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Levels of IL-10 in lambs pre- and post-exposed experimentally to Mycoplasma ovipneumoniae

Ali Hasan Mejbel[1]*, Zainab Ismail Ibrahim[2]

[1, 2]Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
[1]Department of Oral Diagnosis, College of Dentistry, University of Wasit, Wasit, Iraq
Email: Ali.Hasan1207a@covm.uobaghdad.edu.iq (alihalshrify@uowasit.edu.iq)[1], zainabaalrubaei@covm.uobaghdad.edu.iq[2]
* Corresponding Author

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ABSTRACT
Background: Mycoplasma ovipneumoniae is a respiratory pathogen of domestic sheep, mainly, which causes atypical pneumonia and can also predispose to lung infections and recurrent fatal lamb pneumonia outbreaks in subsequent years.
Aims: Isolation of M. ovipneumoniae from the suspected pneumonic sheep, induction of an experimental infection in lambs by inhalation and measurement the levels of interleukin-10 (IL-10) in lambs pre- and post-exposure to the bacterium at different days (0, 6, 14, and 28) as well as in vaccinated lambs by ELISA.
Materialsand 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 used to preparation of infection solution and vaccine. Then, an overall18 lambs of 5-6 months of age were selected, acclimated and divided randomly into infected and vaccinatedgroups. In infected group, thelambs (No=12) were divided equally into three subgroups; (A): non-infected and non-treated, (B): infected but not treated, and (C)infected and antibiotic-treated.In vaccinated group (No=6), the lambswere vaccinated with two doses at 2 weeks intervals. For serological estimation of IL-10, venous bloodwas collected once from the lambs of infected group at day 28; whereas, it was collected from vaccinated lambs at the 0, 6, 14 and 28 days of vaccination.
Results: An overall 13.51%of study sheep were positive to M. ovipneumoniaethat 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. In the vaccinated group, the findings of IL-10 were elevated significantly ($p \leq 0.0306$) in lambs at the 28^{th} day of first-dose vaccination (96.36 ± 3.27 pg/ml) when compared to values of other days; 0 (74.84 ± 3.47 pg/mL), 6 (79.07 ± 2.94 pg/mL), and 14 (74.91 ± 2.18 pg/mL). In infected group, the findings of subgroup B (164.63 ± 532 pg/mL) were elevated significantly when compared to values of subgroups A (82.45 ± 2.06 pg/mL) and C (108.39 ± 4.89 pg/mL).
Conclusion: Mycoplasma ovipneumoniae is primarily prevalent in pneumonic sheep. The expression of IL-10 showed differential patterns across variousvaccination times, with variations observed among infected groups. Subsequently, administration of antibiotic had revealed a significant ability to reduce the overexpression of IL-10 in infected lambs. However, furthermore studies to investigate the prevalence of M.ovipneumoniaeor other mycoplasmas by the molecular tools are necessary. Also, the role of IL-10 and other cytokines in various anatomical sites of respiratory tract throughout disease progression need to moreover studies

Keywords: Mycoplasmosis, Anti-inflammatory cytokine, Inhalation, enzyme-linked immunosorbent assay (ELISA), Iraq

INTRODUCTION

Respiratory syndromes commonly encountered in sheep populations, are often caused by multifactorial agents, including infectious agents like viruses, bacteria, fungi, and parasites, as well as several predisposing management factors such as high stocking densities and low ventilation rates in intensely bred lambs; stress induced by live animal movements, different ecoclimatic factors and season of the year (Peek et al., 2018; Al-

Abediand Al-Amery, 2021; Maksimovićet al., 2022; Al-Juwari, 2023;Ali, 2024). Many advance have been made in the diagnosis and treatment of bacterial infections in animals over the past decades, but understanding of Mycoplasma infections in animals remains inadequate (Maeset al., 2018; Dawoodet al., 2022). The absence of information regarding the role of Mycoplasma as a causative agent in sheep disease is primarily related to the scarcity of studies, including those on Mycoplasma in sheep (Dudeket al., 2022). Moreover, the primary habitat of Mycoplasma in clinically healthy sheep remains unknown (Spaanet al., 2021; Thomaset al., 2024).

