Clinical, histopathological, and molecular characterization of Mycoplasma species in sheep and goats in Egypt

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Received: 06-04-2021, Accepted: 18-08-2021, Published online: 28-09-2021

doi: www.doi.org/10.14202/vetworld.2021.2561-2567 How to cite this article: Mousa WS, Zaghawa AA, Elsify AM, Nayel MA, Ibrahim ZH, Al-Kherajie KA, Elhalafawy HR, El-Shafey D, Anis A, Salama AA (2021) Clinical, histopathological, and molecular characterization of Mycoplasma species in sheep and goats in Menoufiya Governorate, Egypt.

Abstract

Background and Aim: Mycoplasma infection in small ruminants is a serious problem in sheep and goat herds around the world. It is responsible for high economic losses and decreased animal productivity. This study aimed to highlight the clinical, histopathological, minimum inhibitory concentration (MIC), and molecular characterization of Mycoplasma species in sheep and goats in Menoufiya Governorate, Egypt.

Materials and Methods: A total of 234 samples were collected; 104 samples were collected from pneumonia lung tissues from the abattoir, in addition, 10 and 20 samples collected from apparently and diseased sheep, respectively, and 40 and 60 samples were collected from apparently and diseased goats, respectively, which were subjected to isolation on pleuropneumonia-like organism medium. Polymerase chain reaction (PCR), histopathological examination, and determination of the MIC were also performed.

Results: Of 104 samples of lung tissues showing pneumonia lesions, 56 (53.84%) were positive for Mycoplasma isolation. The positive isolation of Mycoplasma from 10 and 20 samples from apparently and diseased sheep was 30% and 40%, respectively as well as the positive isolation of Mycoplasma was 17% and 56.66% out of 40 and 60 apparently healthy and diseased field goat’s cases, respectively. All the diseased sheep and goats showed respiratory manifestations, including cough, bilateral nasal discharge, conjunctivitis, and systemic reaction. Evaluation of the MIC for Mycoplasma ovipneumoniae revealed that lincospectin and tylosin were the most effective antibiotics at 2.5 μg/mL. Histopathological examination of affected lung tissue showed extensive hemorrhagic pneumonia with extensive alveolar hemorrhage. The PCR technique proved to be a rapid, specific, and sensitive method for the detection of M. ovipneumoniae and Mycoplasma arginini at 390 and 326 bp, respectively.

Conclusion: M. ovipneumoniae and M. arginini were the most prevalent species associated with respiratory infections in sheep and goats in the study area. Further studies are needed to investigate the role of these species in dissemination of the disease within herds of small ruminants.

Keywords: goats, minimum inhibitory concentration, Mycoplasma, polymerase chain reaction, prevalence, sheep.

Introduction

Respiratory syndromes are commonly encountered in sheep and goat populations. They are often caused by multifactorial agents, including infectious agents, such as viruses, bacteria, fungi, and parasites, as well as predisposing management factors, such as stress and climatic factors that lead to significant losses [1,2]. Numerous Mycoplasma serotypes are associated with various pathological complications in small ruminants, including respiratory signs, causing major losses, especially in African countries and Egypt [3,4]. Mycoplasma belong to the class Mollicutes, which contains eight genera, of which five are found in animals: Mycoplasma, Ureaplasma, Acholeplasma, Anaeroplasma, and Asteroplasma. Mycoplasma and Ureaplasma are more pathogenic in animals [5]. Indirect economic losses and infertility, high morbidity, and occasionally mortality are associated with acute/subacute or chronic pneumatic Mycoplasma infection [6]. Outbreaks of infection by virulent strains of Mycoplasma ovipneumoniae often occur in lambs from different flocks housed together [7], usually associated with heavy rain, animal transportation, poor climatic conditions,
and introduction of infected animals into susceptible herds [8]. Secretions from diseased or carrier animals have a substantial role in the maintenance and spread of the disease among herds through inhalation of infected droplets from animals in close contact [9,10]. Postmortem and histopathological examination [11] reveals gray or red areas of consolidation in the affected lungs, with marked pleuritis and pleural effusion of yellowish fluid, and fine granular texture with hepa
tization in cross-sections of affected surfaces.

