The role of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in pneumatic lungs of slaughtered sheep

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SUMMARY

*Mycoplasma ovipneumoniae* is one of the most commonly mycoplasma species associated with respiratory diseases in sheep. This microorganism and *Mycoplasma arginini* have been routinely recovered from young lamb with "coughing syndrome". In this study, a polymerase chain reaction (PCR) based 16S RNA gene sequences was used for detection of *M. ovipneumoniae* and *M. arginini* in pneumatic lungs of slaughtered sheep. The lungs of 1000 sheep carcasses were grossly inspected at local abattoir. Pneumonia was detected in 40 (4%) of the carcasses. *M. ovipneumoniae* and *M. arginini* were identified in 20% (n=8/40) and 2.5% (n=1/40) of ovine pneumonic lungs respectively. The pathologic findings associated with *M. ovipneumoniae* were including suppurative bronchopneumonia, fibrinous bronchopneumonia and interstitial pneumonia. The lung with *M. arginini* showed capillary congestion and serous exudate in alveoli. The observed pathologic findings were not diagnostic for mycoplasmal infection. Other bacteria such as *Pasteurella multocida*, *Staphylococcus aureus*, *Corynebacterium pseudotuberculosis* and *Klebsiella pneumoniae* were isolated from pneumatic samples. Our results suggest that *M. ovipneumoniae* is an important agent in respiratory diseases of sheep, whereas the role of *M. arginini* in ovine pneumonia is not significant.

Keywords: *Mycoplasma ovipneumoniae*, *Mycoplasma arginini*, Pneumonia, Sheep, Histopathology, PCR.

Introduction

The respiratory problems particularly different types of pneumonia are common in all species of domestic animals. The causative agents are multifactorial and the disease appears due to the interaction of the infectious micro-organisms (bacteria, mycoplasmas, viruses and fungi), host defense, environmental factors [16] and stress [29]. Respiratory diseases in all major sheep-producing countries results to mortality in lambs, reduced growth rate, condemnation of the carcasses and its consequence is a substantial economic impact on the animal husbandry due to chemotherapeutic and vaccination programs [13].

*Mycoplasma* spp. is one of the major causes of pneumonia in farm animals. Identification of mycoplasmas as the causative agents of disease is not frequently noticed because the lack of rapid diagnostic tests together with similarities in the clinical diseases that they cause. Conventional methods of diagnosis are based on culture and serological tests, such as the complement fixation test [21], enzyme linked immunosorbent assay [5] and immunoblotting [26]. These methods are time-consuming, insensitive, and nonspecific. Recently, PCR has been employed for the laboratory diagnosis of some veterinary mycoplasmas [4, 6, 10, 14].

*Mycoplasma ovipneumoniae* is one of the most commonly isolated microorganisms from ovine respiratory disease in the worldwide. This microorganism associated with *M. arginini* has been recovered from young lambs with a chronic disease that has been termed the "coughing syndrome". This syndrome is widespread in the midwestern states of the United States of America.
USA, with morbidity and variable severity among flocks [19, 23]. The role of mycoplasma, in particular \textit{M. ovipneumoniae} is often overlooked. It can predispose animals to pasteurellosis and viral infections [24]. A few studies are present about the role of \textit{M. ovipneumoniae} and \textit{M. arginini} in ovine pneumonia in Iran. Also, there is no data that shows co-infections of these organisms in sheep. The present study was undertaken to isolation of \textit{M. ovipneumoniae} and \textit{M. arginini} from the pneumatic lungs of slaughtered sheep by PCR and their correlation with pathomorphological changes.

Materials and Methods

SAMPLE COLLECTION

Shahrekord is a land with historical background and beautiful nature in southwest of Iran. This province is 16533 square kilometer at the central part of Zagros' mountains. This study was conducted in winter 2009 from January to March. One thousand lungs of native sheep slaughtered at Shahrekord Slaughterhouse were grossly examined for the presence of pneumatic lesions. The lungs of 40 animals with macroscopic visible pneumatic lesions were obtained. No details of sex breed or husbandry conditions of the sheep were available. These animals were submitted for routine slaughter. Following grossly inspection, the samples of apparently affected lungs were taken for pathologic and microbiologic investigations. In addition, ten apparently healthy lungs were examined for isolation of bacteria as control group.