Mycoplasma belongs to the class Mollicutes, which consist of wall-less prokaryotes that are among the smallest known self-replicating organisms (Rosales et al., 2017). The absence of the bacterial cell wall in Mycoplasma species makes their infection difficult to diagnose and treat (Cazanaveet al., 2012). Mycoplasma-related diseases are undetectable when diagnosis is based solely on clinical indicators such as pathological lesions or serological testing, primarily because of the close taxonomic association of many Mycoplasma species (Tully et al., 2019; Huggins et al., 2022). Numerous Mycoplasma species / serotypes are associated with various pathological complications in small ruminants, including respiratory signs, causing major losses (Kebkiba and Antipas, 2022; Aliet al., 2024). Since it was first isolated from the lungs of sheep with pulmonary adenomatosis in Scotland in 1963, M. ovipneumoniae has been frequently found in diseased and apparently healthy respiratory tracts of sheep and goats (Maksimović et al., 2022). In some cases, M. ovipneumoniae has been responsible for large financial losses mostly due to poor growth rates and productivity of affected animals (Ma et al., 2023). Diseases caused by M. ovipneumoniae infections are known by a variety of names including atypical pneumonia of sheep, non-progressive (atypical) pneumonia of sheep, chronic bronchopneumonia, chronic, non-progressive pneumonia, and proliferating exudative pneumonia (Aliand Ali, 2019; Maksimović et al., 2022; Aliand Abdullah, 2024).

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 (Dawoodet al., 2022; Maksimović et al., 2022; Gardner, 2023). Further surveys are clearly necessary to determine its true prevalence and economic impact. However, should these costs prove to be significant, then the following measures should be considered (Hadi et al., 2020; Welchman et al., 2022; Hamzahand Ibrahim, 2024).Therefore, this study aims to isolation of M. ovipneumoniae from the suspected pneumonic sheep, induction of an experimental infection in lambs by inhalation, and then, measurement the levels of interleukin-10 (IL-10) in lambs pre- and post- exposure to the bacterium at different days (0, 6, 14, and 28) as well as in vaccinated lambs by ELISA.

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 agarwas 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 37ºC in 5% CO_2 for 7-14days. Broths were checked daily for Mycoplasma growth, which was indicated by turbidity and the appearance of the floccule's materials at the bottom ofthe culture tube. All the tubes with former appearance were filtered using 0.45µm and 0.2µm syringe filters to remove debris and the fast-growingmicrobes. The filtrates were cultured onto PPLO agar and incubated at 37ºC in 55% $CO_2$for 7-14 days. The plates were checked three days afterinoculation for the appearance of Mycoplasma colonies indicated by fried egg observations under the light microscope. The suspected colonies werecontinued to be sub-cultured to obtain a single colony pure culture.Giemsa staining (Solarbio, China) wasused to permissively identify the isolates according to the morphology of their colonies (Chen et al., 2024; Mustafa and Jawad, 2024; Salih, 2024).

Vaccine preparation

A stock culture of M.ovipneumoniae was used for inoculation. Initially, the bacteria were cultured on PPLO selective agar, specifically formulated for mycoplasma growth, and incubated at 37°C with 5% CO_2 for 18 hours. After incubation, bacterial colonies were harvested using sterile swabs, rinsed, and re-suspended in sterile phosphate-buffered saline (PBS) to remove residual debris.The bacterial suspension was then transferred to PPLO broth at a 10% inoculum and incubated under the same conditions with slow agitation (75rpm) for 24 hours. The following day, the cultures were further subcultured into 3 liters of fresh PPLO broth (10% inoculum) and incubated at 37°C with 5% CO_2 under slow agitation to promote bacterial growth.After

incubation, M. ovipneumoniae was harvested at the mid-log phase by centrifugation at 4,000×g for 15 minutes at 4°C. The bacterial pellets were washed four times with sterile, cold PBS to ensure purity and remove non-bacterial components.The collected bacteria were pooled, mixed in equal amounts, and inactivated with 1% formalin at 37°C for 24 hours. Inactivation was confirmed by culturing on PPLO selective agar to ensure the absence of viable bacteria. The inactivated antigens were then washed four times with cold PBS and diluted. Based on protein concentration measured by a Nanodrop spectrophotometer, the immunization dose was adjusted to 250µg per 0.5 mL of PBS (Einarsdottiret al., 2018).