Difficulties in the diagnosis of Mycoplasma infection by traditional biochemical and serological tests, due to the fastidious nature of Mycoplasma species, have encouraged researchers to develop modern molecular techniques for rapid and effective diagnosis of Mycoplasma infection, such as polymerase chain reaction (PCR) [10,12], PCR is a valuable, rapid, recent molecular approach for the diagnosis of Mycoplasma infection and genotyping of Mycoplasma species [13].

This study emphasizes that M. ovipneumoniae and Mycoplasma arginini were the most prevalent species associated with respiratory infections in sheep and goats in Giza and El-Menoufiya Governorate, Egypt, as well as reporting the crucial role of PCR for rapid and specific detection of Mycoplasma species. In addition, this study highlights the devastating effects of Mycoplasma species on lung tissue, which shows extensive hemorrhagic pneumonia with extensive alveolar hemorrhage.

This study aimed to determine the prevalence of Mycoplasma species in sheep and goats in Egypt, with molecular detection of the most prevalent species. In addition, it evaluated the minimum inhibitory concentration (MIC) of different antibiotics against the obtained species, as well as performing histopathological examination.

**Materials and Methods**

**Ethical approval**

This study followed the guidelines of the Ethics Committee and current legislation on research and ethical approval of the Faculty of Veterinary Medicine (approval no. VUSC-014-2-21), University of Sadat City, Egypt.

**Study period, sampling, and clinical examination**

The study was conducted from November 2017 to April 2018. A total of 104 samples of lung tissues from rams showing pneumatic lesions were collected from El-Basateen abattoir, Giza Governorate, Egypt. In addition, nasal swabs from 30 sheep (10 apparently healthy and 20 diseased) and 100 goats (40 apparently healthy and 60 diseased) were collected from El-Menoufiya Governorate, Egypt. All examined sheep and goats in the field condition showed respiratory manifestations, including bilateral nasal discharge, cough, conjunctivitis, and fever. The samples were transported to the laboratory under cold conditions (4°C) for bacteriological examination.

**Isolation and identification of Mycoplasma**

The collected samples were cultivated in pleuroneumonia-like organism (PPLO) broth for 3 days, then cultured into PPLO agar medium for another 3 days at 37°C, then examined with a stereo microscope every 2 or 3 days. If the characteristic mycoplasmal “fried egg” colonies appeared on the agar plates, agar blocks with Mycoplasma colonies were transferred into broth medium and incubated at 37°C for 2 or 3 days and then subjected to purification. Mycoplasma species were identified by a digitonin sensitivity disk, and biochemical characterization was performed by a glucose fermentation test and arginine deamination test, according to Valsala et al. [14].

**MIC**

The MIC was determined in a representative field strain (M. ovipneumoniae) for seven antibiotics: danofloxacin 25%, Draxxin 10%, florfenicol 30%, lincomycin 100/50, oxytetracycline 5%, streptomycin 100%, and tylosin 100%. M. ovipneumoniae was sensitive to lincomycin (0.5 μg/mL) and tylosin (0.5 μg/mL). The MIC was determined according to Hannan [15] in 96-well microtiter plates with wells containing growth control (broth medium without antibiotic), sterility control (broth medium without antibiotic and Mycoplasma inoculum), and pH control (broth medium adjusted to pH 6.8). Mycoplasma broth medium (pH 7.8) was supplemented with 0.5% (w/v) sodium pyruvate, 0.5% (w/v) glucose, and 0.005% (w/v) phenol red. The MIC value of each isolate was defined as the lowest concentration of the antibiotic that completely inhibited growth in the broth (no pH and color change) after 1 week. Briefly, 2-fold dilutions were prepared in the range of 0.039-10 μg/mL for fluoroquinolones, 0.125-32 μg/mL for florfenicol, 0.25-64 μg/mL for gentamicin and tetracyclines, 0.5-128 μg/mL for macrolides, and 1-256 μg/mL for lincomycin.

