Detection of *Mycoplasma bovis* Infection in Cattle Mammary Tissue by Immunofluorescence and qRT-PCR Methods

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**ABSTRACT**

Mastitis in cattle causes important economic losses in all over the world and *Mycoplasma bovis* is one of the important agent among pathogens shown to be responsible for this disease. The aim of this study was to determine the presence and prevalence of *Mycoplasma bovis* among the pathological agents implicated in mastitis by immunofluorescence staining and qRT-PCR methods in cattle mammary tissue. For this aim, 120 mammary samples with or without macroscopic lesions as this agent causes subclinical mastitis, were collected. Mastitis was diagnosed in 78 of 120 cases. Chronic mastitis cases in 56 and, acute mastitis in 22 cases were histopathologically diagnosed. Fluorescent positivity was determined 28/120 according to the results of immunofluorescence staining for all sections. Immunpositive signs of *Mycoplasma bovis* were commonly observed in samples with chronic mastitis. These results were confirmed by qRT-PCR. According to these results the presence of *Mycoplasma bovis* agent that an important threat to herd health is 23,3%.

**Keywords:** Cattle, Subclinical Mastitis, *Mycoplasma bovis*, qRT-PCR, Immunofluorescence.

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INTRODUCTION

Bovine mycoplasmosis caused by *M. bovis* has an important place in mastitis disease and cause serious economic losses in cattle worldwide. Mastitis caused by *M. bovis* is highly contagious and results in a rapid decline in milk production (Giovannini et al. 2013; Amram et al. 2013). This agent is associated with a variety of disease involving pneumonia, arthritis, keratoconjunctivitis, otitis media (Tamada et al. 2002; Adamu et al. 2013). *M. bovis* is a high pathogen, especially in young calves less than 4 months, but it always threat animals with other virus such as *Bovine Respiratory Syncytial Virus*, *Parainfluenza Virus Type 3*, *Bovine Herpes Virus*, *Infectious Bovine Rhinotracheitis Virus*, *Bovine Viral Diarrhea Virus* and bacterium such as *Mannheimia haemolytica* serotype A, *Pasteurella multocida* and *Histophilus somni* ( Aebi et al. 2012; Amram et al. 2013). Besides the severity of disease increases by various factors like stress, environmental conditions, immunodeficiency. Treatment application such as vaccination and using antibiotics was reported to be ineffective. In recent years *M. bovis* is shown as the most important pathogen with increasing prevalence values. *M. bovis* infection in animals results in an annual economic loss of body weight gain was reported to be $32 million in the United States. At the same time, $100 million loses reported due to mastitis bigger than due to pneumonia or loss of body weight. *M. bovis* which is highly contagious agents can be found in milk, body fluids, reproductive discharges, semen. Chronically infected cows defined as carrier animals play important role in transferring. *M. bovis* passes from cattle to calves with milk by systemic infection without any clinical signs, therefore this agent threat calves health directly (Nicholas et al. 2002; Haapalaa et al. 2018). Because of the nonspecific clinical signs and pathological lesions of *M. bovis* only laboratory diagnosis by culture and serological methods is necessary for identification. Because of false results due to contamination and long survival time of antigen in blood. In recent years PCR and immunohistochemistry methods have successfully and effectively been employed for *M. bovis* (Radelli et al. 2008; Karahan et al. 2010; Wooley et al. 2012). In this study, we aimed to investigate presence and prevalence of *M. bovis* infection in mastitis cases in Erzurum.

MATERIAL and METHOD

Sampling

The material of this study (120 samples) was obtained from slaughterhouses in Erzurum Province in Turkey. Half of each received mammary tissue samples were stored for 1 day to be fixed in a 10% buffered formalin solution for histopathology and immunohistochemistry and half of each sample were stored -20 °C for qRT-PCR. Routine histopathological process was performed in Shandon Citadel 2000 (USA) tissue system. After the routine histopathology process, all sample were poured into paraffin for blocking and prepared microtome sections in 5µm by using rotary microtome (Leica RM 2255). Haematoxylin Eosin staining applied to all sections. Slides were examined under the light microscopy (Olympus BX52 with DP72 camera attachment).