Preparation of bacterial infectious inoculum

1. Mycoplasma ovipneumoniae was harvested at the mid-log phase by centrifugation at 4,000 × g for 15 minutes. The bacterial pellets were washed and rinsed four times with sterile phosphate-buffered saline (PBS) to ensure purity and remove non-bacterial components.
2. Colony-forming units (CFU) were estimated by plating serial dilutions of M. ovipneumoniae suspensions onto fresh PPLO agar to establish the optical density (OD) corresponding to the infectious dose. The OD was measured for each dilution before incubating the plates at 37°C with 5% CO_2 for 24 hours to accurately determine CFU counts. Once the CFU per mL was established, the OD corresponding to 1×10^6 CFU/mL was determined to be approximately 0.78 OD (measured at 630nm), aligning with the ~0.5 McFarland standard.
3. To ensure precision and accuracy, bacterial concentrations of both the experimental and working inocula were further validated using the standard plate counting method, confirming consistency and reliability in the dosage used for animal experiments. The concentration was also verified via spectrophotometric analysis at 630nm.
4. As described in step 1, M. ovipneumoniae was again harvested at the mid-log phase by centrifugation at 4,000×g for 15 minutes at 4°C. The bacterial pellets were washed and rinsed four times with sterile PBS before diluting the infective inoculum to 1×10^6 CFU/mL in PBS (corresponding tobe approximately 0.78 OD).
5. To induce of M. ovipneumoniae infection in lambs of subgroup B and C, the infective inoculum was given by inhalation using of the nebulizer (Rossmax, Switzerland).

Study design

An overall 18 lambs of 5-6 months of age were selected, acclimated for one week, and divided randomly into two groups; infected (No=12) and vaccinated (No=6). In infected group, the lambs were subdivided equally into three subgroups; (A): non-infected and non-treated, (B): infected but not treated, and (C) infected and antibiotic-treated. In vaccinated group, the lambs were vaccinated with two doses at 2 weeks intervals. Each lambwas injected0.5mL of the vaccine at each time subcutaneouslyin the axilla region.

Blood sampling for serology

An overall 2.5mL of venous blood were collected from each lambs of vaccinated group at the 0, 6, 14 and 28 days of first dose of vaccination; while, lambs of infected group were subjected to single draining of venous blood after 28 days of infection. The samples of blood were drained using a disposable syringe into labeled free-anticoagulant plastic tubes that transported cooled to the laboratory and centrifuged at 5000rpm for 5 minutes. The obtained sera were packed into labeled 1.5mL Eppendorf tubes that kept frozen in refrigerator at -18°Cuntil be examined serologically (Gharban and Yousif, 2020; Gharban and Al-Shaeli, 2021).

Measurement of IL-10

Following the manufacturer instructions (SunLong Biotech, China) of Sheep Interleukin 10 (IL-10) ELISA Kit (SL00128Sp), the contents of ELISA's kit and the serum samples were prepared at room temperature, processed, and the optical density (OD) of Standards and sera were calculated at 450nm using of Microplate ELISA Reader (BioTek, USA). After setting the Blank well to zero, the ODs and concentrations of Standards as well as the ODs of sera were plotted on a diagram to measure the concentration of each marker in sera (Figure 1).

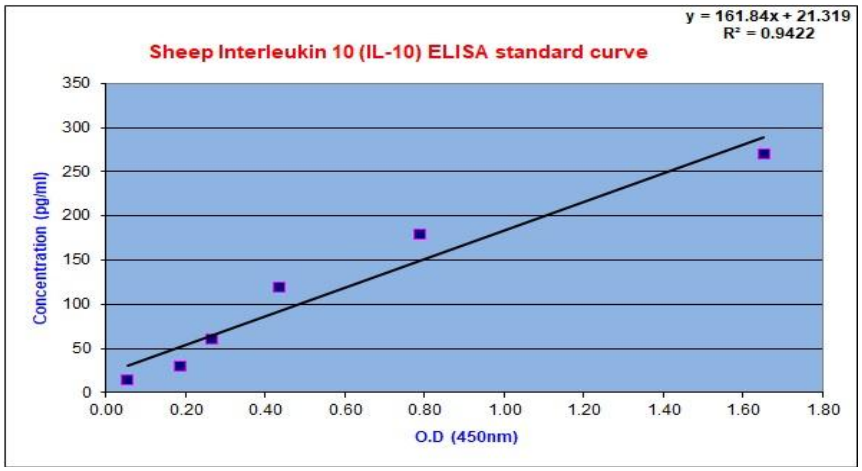

Figure 1: Standard curve for calculation the concentrations of IL-10 in sera of study lambs

Statistical analysis

The obtained results were analyzed by GraphPad Prism version 8.0.2 (GraphPad Software, USA). Two-Way ANOVA and the t-test wereapplied to detect significant variation between values of different study groups at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****). Values were represented as mean ± standard errors (M±SE), (Gharban, 2023).

RESULTS

In current study,nasal swabs of totally 37 pneumonic sheep, cultivated on PPLO agar, revealed that5 (13.51%)were positive to M. ovipneumoniaethat forms distinctive colonies with generally small, appearing as tiny dew drops or resembling fried eggs (Figures 2, 3).