**Histopathological examination**

Lung tissue samples were fixed in 10% neutral buffered formalin (pH 7.4) for 72 h, washed, dehydrated, embedded in paraffin wax, serially sectioned with a microtome at 3 μm thickness, and stained with hematoxylin and eosin for histopathological investigation. Leica DMLB microscopes (Leica Microsystems Wetzlar GmbH Ernst-Leitz-Strasse D-35578 Wetzlar Germany) were used in this study. Histological photographs were taken with a Leica EC3 digital camera as described by Wäsle et al. [16].

**PCR for molecular detection of Mycoplasma strains**

DNA extraction was performed with the GF-1 Tissue DNA Extraction Kit (Vivantis), according to the manufacturer’s instructions. The PCR reaction was performed in a volume of 50 μL, including 25 μL My Taq Red Mix, 2x, 1 μL from each primer (20 μM of each), DNA template 200 ng, and completed with sterile water up to 50 μL. Common
primer 16S RNA gene and specific primers (16-23 S intergenic spacer) were used for molecular detection of *M. ovipneumoniae* and *M. arginini*. The PCR cycle conditions and references for molecular diagnosis of *M. ovipneumoniae* and *M. arginini* are listed in Table-1 [17-19].

**Results**

**Prevalence and bacteriological examination of *Mycoplasma* in lung tissues and nasal swabs from sheep and goats**

Of 104 samples of lung tissues collected from rams at the abattoir, 56 (35.6%) were positive for *Mycoplasma* isolated into PPLO-specific medium. There were 3 and 8 sheep cases positive for *Mycoplasma* out of 10 and 20 apparently healthy and diseased sheep in field cases respectively. On the other hand, 7 and 34 goats cases were positive for *Mycoplasma* out of 40 and 60 field apparently and diseased goat cases, respectively (Table-2). Clinical examination of the diseased cases in sheep and goats showed respiratory manifestations, including cough, bilateral nasal discharge, conjunctivitis, and systemic reaction (fever) (Figures-1a and b). In postmortem examination, the pneumatic lung tissues showed reddening, consolidation, and localized necrosis in different areas of the lung (Figure-1c). *Mycoplasma* in PPLO medium typically appears as “fried egg” colonies (Figure-1d).

**Evaluation of the MIC against field *M. ovipneumoniae* strain**

The MIC was determined in representative field strains of *M. ovipneumoniae* against seven antibiotics: danofloxacin, Draxxin, florfenicol, lincopectin, oxytetracycline, streptomycin, and tyllosin. The results showed that *M. ovipneumoniae* isolates were more sensitive to lincopectin at a concentration of 100/50 and tyllosin 100% in vitro. Resistance was observed for the other antibiotics.

**Table 1:** Primers sequence, PCR cycling conditions for molecular detection of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini*.

| Strain            | Primer sequence | Fragment size (bp) | Reference |
|-------------------|-----------------|--------------------|-----------|
| *M. ovipneumoniae* |                 |                    |           |
| 16S-23-RNA        | F: AGA ACT CTA GGC GAC GTA GAA | 1000               | [17] |
|                   | R: TCT AGA GTC GCC CTC CAT GAA |                     | 18        |
|                   | F: TGA CTA TTA GTC GGT GGA GAG TTC | 390               | [19] |
|                   | R: CAA AAG AGT TCC AAT AGG AGT |                     |           |

**Figure 1:**

(a) Lamb (3 months old) showed unilateral nasal discharges and ocular discharge with depression. (b) Kid (3 months old) showed bilateral mucopurulent nasal discharges. (c) Lung tissue of a 3-year-old ram showed reddening, consolidation, and localized necrosis in different areas of the lung. (d) Fried egg colonies of mycoplasma using Sterio microscopes.
Histopathological findings in sheep and goat lungs infected by Mycoplasma species

