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Original Article

Identification of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in sheep with pneumonia in North East of Iran

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Abstract

Background: Pneumonia due to *Mycoplasma* infections can cause serious health problems and economic losses in small ruminants industry. **Aims:** The aim of this study was isolation and identification of *Mycoplasmas* in sheep naturally infected with pneumonia in Northeastern Iran. **Methods:** This study used histopathology, culture and polymerase chain reaction (PCR) to examine samples from 50 lungs of sheep naturally infected with *Mycoplasmas*. **Results:** Grossly, irregular consolidation with lobular or lobar to diffuse pattern in the cranioventral to caudal lobes of affected lungs were observed. Histopathologically, bronchointerstitial pneumonia in 38 (76%), and purulent to fibrinopurulent bronchopneumonia in 12 (24%) affected sheep were diagnosed. DNA was extracted from lung tissue samples and replicated using genus and species specific primers for *Mycoplasma*. *Mycoplasma* growth was observed in 3 (6%) of a total of 50 lung samples. Genus-specific M*ycoplasma* DNA was identified by PCR in 12 (24%) of samples. Two (4%) and 7 (14%) samples of these 12 cases were positive for reaction with species-specific primers of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini*, respectively. **Conclusion:** Our results showed that *M. ovipneumoniae* and *M. arginini* were the two agents that can be involved in inducing lung consolidation and pneumonia in sheep and PCR was more successful than the culture in detecting *Mycoplasmas.*

Key words: Culture, *Mycoplasma*, PCR, Pneumonia, Sheep

Introduction

Pneumonia and pleuropneumonia due to *Mycoplasma* infections can cause a serious health problem and economic losses in small ruminants industry (Amores *et al*., 2010). *Mycoplasmas* are widely distributed in nature and are able to colonize in humans, animals and plants. This organism belongs to the class *Mollicutes* and does not have the genetic ability to produce a cell wall (Cremonesi *et al*., 2007). *Mycoplasmas* are herd problems with high morbidity, and low mortality and healthy carriers are an important part of the epidemiology of them (Radostits *et al*., 2007).

The pathogenicity of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in sheep is still being defined and specific description of the lesions is necessary. These organisms probably cause disease only in circumstances that host, infectious, and environmental factors create a complex interaction in the pathogenesis of the disease (Lopez and Martinson, 2017).

Mycoplasma ovipneumonia can cause diseases in sheep and can be isolated from the lung, trachea, and nose and occasionally from the eyes of sheep with pneumonia and it can also be found in the respiratory tract of healthy sheep. *Mycoplasma arginini* is frequently isolated with *M. ovipneumoniae* from atypical pneumonia cases in sheep (Ayling and Nicholas, 2007).

The role of *M. arginini* in pneumonia etiology particularly in sheep is still uncertain. However, on several occasions its high frequency of isolation from clinical pneumonia cases has been observed from other animal species (Elfaki *et al*., 2002; Gagea *et al*., 2006; Chazel *et al*., 2010).

The Khorasan Razavi province of Iran is one of the most important provinces in terms of small ruminants breeding, and there is not any study about the role of *Mycoplasmas* in the sheep affected with pneumonia. Therefore, the aim of the present study was isolation and identification of *Mycoplasmas* from sheep naturally infected with pneumonia in this area.

Materials and Methods

Sampling

In this study, lungs of about 1800 sheep slaughtered at industrial slaughterhouse of Mashhad, Iran were examined grossly for the presence of pneumonic lesions. There were not any details about age, sex, breed or husbandry conditions and all the animals were submitted for routine slaughter. Following gross examination, 50 lungs of sheep affected with pneumonia on the basis of texture, exudation and distribution of lesions were selected for pathologic, culture and molecular investigations. In addition, 10 normal lungs without any

pathologic lesion were tested as negative control.

Histopathology

Samples of affected lungs were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, and five-micron sections were taken and stained with haematoxylin and eosin (H&E) for light microscope examination.

