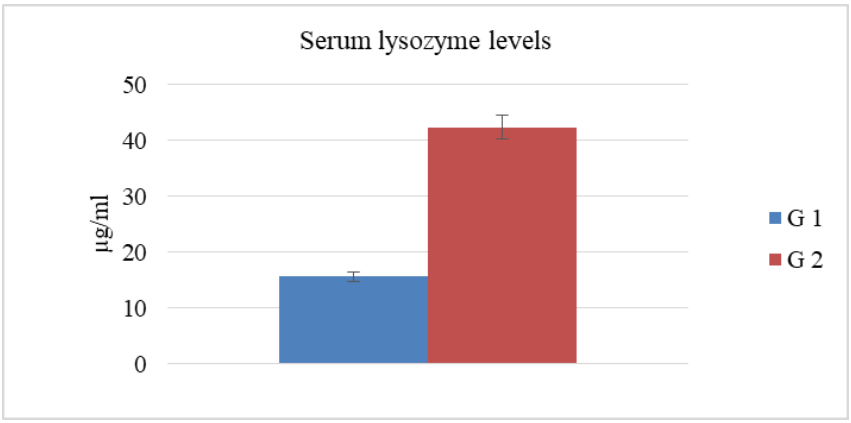

Figure (4). Effect of mixed Mycoplasma infection on the lamb's serum lysozyme activity. Group 1 (G1) are apparently healthy control lambs, group 2 (G2) are lambs with mixed Mycoplasma infection. The data are displayed as the mean values with the SE bar.

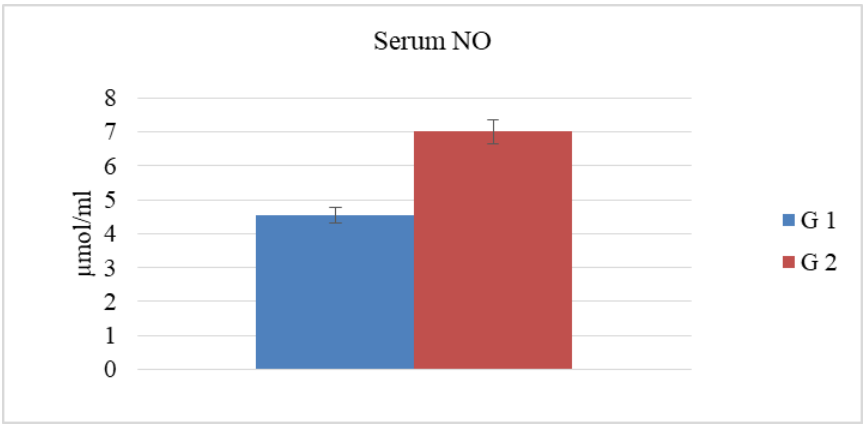

Figure (5). Effect of mixed Mycoplasma infection on the lamb's serum nitric oxide (NO) level. Group 1 (G1) are apparently healthy control lambs, group 2 (G2) are lambs with mixed Mycoplasma infection. The data are displayed as the mean values with the SE bar.

Hematological analysis results

The obtained data of the hematological analysis demonstrated low normal values of erythrocytes' counts (8.56 ± 1.8 x 10^6 /µL, Figure 6), leukocytosis (13.9 ± 2.3 x 10^3 /µL) and neutrophilia (7.65 ± 1.5 x 10^3 /µL, Figure 7) in mixed *Mycoplasma* infected lambs (G2) comparing with the apparently healthy ones, G1 (10.4 ± 1.6 x 10^6 /µL, 11.9 ± 2.1 x 10^3 /µL and 5.59 ± 1.2 x 10^3 /µL, respectively). Mature neutrophils were predominating in stained blood smears of lambs with Mycoplasma infection (G2).

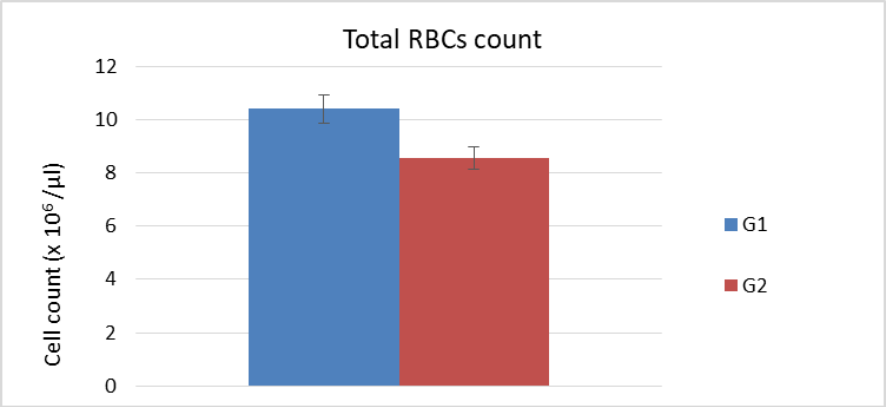

Figure (6). Hematological analysis of apparently healthy control (G1) and mixed Mycoplasma infected lambs (G2). Data are presented as mean values with SE bar; erythrocytes counts x 10^6 / µL.