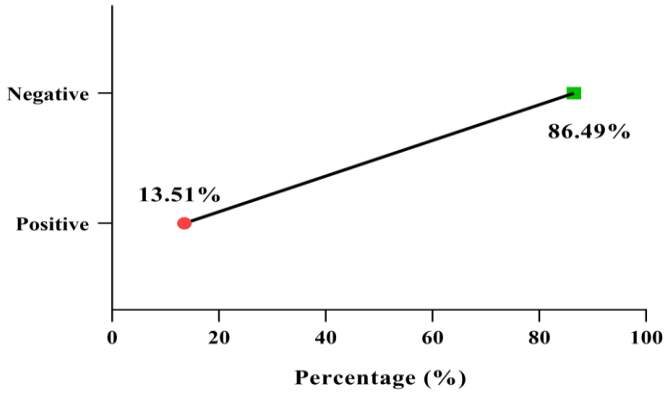

Figure 2: Total results for cultivation the nasal swabs of totally 37 pneumonic sheep

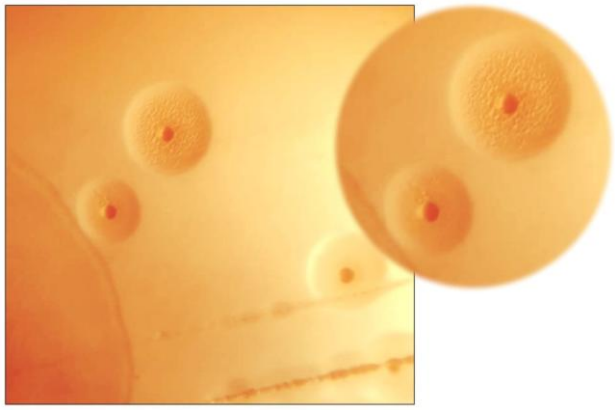

Figure 3: Colonies of M. ovipneumoniae on PPLO agar characterized as round without a central umbilicus(10X)

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 4).

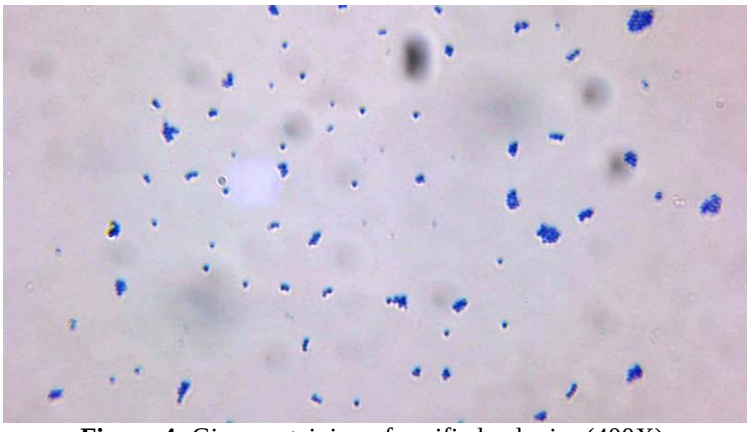

Figure 4: Giemsa staining of purified colonies (400X)

In the vaccinated group, the findings of IL-10 were elevated significantly ($p \leq 0.0306$) in lambs at the 28th day of first-dose vaccination (96.36 ± 3.27 pg/ml) when compared to values of other days; 0 (74.84 ± 3.47 pg/mL), 6 (79.07 ± 2.94 pg/mL), and 14 (74.91 ± 2.18 pg/mL), (Figure 5).

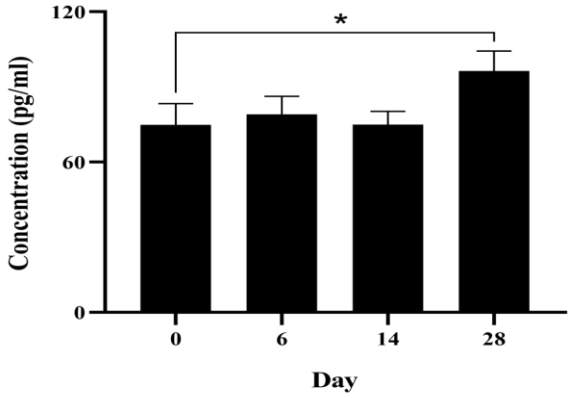

Figure 5: Concentrations of IL-10 among the lambs vaccinated group at various times

In infected group, the findings of subgroup B (164.63 ± 532 pg/mL) were elevated significantly ($p \leq 0.0242$, $p \leq 0.0079$) when compared to values of subgroups A (82.45 ± 2.06 pg/mL) and C (108.39 ± 4.89 pg/mL), (Figure 6).

