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RESEARCH ARTICLE

Diagnosis of *Mycoplasma bovis* Infection in Cattle by ELISA and PCR

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Summary

Mycoplasma bovis is one of the most pathogenic agents in the *Mycoplasma* species that cause disease in cattle. In particular, young calves at less than 4 months of age are a considerable risk from pneumonia caused by *M. bovis*. In this study, we investigated *M. bovis* from tracheal swabs and blood sera of cattle which showed respiratory symptoms. A total of 127 tracheal swab samples were collected from seven different farms in Turkey. In addition, a total 254 acute and convelance sera were collected from the same cattle at intervals 15 days. The materials were collected from cattle between 3-12 months of age that reported respiratory problems such as broncho-pneumonia with coughing, depression, lethargy and fever. *Mycoplasma bovis* was investigated in tracheal swab samples and sera collected from the cattle by using PCR and ELISA respectively. The PCR results showed that *M. bovis* infections were positive in 4 different farms. The rates ranged from 5.3% (1/19) to 37.5% (6/16). Out of the 127 cattle examined, 45 (35.4%) were positive for *M. bovis* antibodies, while 82 (64.6%) were found to be negative. All PCR positive cattle were also found to be positive by ELISA. However by using ELISA, *M. bovis* infections were positive in all farms and the ELISA positive rates ranged from 20% (2/10) to 68.8% (11/16). Considering these results, in especially chronic infections, ELISA is a more useful method than PCR to detect *M. bovis* infection.

Keywords: *Cattle, ELISA, Mycoplasma bovis, PCR*

Sığırlarda *Mycoplasma bovis* Infeksiyonunun ELISA ve PCR ile Teşhisi

Özet

Mycoplasma bovis, *Mycoplasma* etkenleri içerisinde sığırlarda infeksiyona neden olan en patojen etkenlerden biridir. Özellikle, 4 aylık yaşın altındaki genç buzağılarda, *M. bovis*'in neden olduğu pnömonilerde artan bir risk bulunmaktadır. Bu çalışmada solunum sistemi infeksiyonu semptomları gösteren sığırların trachea svapları ve kan serumlarından *M. bovis* infeksiyonunun teşhisi ve *M. bovis* teşhisi için serolojik ve moleküler metodların karşılaştırılması amaçlanmıştır. Türkiye'de bulunan 7 farklı çiflikten gönderilen 127 tracheal svap örneği ile 15 gün arayla aynı sığırlardan alınan 254 adet akut ve konvelesans serum örneği PCR ve ELISA yöntemleriyle incelendi. Bu örnekler 3-12 aylık yaşlar da olan ve bronkopnömoni, öksürük, depresyon, halsizlik ve ateş gibi solunum sistemi infeksiyonu semptomu gösteren sığırlardan toplandı. Tracheal svap örneklerinin PCR sonuçlarına göre 4 farklı çiftik *M. bovis* infeksiyonu yönünden pozitif bulundu. Oranlar %5.3 (1/19) ile %37.5 (6/16) arasında bulundu. *Mycoplasma bovis* antikorları yönünden incelen 127 sığıra ait serumlarda, 45 (%35.4) adeti pozitif olarak saptandı; 82 (%64.6) serum ise negatif olarak saptandı. PCR'da pozitif olarak saptanan tüm sığırlar ELISA yöntemiyle de pozitif olarak saptandı. *M. bovis* infeksiyonu tüm çiftliklerde pozitif olarak saptandı ve ELISA oranları %20 (2/10) ile %68 (11/16) arasında değişkenlik gösterdi. Bu sonuçlar göz önüne alındığında, özellikle kronik infeksiyonlarda *M. bovis* infeksiyonunun teşhisinde ELISA'nın, PCR yöntemine göre daha uygun bir yöntem olduğu sonucuna varıldı.

Anahtar sözcükler: *ELISA, Mycoplasma bovis, PCR, Sığır*

INTRODUCTION

Mycoplasma bovis is one of the most pathogenic agents in the *Mycoplasma* species that cause disease in cattle. *Mycoplasma bovis*-associated pneumonia occurs in cattle, including dairy and beef calves, beef cattle after arrival at a feedlot, and adults at any age [1]. *Mycoplasma bovis* is a particularly important cause of calf pneumonias [2,3]. Especially young calves under 4 months of age are at increased risk for pneumonia caused by *M. bovis* [2].