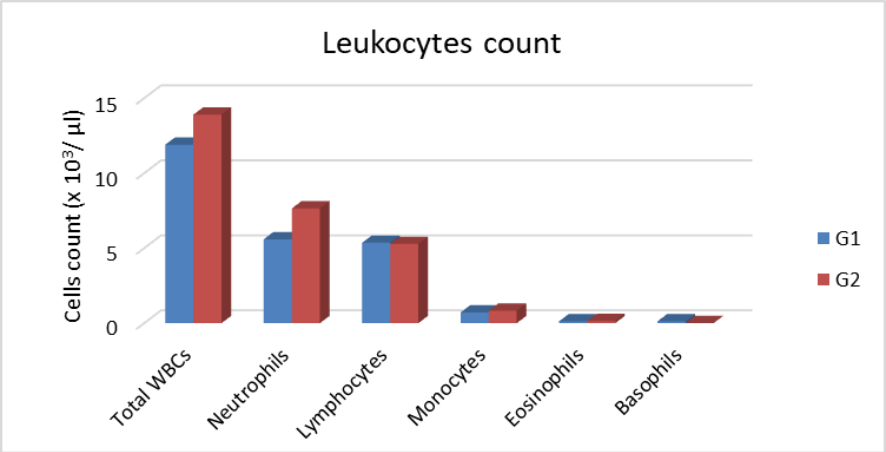

Figure (7). Differential leucocytes' counts of apparently healthy control (G1) and mixed Mycoplasma infected lambs (G2). Data are presented as mean values of hemogram; leucogram x 10^3 µL.

Discussion

Small ruminant mycoplasmal infections are widespread throughout the world and provide a significant socioeconomic challenge, especially in areas, where the population depends heavily on small ruminants for providing milk and meat **(Frey, 2002)**. In the current study, a total of seventeen Mycoplasma isolates were isolated from the examined lambs, identified by biochemical tests and confirmed and typed by PCR using specific primers. Nine isolates were confirmed as *M. arginini* and four isolates as *M. ovipneumoniae.* Among the infected lambs, there were four in group 2 co-infected with both *M. arginini* and *M. ovipneumoniae*. These data demonstrated the high prevalence of these Mycoplasma species in sheep farms in Menofia Governorate, which come in accordance with previous reports, where high occurrence of *M. ovipneumoniae* and *M. arginini* infection were recorded in sheep and goats farms in Egypt, and also the young animals was found to be more susceptible than the adult ones **(Ibrahim *et al.* 2018**

and **Dardeer *et al.* 2006)**. The obtained data of the innate immune mediators, serum lysozyme and NO, revealed a marked increase in both parameters (42.3 ± 6.9 and 7.01 ± 0.15 respectively) in mixed Mycoplasma infected lambs (G2) compared to apparently healthy controls (G1) (15.6 ± 2.6 and 4.53 ± 0.12 respectively) (Figures 4 and 5). These results imply that *Mycoplasma* infection in lambs contributes significantly to the deregulation of the host immune response by inducing the generation of innate immune markers. This finding agrees with earlier authors (**Ramadan *et al.* 2009**, **Ahmed *et al.* 2019,** and **Fathi, 2022)**, who stated that *M. bovis* infection induced a significant elevation in NO and lysozyme levels in both mastitic cattle and experimentally infected mice. Lysozyme and NO are key constituents of the antimicrobial responses of innate immunity. However, excessive production of these immune mediators as occurred in co-infected group (G2) may potentially contribute to immune dysregulation, inflammation and tissue damage rather than effective pathogen clearance **(Camp and Jonsson, 2017)**. The increased lysozyme and NO in G2 may be attributed to the immune defense against *Mycoplasma* antigens, metabolites and biofilms. Infection with *M. ovipneumoniae* antigens was found to stimulate a strong immune response in the lungs, trachea, and bronchi **(Xu *et al.* 2022)**. The main virulent component of *M. ovipneumoniae* is the capsular polysaccharide, which activates toll-Like Receptor (TLR) signaling, thereby triggering an inflammatory response in sheep airway epithelial cells. This was achieved via activation of both TLR4-MyD88-NFκB and TLR4-TRIF-IRF3 signaling pathways **(Jiang *et al.* 2017)**. This response is linked to the attraction of macrophages, neutrophils, lymphocytes and natural killer cells **(Waites and Talkington, 2004)**, which generate lysosomal enzymes, reactive oxygen species and reactive nitrogen species like NO (**McAuliffe *et al.* 2006** and **Hermeyer *et al.* 2011**). Furthermore, *Mycoplasma arginini*, in particular its lipoprotein, has been shown to change the function of the human monocyte and promote the inflammatory response, which may also be the cause of the elevated immune parameters (**Herbelin *et al.* 1994**).

In the same regard, the hematological results (Figures 6 and 7) showed leucocytosis characterized by neutrophilia in co-infected lambs as previously proved **(Abed and Alsaad, 2017)**, where *Mycoplasma ovipneumoniae* caused an increase in the leucocyte cells' counts in infected lambs. The altered hemogram in infected group may be attributed to the increased inflammatory and immunological response towards *Mycoplasma* infection **(Waites and Talkington, 2004)**.

Conclusion

Combined infection with *M. ovipneumoniae* and *M. arginini* caused a negative impact on both the innate immune response and hematological picture of lambs, which may be responsible for the disease progress. Further studies are needed to better understand the pathogenic effect of such co-infection in small ruminants.

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