Immunofluorescence staining method

After the routine histopathology process, Paraffin sections 4 µm were taken on to lysine-coated slides. These slides put in the oven for deparaffinization in 57° C for 1 hour. For indirect immunofluorescence staining, paraffin sections in 4 µm were placed on lysine-coated slide after primary Anti-rabbit *M. bovis* polyclonal antibody, (Pendik Veterinary Control Institute) antibody application which was performed according to the protocol, 1\10 diluted seconder immunofluorescence antibody Goat Anti-Rabbit IgG H&L (FITC) (Cat No: ab 6717, 1/100 dilution, Abcam, Cambridge, UK) dropped 12 µl by using micropipette to each slides and waited 45 min. in the darkness. After standing in the dark, slides were washed with distilled water and covered with mounting medium (glycerol, 9 volumes; PBS 1 volume). All slides were examined in fluorescence microscope (Carl Zeiss axioskop A1 with Calibri 2 led fluorescence attachment).

Total RNA Isolation

Total RNA isolation was realized from the collected mammary tissue samples through the utilization of Trizol (Invitrogen, USA). Total RNA isolation was realized in line with the manufacturer’s protocols. Following the total RNA isolation, the RNA concentration was measured by virtue of NanoDrop (Epoch Microplate Spectrophotometer, USA). RNAs were run in a 1.5% agarose gel in 1XTE solution for one hour at 80 volts with a view to control total RNA quality and visualized by gel imaging system and their RNA quality was determined.

DNase I treatment and cDNA Synthesis

DNase I (Thermo Scientific, USA) was performed against DNA contamination in isolated RNA samples. Dnaz I treatment was performed in line with the protocol provided in the kit. Subsequently, 1 µg was taken from these RNAs and cDNA was synthesized through utilization of the miScript Reverse Transcription Kit (Qiagen, Germany) in line with the protocol provided. The purity and quantity of the obtained cDNA was measured by virtue of spectrophotometer (Epoch Microplate Spectrophotometer, USA), and the cDNAs were diluted at the same ratios. Subsequently, the cDNA samples were stored at -20 °C for utilization in Real Time PCR studies.
Real-time PCR

qRT-PCR was performed through utilization of the CFX96 BioRad device in order to detect M.Bovis. The β-actin gene was employed for internal control. Master mix content created in real time PCR experiments is as follows: Syber Green 2X Rox Dye Master mix (Qiagen Germany), forward and reverse primers designed for genes, cDNAs as template and nuclease-free water. Reaction conditions and primer sequences of the genes (Fan et al. 2018) are shown in Table 1.

Statistical analysis

IBM SPSS 20 program was performed for statistical analysis. The Cp values of each virus were evaluated using a linear mixed model (Thonur et al. 2012).

RESULTS

In this study 120 cow mammary tissue were collected from the slaughterhouse. Mastitis was diagnosed in 78 of 120 (65%) cases. Chronic mastitis cases in 56 and, acute mastitis in 22 cases were histopathologically diagnosed. Fluorescent positivity was determined 28/120 according to the results of immunofluorescence staining for all sections. Positive immun signs of M. bovis were observed in 24 samples with chronic mastitis, 4 samples with acute mastitis. qRT-PCR test for M. bovis agent extracted from mammary tissue were applied, 28 out of 120 lung tissue samples examined were positive at qRT-PCR test. For positive reactions of immunofluorescence stain frequently cytoplasm of inflammatory cells was first target also epithelium and exudate in the lumen of mammary gland.

Histopathology

Histopathological analysis of 56/120 samples were diagnosed with chronic mastitis and 22/120 cases were diagnosed with acute mastitis. In chronic cases, mononuclear cell infiltration in interstitial tissue(Fig.1 A,B) and interalveolar septum thickness due to increased connective tissue were observed. In acute cases, presence of exudates containing neutrophil leukocytes in alveoli of mammary gland was observed.

Macrophage infiltration with mononuclear cells was observed. Besides, diffuse and common plasma cell infiltration (Fig.1C) was the most striking finding in M.bovis positive tissues.

Immunofluorescence staining results

Positive staining for M. bovis antigen was detected in 28 out of 120 by indirect immunofluorescence. M. bovis antigen has been localized in the cytoplasm of macrophages around the alveoli and lumen of alveoli containing inflammatory cells in exudate. Positive reactions for immunofluorescence were located in frequently cytoplasm of inflammatory cells and epithelial cells.

qRT-PCR Results

A ten-fold serial dilution of each of the in vitro transcribed RNAs of M. bovis was triplicate analysis. M. bovis was detected 20 of 120 samples. M. bovis nucleic acid signals were shown in Figs. 2 and 3.