PATHOLOGICAL INVESTIGATION

Tissue samples of 1 cm$^3$ in thickness were fixed in 10% neutral buffered formalin for histopathological examination. The samples were then dehydrated in graded ethanol and embedded in paraffin. Sections of 5 μm in thickness were stained with hematoxylin and eosin and examined by an ordinary light microscope.

BACTERIOLOGIC CULTURE

For bacterial isolation and identification, samples of the affected areas were aseptically taken and placed in sterile plates, kept in an icebox and were submitted to the Bacteriology Department. The outer surface of the pneumatic lungs were first seared with a heated spatula before the cut inner surface of the lungs were cultured on blood agar by contact with addition of 5% sheep blood and McConkey agar. The plates were then aerobically incubated at 37°C for 24-48 h. Subcultures were made and pure cultures of each strain were obtained. Identification of the isolated bacteria was performed according to the standard procedures and included morphology of the colonies on blood agar plates, presence and type of haemolysis, Gram staining, cytochrome oxidase, catalase, indole production, urease production, sulfhydric acid production (TSI), oxidation/fermentation, motility and growth ability under aerobic condition [27]. Biochemical characters of isolates were determined with commercial test kits.

POLYMERASE CHAIN REACTION (PCR)

DNA was extracted from frozen lung samples with genomic DNA purification kit (Fermentazs) according to the manufacturer's instructions. \textit{M. ovipneumoniae}-specific 16S rRNA gene sequences were identified by PCR using primers LMF1: 5’-TGAACGGAATATGTTAGCTT-3’ and LMR1: 5’-GACTTCATCCTGC ACTCTGT-3’ (360 bp). The programmed thermocycler was including a preliminary denaturation step, one cycle at 95°C for 5 min, 35 cycles at 94°C for 60 s, 56°C for 45 s, and 72°C for 60 s and a final step at 72°C for 5 min [7].

PCR for detection of \textit{M. arginini} was performed with specific primers: MAGF 5’ GCATGGAATCGCATGATTCCT 3’, GF4R 5’ GGTGTTCTTCCTTATATCTACGC 3’ (545 bp). The thermocycler was programmed for one cycle at 95°C for 5 min and was followed by 35 amplification cycles consisting of denaturation at 94°C for 60 s, annealing at 46°C for 60 s, and extension at 72°C for 60 s. The last cycle was followed by a step at 72°C for 10 min [32].

Each set of samples was run with a positive and a negative reaction control. In the negative extraction control, an equal volume of sterile demonized water was used. PCR reactions were analyzed in 2% agarose gel containing ethidium bromide and then the results analyzed. Ten apparently healthy lungs were examined for isolation of \textit{Mycoplasma} spp as control group.

Results

In this research, the pathologic findings of pneumatic sheep lungs in local abattoir were compared to PCR results for \textit{M. ovipneumoniae} and \textit{M. arginini} (Table 1).

IDENTIFICATION OF \textit{M. OVIPNEUMONIAE} AND \textit{M. ARGININI}

On PCR technique, amplicons of the expected size for \textit{M. ovipneumoniae} and \textit{M. arginini} were detected in 8/40 (20%) and 1/40 (2.5%) of ovine pneumatic lungs respectively (Fig. 1 and 2). No amplicons of \textit{M. ovipneumoniae} and \textit{M. arginini} were obtained in 10 control sheep.

PATHOLOGIC FINDINGS AND BACTERIAL CULTURE

\textit{Mycoplasma ovipneumoniae}

In this study, \textit{M. ovipneumoniae} was isolated from different types of pneumonia including suppurative bronchopneumonia (n=4/40), interstitial pneumonia (n=3/40) and fibrinous
pneumonia (n=1/40). The gross appearance in bronchopneumonia showed irregular consolidation in lobular form. The cranial, middle and accessory lobes were the main affected areas. Depending on the age and nature of the process, consolidated lungs varied from dark red in acute to gray-pink, and gray in chronic form. Histopathologically, neutrophil-rich exudates were noted in the alveolar spaces and lumens of Airways. In chronic cases, varying degrees of bronchiolar lymphatic tissue hyperplasia (BALT) was commonly observed. Pasteurella multocida, Staphylococcus aureus, Corynebacterium pseudotuberculosis and Klebsiella pneumoniae were isolated in bacterial culture of lungs associated with suppurative bronchopneumonia.