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Mycoplasma bovis infections can be explained as chronic and polymicrobial [1-5]. Animals can be infected via the respiratory system [2]. Respiratory tract and nasal secretions are important for epidemiology of infection [1,6]. Beside this, the importance of aerosols in calf-to-calf transmission of *M. bovis* is unknown but recently Maunsell et al.[1] reported that *M. bovis* has been isolated from air in shed containing diseased calves and calves may be experimentally infected by inhalation of *M. bovis*. Infected cattle spread *M. bovis* to enviroment through respiratory sectertions for many months as reservoir [4].

The severity of pneumonia could be varried depending on the maintenance and environmental conditions. The effects of antibiotics and vaccines are not known to have negligible effects in calves [3]. Control of infection is difficult and economic loses is imminent [7,8]. The first condition is to ensure a high level of hygiene in the prevention of *M. bovis* infection. Because of non-specific clinical manifestations, and a wide range of variation in the epizootiology and pathogenesis, all *M. bovis* infections are have to be had specific diagnostic and control procedures [7].

Mycoplasma bovis infections could be diagnosed by bacteriological culture and serological methods [2,6,9,10]. Nevertheless, these methods are time consuming and false-negative results could be common [9]. Serological methods are less time consuming than the cultural methods and also more samples can be investigated. Recently, new molecular identification methods were improved and used in diagnosis of *M. bovis* infections worldwide by several authors [11-13]. PCR is much shorter in comparison to the conventional culture method for the identification of *M. bovis* infection [12].

In Turkey there are few reports about the *M. bovis* infections in cattle [14]. In this study we investigated *M. bovis* from tracheal swabs and blood sera of cattle that showed respiratory symptoms for the situation of *M. bovis* in Turkey. Also we aimed to compare the efficiency of molecular and serological methods for detection of *M. bovis* infections.

MATERIAL and METHODS

A total of 127 tracheal swab samples and 254 acute and convelance sera (15 day intervals) were collected from 6-12 months age cattle located in seven different geographically distinct farms in Turkey that had respiratory problems such as broncho-pneumonia with symptoms coughing, depression, lethargy and fever *(Table 1)*. All farms were beef farms and the capacities were between about 100 and 14.000 cattle. All the samples were transported to the laboratory in cold chain and were stored at –20ºC.

Molecular Identification of Mycoplasma bovis

DNA extraction was performed by the boiling method from directly swab samples [14,15]. The swab samples were analyzed by PCR using *M. bovis* spesific primers derived from the *mb-mp*81 gene, as described by Foddai et al.[13,14]. *Mycoplasma bovis* spesific primers were used to amplify 447 bp of *mb-mp* gene of *M. bovis* (mb-mp1F: 5-TAT TGG ATC AAC TGC TGG AT-3; mb-mp1R: 5-AGA TGC TCC ACT TAT CTT AG-3). Amplification was performed in a total reaction volume of 50 µl, containing 5 µl 10x PCR buffer, 5 µl 25 mM $MgCl_2$, 250 µM of each dNTP, 1.25 U Taq DNA polymerase, 20 pmol of each primer and 25 ng of template DNA. The reaction conditions were as follows: denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min for 30 cycles, followed by a final extension step at 72°C for 10 min. The amplified products were detected by staining with 10 mg/ml ethidium bromide after electrophoresis at 80 V for 2 h in 2% agarose gels [14]. The results were screened from agarose gel by the molecular imaging system (Gene Genius, Syngene, England). *Mycoplasma bovis* DNA, which is used as positive control in PCR tests, was obtained from Prof. Dr. Burhan ÇETİNKAYA from Fırat University Veterinary Faculty Department of Microbiology Elazığ/Turkey.

Table 1. *Origins of sera and swap samples*
Tablo 1. *Serum ve svap örneklerinin orijinleri*

Farm	Number of Samples		Age (Months)
	Swabs	Sera	
Farm 1	16	32	6-12
Farm 2	10	20	6-12
Farm 3	16	32	6-12
Farm 4	19	38	3-12
Farm 5	10	20	6-12
Farm 6	27	54	6-12
Farm 7	29	58	6-12

ELISA

Bio-X *M. bovis* ELISA kit (BIO K 260, Belgium) was used in the serological analysis. The test was carried out according to the manufacturer's instructions. After the test, the absorbance values were read at 450 nm with a Titertec Multiscan MS plate reader.

RESULTS

The PCR results showes that *M. bovis* infections were positive in 4 different farms. The rates ranged from 5.3% (1/19) to 37.5% (6/16). The overall percentage, with a mean of a 12.6% (16/127) *(Table 2, Fig. 1)*.