Figure 1. A) Mononuclear (macrophage) cell infiltrations in the interstitial space (arrows), H & E. 20 μm. B) Plasma cell infiltrations (arrows), H & E. 20 μm. C) Plasma cell accumulation (arrows). Macrophages in gland (arrowhead). H & E. 20 μm. D) M. bovis positivity in cytoplasm of mononuclear cells (arrows). IF. 20 μm E) Positivity (arrows) in mononuclear cells. IF. 20 μm. F) Positive reaction for M. bovis (arrows) in mononuclear cells. IF. 20 μm
Figure 2. *M. bovis* nucleic acid signals in mammary tissues.

Figure 3. *M. bovis* primer melt peak and melting curve analysis.

Table 1. Primer sequences of *M. bovis/uvrC*

| Gen Name       | Primer sequences                      | Annealing | Reference          |
|----------------|---------------------------------------|-----------|--------------------|
| *M. bovis/uvrC*| F:CCTGTCGGAGTTGCATTGTT                | 60        | Fan et al., 2018   |
|                | R:CGGTCAAC1TCAACTTTGAATTG             |           |                    |
Mastitis diseases in cattle is widespread throughout the world, as are diseases which often result in Turkey (Karahan et al. 2010). Mycoplasmal agents are reported to be the most common cause of mastitis with the increasing prevalence values. A lot of studies usually built on serological tests carried out in the presence of M. bovis in the world; in France (Grand et al. 2002) 10-20 %, England (Ayling et al. 2005) 22%, United States (Soehnlen et al. 2011) 41.1%. In our country, it is noted that a small number of studies on this subject as reported of mycoplasmal mastitis in cattle, the prevalence of M. bovis was reported to be 7.5 % in the Marmara region and (Erdağ et al. 1998). In this study the presence of M. bovis factors in cattle with mastitis was found in 23.3 % by immunofluorescence and qRT-PCR. Although the pathogenesis of the disease is not fully explained, the data reported in different studies are as follows; In a study conducted by investigating M. bovis in tissues by immunohistochemical methods, M. bovis cause necrosis in the cells by providing oxidative stress and nitrate stress markers via increasing the production of H₂O₂ (Hydrogen peroxide) (Schott et al. 2013). M. bovis infection was found to slow cellular metabolism in an experimental study. In addition, decreased neutrophil degranulation and ROS (Reactive Oxygen Species) factors caused by decreasing the growth of leukocytes, the immune system was seen to suppress the immunosuppression and therefore it is revealed that M. bovis infections are chronic. In experimental studies, it has been found that M. bovis agents have been placed in the cytoplasm of phagocytic cells such as tissue macrophages and can hold onto the surface of macrophages and proliferate here (Kleinschmidt et al. 2013). Obtained findings of this study especially immunofluorescence staining results support these informations.

In recent studies, diagnostic methods such as PCR, culture, ELISA, SDS-PAGE, nucleic acid hybridization were compared and PCR techniques were described as more sensitive and specific method than any other technique. Besides, many researchers have used immunohistochemistry successfully in operation to demonstrate the presence of the agent in lesion. Last research claim that the first step to control and to prevent the spread of mycoplasmal disease is recognizing but mycoplasmal agents are known to be difficult in the early and rapid diagnosis due to difficulty in isolation and identification (Giovannini et al. 2013). This disease not only threatens the cows with mastitis but also threatens the calves due to their subclinical progression in adults and contaminating milk (Stipkovits et al. 2000; Rosetti et al 2010).

In conclusion our findings demonstrate a high prevalence of M. bovis infection in adult cattle in the east of Turkey. Suitable environment conditions for the animals waiting to be able to create an infectious disease in the normal flora and subclinically infected cows of the factors show that the continuity of this threat. There is a critical need for preventative strategies in the farm for this pathogen. According to the results obtained, RT-PCR is an effective method for identification but optimizing processes in the RNA extraction phase for detection M. bovis factor by using this method hold an important place for obtaining reliable results.

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**REFERENCES**

Giovannini S, Zanoni MG, Salogni C, Cinotti S, Alborali GL. Mycoplasma bovis infection in respiratory disease of dairy calves less than one month age. Research in Veterinary Sciences, 2013; 95: 576-579.

Amram E, Freed M, Khateb N, Mükula I, Blum S, Spersger J, Sharir B, Ozeri R, Harrus S, Lysnyansky I. Multiple locus variable number tandem repeat analysis of Mycoplasma bovis isolated from local and imported cattle, The Veterianary Journal, 2013; 197: 286-290.