Culture

Samples of consolidated areas of selected pneumonic lungs were aseptically taken and placed into the transport medium contained pleuropneumonia-like organisms (PPLO) broth (HiMedia, Hungary) and horse serum 7% decomplemented by heating at 56°C for 10 min and yeast extract 1% (Scharlau, Spain), kept two days in refrigerator and then transported on ice to the laboratory. The samples were diluted, filtered and then inoculated to fresh PPLO broth containing PPLO broth 21 g/L and yeast extract 12 g/L and penicillin 4 ml 200000 U/ml and thallium acetate (Sigma-Aldrich) 10 ml (1 ml/L) and kept for two days in incubator at 37°C and 98% humidity.

They were plated onto PPLO agar medium containing 36 g/L PPLO agar and PPLO broth 21 g/L and yeast extract 12 g/L and penicillin 4 ml 200000 U/ml and thallium acetate 10 ml (1 ml/L) and horse serum 150 ml/L. All media were incubated at 37°C in 5% CO_2 atmosphere and 98% humidity atmosphere and observed for 3 to 14 days. The broth media were examined daily for any signs of *Mycoplasma* growth, such as slight turbidity, swirls of growth, a change in pH, or opalescence. The agar media were examined for *Mycoplasma*-like colonies and typical "fried-egg" colonies using a microscope (Kilic *et al*., 2013 with some modifications).

DNA extraction

Two g of each tissue sample were homogenized and then DNA extraction was carried by HiPurA™ DNA Purification Kit (HiMedia, Hungary).

Because of the difficulty in scrape of *Mycoplasma* colony from the surface of agar, a piece of agar containing *Mycoplasma* colony was cultured into the PPLO broth. The cultured were repeated with a piece of same agar medium that was not cultivated, as negative control.

The PPLO broth containing M*ycoplasma* and negative control media were centrifuged at 11200 g for 15 min. The supernatant was removed and discarded as much as possible by aspiration. Five hundred µL Triss-EDTA (TE) buffer was added to the previous step pellet. Five hundred µL of the suspension was transfered to 2.0 ml screw tap conical microtube and boiled for 20 min, then 100 µL of it mixed with 100 µL of chloroform slowly in microcentrifuge tube and were centrifuged at 11200 g for 5 min. Five µL of the supernatant were used for polymerase chain reaction (PCR) amplification (Kilic *et al*., 2013 with some modifications).

PCR amplifications

All PCR amplifications were carried out in 25 µL reactions containing 15 µL of master mix (ampliqon, Denmark), 1 µL of each primer (10 pmol/µL), 5 µL genomic DNA template, and 3 µL deionized water.

All reactions were run on a Bio-Rad T100 thermal cycler. Amplification and detection of *Mycoplasma* genus were carried out by universal PCR primers of GPO-1 (5´-ACT CCT ACG GGA GGC AGC AGT A-3´) and MGSO (5´-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3´) based on species specific sequence of *16S rRNA* gene (Kilic *et al*., 2013).

The temperature protocol was as follows: after an initial denaturation step at 94°C for 3 min, 35 cycles were performed consisting of three steps: denaturation (94°C, 45 s), annealing (60°C, 30 s), and extension (72°C, 45 s) and final extension at 72°C for 5 min.

The primers of LMF1 (5´- TGA ACG GAA TAT GTT AGC TT- 3´) and LMR1 (5-´ GAC TTC ATC CTG CAC TCT GT -3´), and MAGF (5-´ GCA TGG AAT CGC ATG ATT CCT- 3´) and GP4R (5´- GGT GTT CTT CCT TAT ATC TAC GC- 3´) were species-specific for detection of *M. ovipneumoniae* and *M. arginini*, respectively (Kilic *et al*., 2013).

The temperature protocol was the same for both species as follows: after an initial denaturation step at 94°C for 3 min, 37 cycles were performed consisting of three steps: denaturation (94°C, 45 s), annealing (55°C, 30 s), and extension (72°C, 45 s) and final extension at 72°C for 5 min.

The fragments size of PCR products was 715, 361, and 545 bp for *Mycoplasma* genus, *M. ovipneumoniae* and *M. arginini*, respectively.

The PCR products were run on 1.5% agarose gel and subjected to electrophoresis for about 2 h at 80 V. After the bonds of amplified fragments were visualized and photographed under UV transilluminator.