In the acute stage of pneumonia in Mycoplasma-positive samples, sheep lung tissues showed a widespread homogenous eosinophilic inflammatory exudate inside the alveoli with alveolar hemorrhages and marked active alveolar macrophages (Figures 2a and b). In addition, extensive hemorrhagic pneumonia was detected in some cases (Figure 2c), and hydropic degeneration and/or necrosis of the epithelial lining of the bronchioles were recorded (Figure 2d). In the subacute stage of pneumonia in Mycoplasma-positive samples, goat lung tissues showed interstitial pneumonia, active alveolar macrophages, thick interalveolar septa by mononuclear cell infiltration, and alveolar-capillary dilatation (Figures 3a and b). In the chronic stage of pneumonia in Mycoplasma-positive samples, sheep lung tissues showed multifocal nodules of mononuclear cell infiltration, mononuclear cells aggregated in the bloodstream, peribronchiolar lymphoid cell infiltration, and desquamation of necrotic epithelial cells of the bronchioles inside the lumen (Figures 3c and d).

Molecular identification of Mycoplasma species in sheep and goats by PCR

The identification of Mycoplasma species recovered from sheep and goats in this study was an efficient tool for the detection of Mycoplasma species at 1000 bp using common universal 16S rRNA primer, as shown in Figure-4. The molecular identification of M. ovipneumoniae was successfully amplified using 16S-23S intergenic spacer gene at 390 bp (Figure-5). M. arginini was molecularly identified by a specific primer in which the amplified band was detected at 326 bp (Figure-6).

Discussion

Respiratory infections are responsible for great economic losses in small ruminants [20]. Although many etiological agents are involved, Mycoplasma species are considered a particularly substantial cause of such infections and exert a significant socioeconomic effect, particularly in areas where small ruminants are an important source of milk and meat [4]. Various serious problems are associated with Mycoplasma infection, such as contagious caprine pleuropneumonia, conjunctivitis, arthritis, mastitis, and mild respiratory distress [21]. M. ovipneumoniae and M. arginini are frequently present in pneumonia lesions among small ruminants [14,22].

Table-2: Results of bacteriological examination of Mycoplasma from lung tissues and nasal swabs collected from sheep and goats.

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| Lung tissue | Apparently healthy | Diseased | Total samples |
|-------------|--------------------|----------|--------------|
| Rams        | 104                | 20       | 124          |
| Sheep       | 56                 | 8        | 64           |
| Goats       | 36                 | 34       | 70           |

Available at www.veterinaryworld.org/Vol.14/September-2021/33.pdf
In the current study, of a total of 234 samples (104 samples of pneumonic lung tissues, 30 samples from diseased and apparent healthy sheep, and 100 samples from diseased and apparent healthy goats), 108 (46.2%) were positive in bacterial isolation. A similar finding was reported by Abdou [24], who reported a 42.5% prevalence rate of *Mycoplasma*. On the other hand, prevalence rates of *Mycoplasma* were 40% and 17.85% in apparently healthy sheep and goats respectively in Egypt [25]. Mostafa [26] reported that the prevalence rates of *Mycoplasma* in apparently healthy sheep and goats were 14.67% and 20.39%, respectively. The higher prevalence rates of *Mycoplasma* in our survey may be due to the bad hygienic measures applied in animal management and husbandry practices.

With regard to the use of the MIC as the reference point for comparison to determine the efficacy of antibiotics [15], in our study, the MIC for representative field strains of *M. ovipneumoniae* using seven different antibiotics showed that *M. ovipneumoniae* was more susceptible to lincospectin and tylosin and was resistant to other antibiotics. This result was supported by Al-Momani et al. [27] and Tatay-Dualde [28] who showed that tylosin, erythromycin, and lincosamides were the most effective antibiotics against *Mycoplasma* species. On the other hand, an earlier study by Otlu [29] reported that *Mycoplasma* species were sensitive to enrofloxacin and resistant to streptomycin. Furthermore, Eissa et al. [30] reported that enrofloxacin was effective against *M. ovipneumoniae* isolates due to its wide spectrum of activity, lipid solubility, and weakly basic reaction.

