

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. Immunopositive 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.

Sığırların Memelerinde *Mycoplasma bovis* Enfeksiyonunun İmmunofloresan ve qRT-PCR Yöntemleriyle Araştırılması

ÖZ

Sığırlarda Mastitis tüm dünyada önemli ekonomik kayıplara neden olmaktadır. *Mycoplasma bovis* ise bu hastalıktan sorumlu olarak gösterilen patojenler arasında en önemli bakteriyel etkenlerden biridir. Bu çalışmanın amacı, sığırların memelerinde oluşan mastitiste rol oynayan patolojik ajanlar arasında *Mycoplasma bovis*'in varlığı ve prevalansını immunofloresan boyama ve qRT-PCR yöntemleriyle ve saptamaktır. Bu amaçla, etkenin subklinik mastitise yol açtığı düşünülerek, makroskopik olarak lezyonlu ya da lezyonsuz olunmasına bakılmaksızın 120 adet meme örneği toplandı. Toplanan 120 örneğin 56 kronik ve 22 akut olmak üzere toplam 78 örnekte histopatolojik olarak mastitis tanısı kondu. Tüm kesitlere uygulanan immünofloresan boyama sonuçlarına göre floresan pozitifliği 28/120 olarak belirlendi. *Mycoplasma bovis* immünopozitiflikleri daha yoğun olarak kronik mastitisli örneklerde gözlemlendi. Bu sonuçlar örneklere uygulanan qRT-PCR sonuçları ile uyumludur. Bu sonuçlara göre, Erzurum'da sürü sağlığı için önemli bir tehdit olan *Mycoplasma bovis* etken varlığı %23,3 oranında tespit edilmiştir.

Anahtar Kelimeler: Sığırlar, Subklinik mastitis, *Mycoplasma bovis*, qRT-PCR, İmmunofloresan.

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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\ 50 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 florescence 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 manufacturers'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 1XTBE 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.

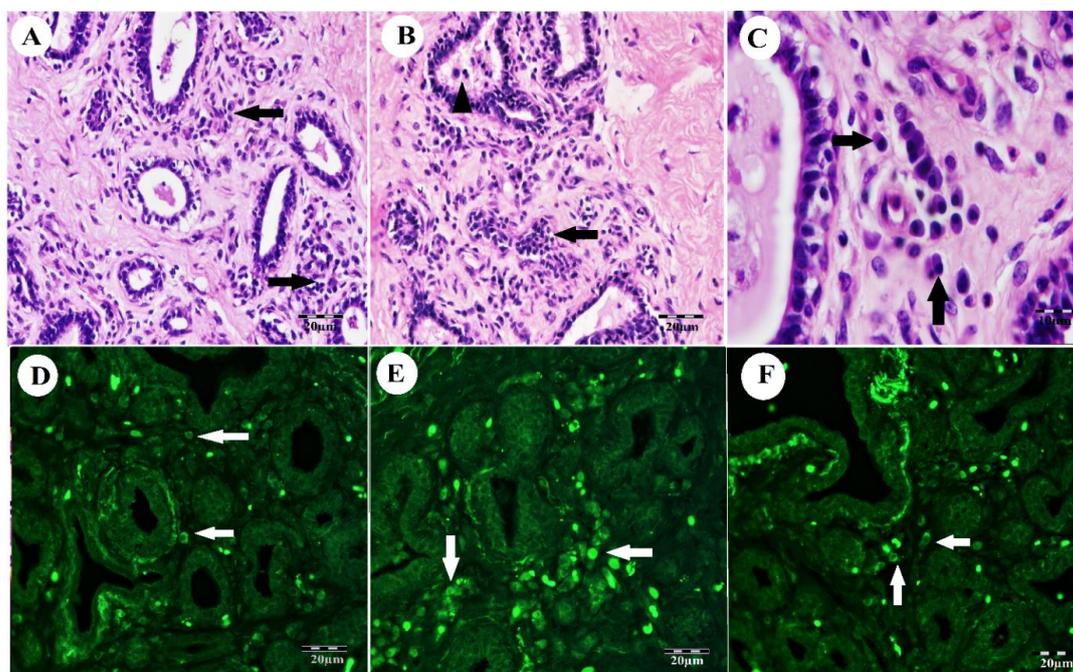


Figure 1. **A)** Mononuclear (macrophage) cell infiltrations in the interstitial space (arrows). H & E. 20 μ m. **B)** Plasma cell infiltrations (arrows), H & E. 20 μ m. **C)** Plasma cell accumulation (arrows). Macrophages in gland (arrowhead). H & E. 20 μ m. **D)** *M. bovis* positivity in cytoplasm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