Results

Pathological findings

Grossly, irregular consolidation with lobular or lobar to diffuse pattern in the cranioventral to caudal lobes of affected lungs were observed. The color of consolidated areas was varied from dark red to grey pink or grey (Fig. 1). In some affected lungs, various lesions including presence of a thin layer of fibrin with yellow color covered the pleural surfaces, rib impressions on the costal surfaces of the diaphragmatic lobes and abscesses containing odorless pus were seen.

Histopathologic examination of affected lungs showed bronchointerstitial pneumonia in 38 sheep (76%), and purulent to fibrinopurulent broncho-pneumonia in 12 sheep (24%). In fibrinopurulent bronchopneumonia, multifocal areas of necrosis associated with variable amounts of fibrin, neutrophils and macrophages were observed in the bronchioles and alveoli (Fig. 2). In bronchointerstitial pneumonia, thickening of interstitial tissue between alveoles mainly by lymphocytes and plasma cells is associated with

bronchiolar associated lymphoid tissue (BALT) hyperplasia (Fig. 3), and accumulation of fibrinopurulent exudates within alveoles and bronchioles were seen.

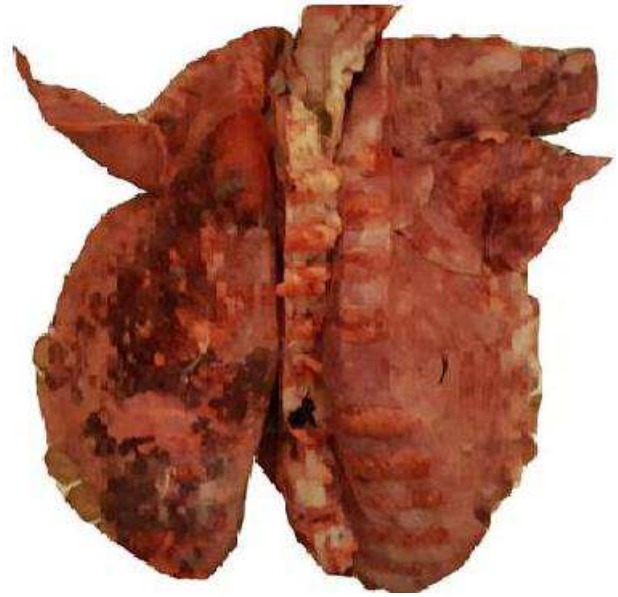

Fig. 1: Gross appearance of lobar pneumonia. Irregular red consolidations are seen in affected right lobe

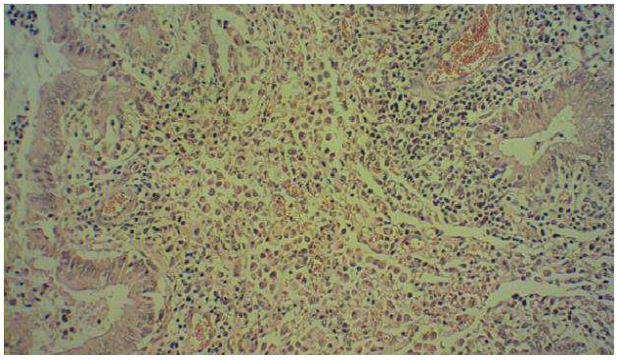

Fig. 2: Accumulation of exudate including neutrophils and macrophages are observed within affected bronchiole and alveoli associated with cuffing of lymphocytes around vessels and bronchioles (H&E, ×600)

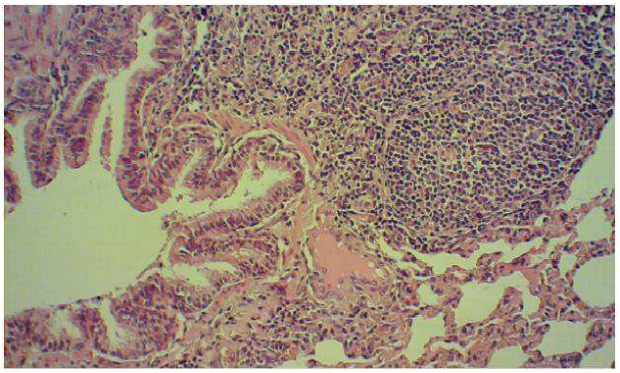

Fig. 3: BALT hyperplasia are seen in a sheep lung affected with fibrinous pneumonia (H&E, ×600)

Culture results

The evidence of *Mycoplasma* growth such as turbidity in the mycoplasma broths and *Mycoplasma* colonies on the *Mycoplasma* agar plates was observed in 3 sheep (6%) (Fig. 4).