The histopathological examination of 10 randomly selected samples that were positive for *Mycoplasma* isolation showed extensive hemorrhagic pneumonia with eosinophilic exudate and extensive alveolar hemorrhage with infiltration of mononuclear cells. In addition, degeneration and deciliation of the surface epithelium of bronchiolar mucosa were observed during the histopathological examination. This was previously described by Adehan et al. [6], who reported that most alveoli and bronchioles were filled with a mixture of neutrophils and macrophages, whereas other alveoli were filled with edema fluid and fibrin. In addition, Hernandez et al. [9] observed addition to fibrinopurulent membrane on the pleural surface and serofibrinous fluid in the thoracic and abdominal cavities.
necrotizing vasculitis in vessel walls, with infiltration by inflammatory cells and thrombus formation.

The definitive diagnosis of Mycoplasma infection is based on typical isolation into a specific medium, which is time-consuming and requires special procedures. Molecular approaches, such as PCR, are rapid, specific, and accurate for diagnosis of infection by Mycoplasma species, as shown by Amores et al. [30] and Settypalli et al. [31], by targeting specific genes [32]. In addition, Besser et al. [33] successfully detected *M. ovipneumoniae* recovered from bronchoalveolar lavage fluid in sheep based on 16S rRNA species-specific gene.

In the current study, *M. ovipneumoniae* was detected with a prevalence of 3.8% (4/104) and *M. arginini* was detected with a prevalence of 4.8% (5/104) in lung tissues of sheep. Similar findings in Egypt were reported by Abdel-Halium et al. [3], who identified both *M. arginini* and *M. ovipneumoniae* from sheep and goats with pneumonic lesions. On the other hand, higher prevalence rates were reported in Nigeria [34] *M. ovipneumoniae* and *M. arginini* were detected with prevalence rates of 61.5% and 30.8%, respectively, in lung tissues of sheep. In addition, in Benin [6], *M. ovipneumoniae* and *M. arginini* were detected with prevalence rates of 44.4% and 11.1%, respectively, in lung tissues of sheep. In a comparative study in Turkey [35] *M. ovipneumoniae* and *M. arginini* were detected with prevalence rates of 65% and 35%, respectively. Rekha et al. [36] detected only *M. arginini* in sheep with caprine pneumonia in India.

**Conclusion**

*M. ovipneumoniae* and *M. arginini* are the most common Mycoplasma species in sheep and goats with respiratory infections in Giza and El-Menoufiya Governorates in Egypt. Lincospectin and tylosin are the most effective antibiotics for the treatment of *Mycoplasma* infection in small ruminants. PCR is an effective method of detection of *Mycoplasma* species. Histopathological examination shows the devastating effects of *Mycoplasma* infection in lung tissue, including extensive hemorrhagic pneumonia and alveolar hemorrhage with degeneration and deciliation of the surface epithelium of the bronchiolar mucosa. Further studies are needed for a better epidemiological picture of disease dissemination by *Mycoplasma* species in small ruminants in Egypt.

**Authors’ Contributions**

WSM, AAZ, AAS, MAN, AME, AAS, and HRE: Involved in the conception of the research idea and methodology design, performed the data analysis and interpretation, and prepared the manuscript for publication, HRE, DE, ZHI, and KAA: Participated in the design of the methodology and involved in laboratory work, and AA: Participated in the histopathology work and data analysis and contributed their scientific advice during the work and revision. All authors read and approved the final manuscript.

**Acknowledgments**

The authors would like to thank the staff members of Animal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, University of Sadat City, as well as Animal Health Research Institute, Agricultural Research Center for the help during the study. The authors did not receive any funds for this study.

**Competing Interests**

The authors declare that they have no competing interests.

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