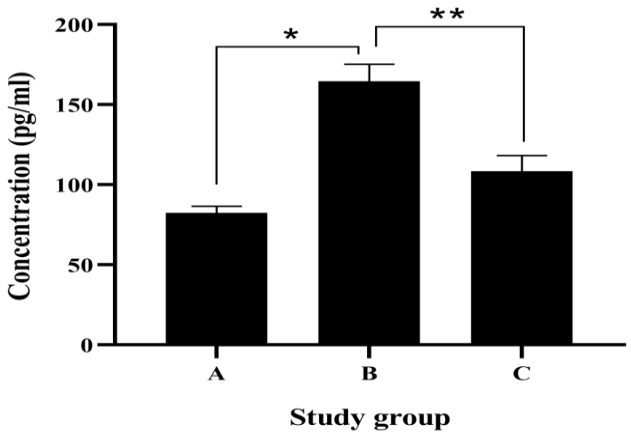

Figure 6: Concentrations of IL-10 among the lambs of infected subgroups

DISCUSSION

Mycoplasmaovipneumoniaeinfection in small ruminants is a serious problem in herds of small ruminantsaround the worldas it is responsible for high economic losses and decreased animal productivity. This study revealed that the prevalence rate of M. ovipneumoniaein sheep 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 recordedthe prevalence rates of Mycoplasma were 40% in apparently healthy sheep, and Mousaet 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 (Gaetaet al., 2022). In Italy, Pavoneet al.(2023) found that 47.5% out of the 202 tested sheep were positive to Mycoplasmal infections thatinvolvedM. 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%), (Chenet al., 2024).

Numerous studies have shown that inflammatory elements play a significant role in initiating and extending Mycoplasma-associated diseases (Niet al., 2015; Hoelzleet al., 2020; Xiuet al., 2024). In this study, the findings of infected group showed that the concentrations of IL-10 were elevated significantly in lambs of subgroups B and C; however, the administration of antibiotic was contributed significantly in decreasing the concentration of IL-10 in subgroup C. Throughout the various periods in vaccinated group, lambs received two doses of vaccination was revealed a higher values of IL-10 (28 days) when compared to other study periods (0, 6 and 14 days). The pathogenesis of M. ovipneumoniaemainly includes direct damages and immune damages, which are highly correlated (Sarayaet al., 2014; Heet al., 2016). Mycoplasmaovipneumoniaecan adhere to the host cell membrane via the apical structure. This process is helpful in nutrient acquisition and avoiding respiratory clearance (Shimizuet al., 2005; Liet al., 2023). Mycoplasmaovipneumoniaelipoproteins can be recognized by toll-like receptor (TLR) heterodimers (TLR2/6 and TLR2/1), resulting in up-regulation of pro-inflammatory cytokines (Xueet al., 2015; Baiet al., 2020). The induction of pro-inflammatory cytokines can stimulate a distinctive innate immune response in the lung (Wanget al., 2024). In the bronchoalveolar lavage fluid (BALF) of M.ovipneumoniae, the levels of IL-10 were shown to be up-regulated (Koet al., 2023). Cytokines changes in serum were also detected (Yiwenet al., 2021). Several cytokines were reported to correlate with the severity of M.ovipneumoniae, but in most studies only few cytokines were examined, which makes it difficult to analyze the immune response patterns of the host (Baiet al., 2020; Liet al., 2023).Jianget al.(2017) suggested that capsular polysaccharide of M. ovipneumoniae could trigger inflammatory responses in sheep bronchial epithelial cells through a mechanism by which it activated both MyD88-dependent and MyD88-independent signaling pathways and subsequently enhanced the production of various pro-inflammatory cytokines, such as IL1β and TNFα, and meanwhile increased the expression of anti-inflammatory cytokines, such as IL10 and TGFβ. This attribution of capsular polysaccharide makes it a promising vaccine candidate for M. ovipneumoniae infection as proinflammation may be an important step in the establishment of disease. Besides, considering inhibition of inflammatory mediators serves as a key strategy to control inflammation; specific agents that can suppress the transcription and translation of inflammation-associated genes may have a therapeutic potential for M. ovipneumoniae infections.

CONCLUSION

Mycoplasma ovipneumoniae is primarily prevalent in pneumonic sheep. The expression of IL-10 showed differential patterns across variousvaccination times, with variations observed among infected groups. Subsequently, administration of antibiotic had revealed a significant ability to reduce the overexpression of IL-10 in infected lambs. However, furthermore studies to investigate the prevalence of M.ovipneumoniaeor other mycoplasmas by the molecular tools are necessary. Also, the role of IL-10 and other cytokines in various anatomical sites of respiratory tract throughout disease progression need to moreover studies.

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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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