Tamada Y, Ohura K, Kato K, Kimura K, Maeda T. Mycoplasma bovis infection in calves with ear drops. Journal of the Hokkaido Veterinary Medical Association. 2002; 46: 308-311.

Adamu JY, Wawegama NK, Browning GF, Markham PF. Membrane proteins of Mycoplasma bovis and their role in pathogenesis. Research in Veterinary Science, 2013; 95: 321-325.

Aebi M, Bodmer M, Frey J, Pilo P. Herd-specific strains of Mycoplasma bovis in outbreaks of mycoplasmal mastitis and pneumonia. Veterinary Microbiology, 2012; 157: 363–368.

Nicholas RAJ, Ayling RD, Stipkovits LP. An experimental vaccine for calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and pathological findings, Vaccine, 2002; 20: 3569-3575.

Karahan M, Kalin R, Atel E, Çetinkaya B. Detection of Mycoplasma bovis in cattle with mastitis and respiratory problems in eastern Turkey. Veterinary Record, 2010; 166: 827-829.

Radelli E, Luini M, Loria GR, Nicholas RAJ, Scanziani E. Bacteriological, serological, pathological and immunohistochemical studies of Mycoplasma bovis respiratory infection in veal calves adult cattle at slaughter. Research in Veterinary Sciences, 2008; 85: 282-290.

Wooley L K, Fell S, Gonsalves JR, Walker MJ, Djordjevic SP, Jenkins C, Eamens GJ. Evaluation of clinical, histological and immunological changes and qPCR detection of Mycoplasma hypneumoniae in tissue during the early stages of mycoplasmal pneumonia in pigs after experimental challenge with two field isolates. Veterinary Microbiology, 2012, 161: 186-195.
Grand DL, Calavas D, Brank M, Citti C, Rosengarten R, Be'zille P, Poumarat F. Serological prevalence of Mycoplasma bovis infection in suckling beef cattle in France Veterinary Record, 2002; 150: 268-273.

Ayling R, Nicholas R, Hogg R, Wessels S, Scholes B, Byrne W, Hill M, Moriarty J, Brien T. Mycoplasma bovis isolated from brain tissue of calves, 2005; 156: 391-392.

Soehnlen MK, Kunze ME, Karunathilake E, Henwood BH, Karyawasam S, Wolfgang DR, Jayarao BM. In vitro antimicrobial inhibition of Mycoplasma bovis isolates submitted to the Pennsylvania Animal Diagnostic Laboratory using flow cytometry and broth microdilution method. Journal of Veterinary Diagnostical Investigation, 2011, 23(3); 547-551.

Erdağ Ö, Erdoğan G, Türkaslan J, Gürel A. Buzağı ve dana pneumonialerinde Mycoplasma ve bakteriyel etkenlerin izolasyon, identifikasyon ve antibiyotik duyarılıkları. Animal Information, 1995; 112: 115-119.

Schott C, Cai H, Parker L, Bateman K G, Caswell L. Hydrogen peroxide production and free radical mediated cell stress in Mycoplasma bovis pneumonia. Journal of Comparative Pathology, 2013; 1-11.

Kleinschmidt S, Spergser J, Rosengarten R, Hewicker Trautwein M. Long-term survival of Mycoplasma bovis in necrotic lesions and in phagocyte cells as demonstrated by transmission and immunogold electron microscopy in lung tissue from experimentally infected calves. Veterinary Microbiology, 2013; 162: 949-953.

Stipkovits L, Ripley P, Varga J, Pálfi V. Clinical study of the disease of calves associated with Mycoplasma bovis infection. Acta Veterinaria Hungarica, 2000; 48(4):387-95.

Rosetti BC, Frey J, Pilo P. Direct detection of Mycoplasma bovis in milk and tissue samples by real-time PCR. Molecular and Cellular Probes, 2010; 24: 321-323.

Haapalaa V, Pohjanvirta T, Vähänikilä N, Halkilahto J, Simonend H, Pelkonen T, Soerens T, Simojoki H, Autio T. Semen as a source of Mycoplasma bovis mastitis in dairy herds. Veterinary Microbiology, 2018; 216: 60–66.

Fan Q, Xie Z, Xie Z, Xie L, Huang J, Zhang Y, Zeng T, Zhang M, Wan S, Luo S, Liu J, Deng X. Development of duplex fluorescence-based loop-mediated isothermal amplification assay for detection of Mycoplasma bovis and bovine herpes virus 1. J Virol Methods. 2018; 261:132-138.