Fibrinous brochopneumonia was detected in three cases. Distribution the lesions were almost anteroventral, and the apical and cardiac lobes were the mostly affected parts. Microscopically, the affected lung was dominated by diffuse capillary congestion. Variable amounts of fibrinous exudate in the lumen of the alveoli and bronchioles were a conspicuous and predominant feature. Interlobular septa and pleura were thickened by fibrin, neutrophils and edema. Some necrotic areas were usually surrounded by a rim of elongated cells, often referred to as "oat cells" which were neutrophils mixed with alveolar macrophages. Fibrinous pleurisy was observed. Extensive and wide spread vascular thrombi were evident in small blood vessels, capillaries and lymphatics of pneumonic lungs (Fig. 3). No bacterium was isolated in culture.

In interstitial pneumonia, the gross lesions were distributed diffusely, often with involvement of dorsocaudal regions. Affected lungs were voluminous, enlarged, diffusely red to

<table>
<thead>
<tr>
<th>Other bacteria</th>
<th>M. arginini</th>
<th>M. ovipneumoniae</th>
<th>No of pneumonic lungs</th>
<th>Type of pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. multocida</td>
<td>—</td>
<td>4</td>
<td>25</td>
<td>Suppurative bronchopneumonia</td>
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<tr>
<td>S. aureus</td>
<td>—</td>
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<tr>
<td>K. pneumoniae</td>
<td>—</td>
<td>1</td>
<td>3</td>
<td>Fibrinous bronchopneumonia</td>
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<tr>
<td>C. pseudotuberculosis</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>—</td>
<td>3</td>
<td>11</td>
<td>Interstitial pneumonia</td>
</tr>
<tr>
<td>S. aureus</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>Pulmonary congestion and edema</td>
</tr>
</tbody>
</table>

Table I: Mycoplasmas isolated from the pneumonic lungs of sheep.

FIGURE 1: PCR detection of Mycoplasma ovipneumoniae in pneumonic lung of sheep. M.: 100 bp molecular weight markers, Lane 1: negative control, Lane 2: positive control, Lane 3, 4 and 5: positive amplification (360 bp).

FIGURE 2: CR detection of Mycoplasma arginini in pneumonic lung of sheep. M.: 100 bp molecular weight markers, Lane 1: negative sample, Lane 2: positive amplification (545 bp).
pale appearance, failed to collapse and rib impressions were seen on the costal surfaces of diaphragmatic lobes (Fig. 4). No evidence of exudates could be detected in cut surfaces and air passages. The lungs were rubbery in consistency. The histopathologic features showed a marked increase in mononuclear cells and occasionally mild fibrosis in the inter-alveolar septa and presence of varying numbers of macrophages within the alveolar lumina. Also, hyperplasia of pneumocyte type II was seen. There was no obviously exudate in alveolar spaces and airways. *Klebsiella pneumoniae* was obtained from one lung specimen.

### Mycoplasma arginini

*M. arginini* was isolated from one examined lung. Macroscopically, the lung showed red consolidation in cranial lobes and was edematous. Histopathologic sections revealed capillary congestion and serous exudate in alveoli. In bacterial culture, *Staphylococcus aureus* was isolated.

#### Discussion

Mycoplasmas are highly fastidious bacteria, difficult to culture and slow growing belongs to the class *Mollicutes*. Many species are veterinary pathogens causing respiratory infection, mastitis, conjunctivitis, arthritis, and occasionally abortion. Mycoplasma infections have caused indirect economic losses as a result of emaciation, delayed market weight and infertility, due to the subacute or chronic pneumonia in ruminants [25]. In sheep, mycoplasmal pneumonia has been associated with *M. ovipneumoniae*, *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *mycoides* LC and *M. arginini* [9, 15]. *M. ovipneumoniae* is the most commonly isolated mycoplasma from the upper respiratory tract of normal sheep, and can be significant in respiratory disease in both sheep and goats [3, 11]. During times of stress, subclinical infection may predispose sheep to atypical pneumonia with paroxysmal coughing [22]. The infection is sometimes in association with *Mannheimia haemolytica*, Parainfluenza-3 virus or *M. arginini* [23, 30]. In some studies, *M. ovipneumoniae* and *M. arginini* were recognized as organisms associated with ovine pneumonia. Co-infection of these bacteria in the USA causes a long-term coughing syndrome as well as rectal prolapse [23]. In New Zealand and Australia, *M. ovipneumoniae* has been linked to a chronic non-progressive pneumonia in young sheep [3].