Fig. 4: Positive growth of M*ycoplasma* colonies as typical fried-eggs after culture on agar media

Molecular findings

The lung samples were examined by PCR for finding *Mycoplasma* genus and 12 samples (24%) were positive. Then, isolated DNA samples were tested to identify *M. ovipneumoniae* and *M. arginini* by PCR. The results showed that 2 sheep (4%) and 7 sheep (14%) were positive for *M. ovipneumoniae* and *M. arginini*, respectively (Figs. 5-6).

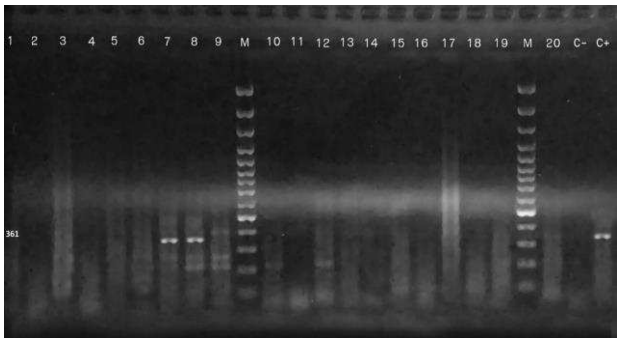

Fig. 5: *Mycoplasma ovipneumoniae* PCR electrophoresis analysis in 1.5% gel agarose. M: Marker (100 bp DNA ladder), Lane C+: Positive control (361 bp band), Lane C-: Negative control, and Lanes 7 and 8: positive samples of *M. ovipneumoniae*

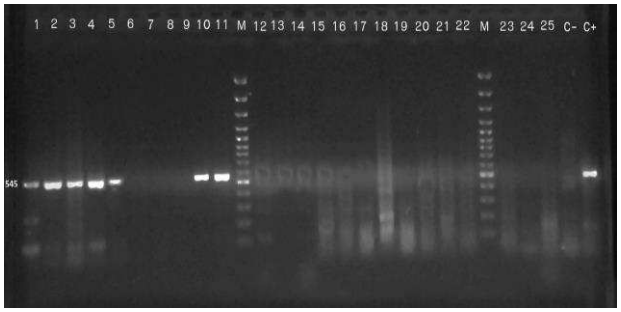

Fig. 6: *Mycoplasma arginini* PCR electrophoresis analysis in 1.5% gel agarose. M: Marker (100 bp DNA ladder), Lane C+: Positive control (545 bp band), Lane C-: Negative control, and Lanes 1 to 5 and 10 to 11 positive samples of *M. arginini*

Genomic DNA was purified from colonies of broth culture media, all of them were identified as *M. arginini*. None of the negative control culture media were detected with *Mycoplasma* genus.

Discussion

Mycoplasma pneumonia causes subtle and unnoticed clinical signs and infected animals have good appetites until severe damage associated with secondary bacterial infections. Several *Mycoplasma* species including *M. ovipneumoniae*, *Mycoplasma mycoides* subsp. *Mycoides* (large colony type), *Mycoplasma capricoluum capripneumoniae*, *Mycoplasma agalactiae*, *M. arginini* and *Mycoplasma conjunctivae* were isolated from ovine pneumonias (Adehan *et al*., 2006; Radostits *et al.*, 2007).

Fatal pneumonia due to *M. ovipneumoniae* and *M. arginini* have been reported in sheep (Carmichael *et al*., 1992; Mohan *et al*., 1992). In the present study, *M. ovipneumoniae* and *M. arginini* were detected from pulmonary lesions of sheep by PCR and culture methods. Positive culture was seen only in 3 of 12 positive PCR samples. The results showed that PCR was more successful than the culture in detecting of *Mycoplasma* sp. and it was in agreement with the results of other researchers (Tola *et al*., 1997; Amores *et al*., 2010; Kheirkhah *et al*., 2011; Abtin *et al*., 2013; Pooladgar *et al*., 2015).