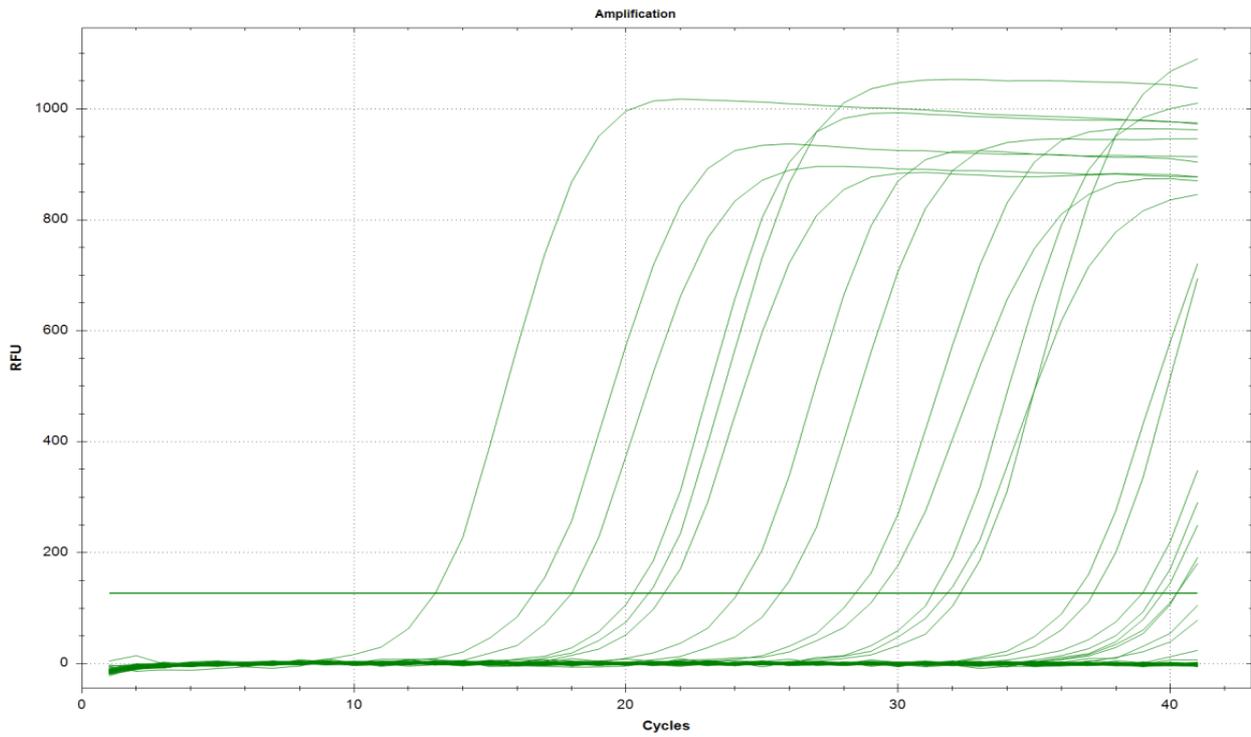


Figure 2. *M. bovis* nucleic acid signals in mammary tissues.

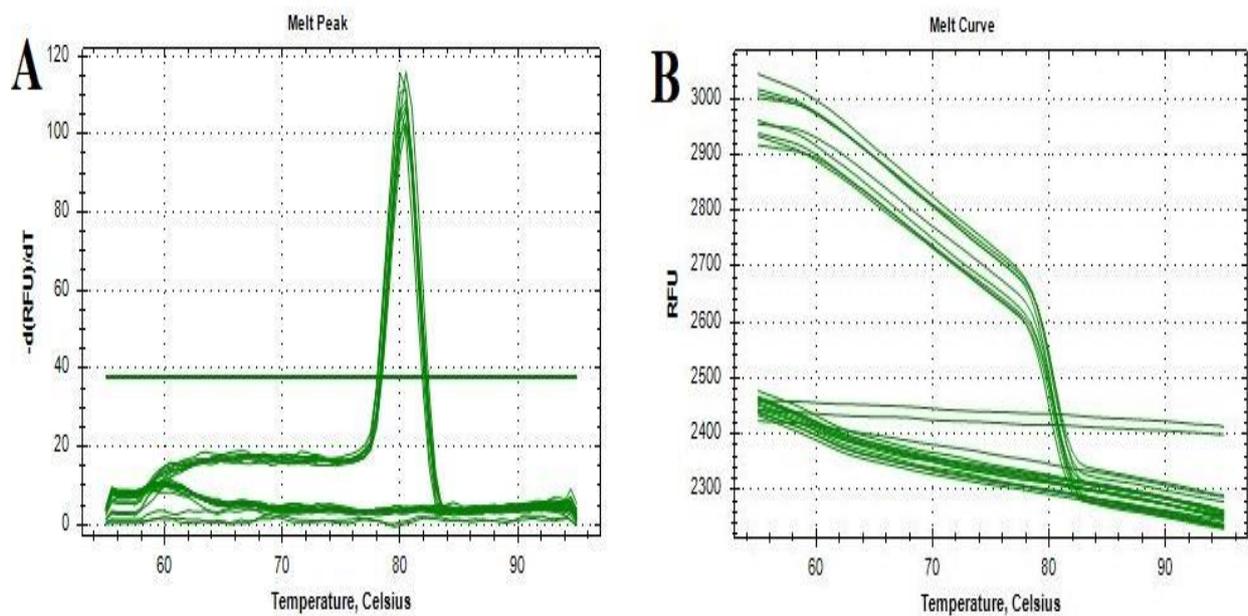


Figure 3. *M. bovis* primer melt peak and melting curve analysis.

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

Gen Name	Primer sequences	Annealing	Reference
<i>M. bovis</i> /uvrC	F:CCTGTCTGGAGTTGCAATTGTT R:CGGTCAACTTCAACTTGAATTTG	60	Fan et al., 2018

DISCUSSION

Mastitis diseases in cattle is widespread throughout the world, as are diseases which often results 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 (Soehnlén 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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