In this investigation, two mycoplasma species, *M. ovipneumoniae* and *M. arginini*, were identified from the pneumatic lung tissues of sheep slaughtered at the abattoir in southwestern Iran, by PCR technique. This method has been shown by others as an extremely sensitive and specific technique that allows direct detection of antigen and overcomes to cross-reactions of conventional tests. A comparison of the PCR technique with the microbiological culture, DNA fluorochrome staining, and hybridization techniques indicated that the PCR is a rapid, sensitive, and efficient method [6, 33]. In present study, the frequency of *M. ovipneumoniae* and *M. arginini* in pneumatic lungs of slaughtered sheep were obtained 20% (n=8/40) and 2.5% (n=1/40) respectively. These organisms were isolated from fibrinous pneumonia, suppurative bronchopneumonia and interstitial pneumonia. The observed pathologic findings were not diagnostic for mycoplasma that caused pneumonia in sheep. Moreover, during the course of this study, we were able to isolate other bacteria including *Pasteurella multocida*, *Staphylococcus aureus*, *Corynebacterium pseudotuberculosis* and *Klebsiella pneumoniae* from the same specimens. It is believed that a primary infection with *M. ovipneumoniae* may predispose sheep to invasion of the lower respiratory tract by other organisms such as respiratory viruses and secondary bacteria [6, 24, 30]. In most animals, polymicrobial infections were present in respiratory disease, and *M. ovipneumoniae* may more...
commonly act as a primary agent that increases the susceptibility of infected bighorn sheep to secondary bronchopneumonia. [7]. The obtained observations here suggest an etiologic role for *M. ovipneumoniae* in ovine pneumonia. In according to the isolation of *M. arginini* from one pneumonic lung, it seems this organism has not an important agent in pneumonia of sheep. Our results were in agreement with the findings of GOLTZ et al [12]. They studied pathogenicity of *M. ovipneumoniae* and *M. arginini* in experimentally infected goats and stated *M. ovipneumoniae* is a pathogenic agent for goats and caused pneumonia and pleuritis, but *M. arginini* is not a significant primary pathogen in respiratory tract of goats. Previous reports confirm these results in goats and sheep [1]. Also, LIN et al [17] detected *M. ovipneumoniae* antibody in serum samples of affected sheep to atypical pneumonia but no positive results for *M. arginini* obtained. In contrast, some studies showed these two organisms have been produced a fatal pneumonia in sheep [8, 20, 28].

In present investigation, the prevalence of *M. ovipneumoniae* and *M. arginini* in pneumonic lungs was rather low and mixed infections of these organisms were not diagnosed. In Iran, TABATABAYI et al [31] isolated *M. arginini* from 79% (49 out of 62) of sheep pneumonic lungs by culture. They also identified other bacteria such as Pasteurella multocida, Staphylococcus spp. and Corynebacterium spp. ADEHAN et al [2] isolated different species of mycoplasmas from pneumonic lungs of sheep and goats in abattoirs. They showed incidence of *M. ovipneumoniae* and *M. arginini* 44.4% (8/18) and 11.1% (2/18), respectively. IKHELOA et al [15] reported incidence of these organisms 61.5% (8/13) and 30.8% (4/13) respectively, in Nigeria. Several factors can influence the precision of the reported prevalence of a disease or isolated microorganism such as the diagnostic tests, sampling methods, characteristics of the animals and meteorological situation.

Newer molecular methods, such as PCR and the 16S rDNA PCR and denaturing gradient gel electrophoresis offer a rapid method for detecting mycoplasma and can detect multiple mycoplasma infections [18]. They avoid the antigenic cross-reactivity and variability that hinder serological methods, and they allow easier standardization between laboratories [11]. They have been limited, since little interspecific variation in 16S ribosomal DNA were identified (rDNA) [19].

In present study, the specific primers that used for detection of *M. arginini* were designed by TIMENETSKY et al [32] for identification of mycoplasmal infection in cell culture. Further studies need to investigate the pathogenicity of these bacteria. A precise diagnosis for mycoplasmoses must be established, since the control strategies and consequences differ widely depending on the etiological agent. Our results suggest that *M. ovipneumoniae* is an important agent in respiratory disease of sheep, whereas the role of *M. arginini* in ovine pneumonia is not significant.

References