Mycoplasma arginini is routinely recovered along with *M. ovipneumoniae* from young lambs with respiratory disease (Niang *et al.*, 1999). Higher incidence of *M. arginini* in pneumonic sheep as compared to normal sheep may be suggestive of its pathogenic potential.

Our results showed that, culture of 6% and *Mycoplasma* genus PCR of 24% of lung samples were positive. Abtin *et al*. (2013) have reported from total of 102 samples from milk, eye, ear and joint exudates of sheep in Qom province of Iran, 19 (18.63%) and 59 (57.8%) samples were positive for *M. agalactiae* by culture and *Mycoplasma* genus PCR, respectively.

In another similar study, Pooladgar *et al*. (2015) reported 34 (37.36%) and 47 (51.65%) of a total 91 samples were positive by culture and *Mycoplasma* genus PCR methods in sheep of Khuzestan province of Iran.

Pathogenic *Mycoplasmas* have a predilection for the respiratory system, urogenital tract, mammary gland and serous membranes. They adhere to epithelial cells and this adhesion is a prerequisite for colonization and infection (Smith *et al*., 2019). The mechanism of pathogenesis is not well understood and activation of the immune system of the host probably plays a major role (Radostits *et al*., 2007). Certain conditioning factors are known to be required for the development of the disease such as age, gestation, parturition, crowd and poor ventilation (Gutierrez *et al*., 1999).

A high prevalence of *M. ovipneumoniae* in association with *Mannheimia haemolytica* has been reported in the lungs of lambs in Turkey (Haziroglu *et al*., 1994) and Italy (Ettorre *et al*., 2007). *Mycoplasma ovipneumoniae* and M. *haemolytica* biotype A serotypes act synergistically to produce chronic atypical pneumonia principally in lambs up to 12 months of age. Mortality rarely exceeds 10% even under experimental conditions, but considerable economic losses due to unthriftiness can occur. *Mycoplasma ovipneumoniae* facilitates lung colonization by other organisms, such as *M. haemolytica* producing a more severe pathology (Ayling and Nicholas, 2007).

Mycoplasma ovipneumoniae is the most commonly isolated *Mycoplasma* from the respiratory tract of sheep often in association with *M. arginini* that may exacerbate disease signs (Ayling *et al*., 2004; Ayling and Nicholas, 2007). Respiratory disease involving *M. ovipneumoniae* has also been reported in goats in Spain, Nigeria, Sudan, Canada and Portugal (Goncalves *et al*., 2010). The presence of infection with *Mycoplasma* species including *M. ovipneumoniae* and *M. arginini* in association with lung consolidation, environmental temperature and relative humidity has been investigated in lambs in Spain and the results have showed higher temperatures and lower relative humidity favour the presence of *Mycoplasma* species. *Mycoplasma arginini* has also been associated with lung consolidation (Fernandez *et al*., 2016). In UK, *M. ovipneumoniae* has been detected in higher percentages from nasal swab and lung samples of sheep vaccinated against *Pasteurella* sp. (McAuliffe *et al*., 2003). Haziroglu *et al*. (1994) have reported the prevalence of 3.6% pneumonia among 13588 lambs including 43% *M. ovipneumoniae*, 51.6% *M. hemolytica* and 26.2% mixed infection by both organisms. In a slaughterhouse study on 40 pneumonic lungs of sheep in Southwest of Iran, *M. ovipneumoniae* (20%) and *M. arginini* (2.5%) were detected by PCR method (Azizi *et al*., 2011).

Polymerase chain reaction can be used more reliably on *Mycoplasma* growing in culture in the appropriate medium which greatly facilitates PCR detection even in the presence of bacterial contamination (Nicholas *et al*., 2008). This study was conducted on the suspected samples, therefore, the results can be more reliable than other studies that were conducted on the samples with or without gross pneumonia lesions.

In conclusion, the results of this study showed that *M. ovipneumoniae* and *M. arginini* were the two agents that can involve in inducing lung consolidation and pneumonia in sheep and PCR was more successful than the culture in detecting *Mycoplasmas.*

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Conflict of interest

We declare that we have no conflict of interest.

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