The ELISA results showed in *Table 3*. Out of the 127 cattle examined, 45 (35.4%) were positive for *M. bovis* antibodies, while 82 (64.6%) were found to be negative. All PCR positive cattle were also found positive by the ELISA. *Mycoplasma bovis* infections were positive in all farms and the ELISA positive rates ranged from 20% (2/10) to 68.8% (11/16). The

Table 2. *PCR findings of tracheal swab samples*
Tablo 2. *Tracheal svap örneklerine ait PCR bulguları*

Farm	Number of Samples	Positive Numbers (%)
Farm 1	16	6 (37.5)
Farm 2	10	0
Farm 3	16	0
Farm 4	19	1 (5.3)
Farm 5	10	3 (30)
Farm 6	27	0
Farm 7	29	6 (20.7)
Total	127	16 (12.6)

with both mastitis and respiratory problems. In this study, the PCR results shows that 12.6% (16/127) positive samples were detected and ELISA results showed 35.4% (45/127) positive rates. In this study, we found approximately same detection rates and this finding supports the Karahan et al.[14].

PCR was reported to have effective specificity and sensitivity in diagnosis of *M. bovis* infections [11]. Previous studies have shown that several species can be detected via two-stage nested PCR. However, in these procedures it is need to be looked carefully to their sensitivity characteristics. Sung et al.[12], reported that the optimization of primer sequences and the reaction conditions presented here

Fig 1. Agarose gel electrophoresis of PCR. Line 1, molecular weight markers (Fermentas); Line 2, positive control; L3-L20, swab samples

Şekil 1. PCR sonucu elde edilen agaroz jel elektroforez görüntüsü. Sıra 1, moleküler ağırlık marker'ı (Fermentas); Sıra 2, pozitif kontrol; L3-L20, svap örnekleri

Table 3. *ELISA results in comparison with PCR findings*
Tablo 3. *PCR bulguları ile karşılaştırmalı ELISA sonuçları*

Farm	Number of Cattle	PCR Positive (%)	ELISA Positive			
			Total (%)	++	+++	++++
Farm 1	16	6 (37.5)	11 (68.8)	0	5	6
Farm 2	10	0	2 (20)	2	0	0
Farm 3	16	0	9 (56.2)	5	4	0
Farm 4	19	1 (5.3)	4 (21.1)	1	2	1
Farm 5	10	3 (30)	4 (40)	1	0	3
Farm 6	27	0	7 (25.9)	6	1	0
Farm 7	29	6 (20.7)	9 (31)	0	3	6
Total	127	16 (12.6)	45 (35.4)	15	14	16

overall percentage was found positive as 35.4 % (45/127) *(Table 3)*.

DISCUSSION

This research showed that *M. bovis* infection is a common respiratory problem in cattle in Turkey. *Mycoplasma bovis* infections are causing various economic loses such as treatment, laboratory diagnosis and product for dairy and beef cattles.

In Turkey, Karahan et al.[14] were investigated a total of 148 samples (3 lungs, 4 eye swabs, 51 nasal swabs and 90 milk samples) from three different farms in Eastern Turkey. They found 23% (34/148) samples to be positive. These samples were 3 lung, 12 to 51 nasal swabs and 19 to 90 milk. Authors concluded that *M. bovis* was relatively common in eastern region of Turkey especially in cattle

enhanced the sensitivity and ensured the high degree of specificity of the two-stage nested PCR. It was revealed that the specificity of the method presented their study was sufficient for the discrimination of mycoplasma contamination from other probable contaminants, including *E. coli*, *S. aureus*, and budding yeasts. It was showed in this study that the use of PCR makes the identification of *M. bovis* infection much shorter comparing to the conventional culture method.

The other method for detection of *M. bovis* is serology. Serological tests shows increasing antibody titres ten to fourteen days after the onset of clinical symptoms. Consequently, the pathogen can not be detected during the incubation period [7,9]. Sachse et al.[7] reported that the authors developed an ELISA for *M. bovis* antibodies using whole-cell antigen of the agent for solid-phase coating. The assay has proved sufficiently specific and the sensitivity of detection (10^5-10^6 cfu/ml) was 100 times greater than

with other serological methods [7]. The critical problem in these methods is the serological cross reactions between *Mycoplasma* strains.

Comparison of the PCR and the ELISA results in this study showed that positive rates in PCR were less then the ELISA in all farms. The results can be explained as sampling: It must be taken into consideration with respiratory sampling that *M. bovis* can be better recovered from broncho-alveolar lavages than nasal swabs, although this method is much more diffucult [8]. All animals in these farms were treated with different antibiotics. PCR results could be affected negatively due to regular treatment with antibiotics at a high dosage (mycoplasma cell numbers in materials may be less then detectable limits). Antibodies to *M. bovis* persist for several months and can be detected easily with the ELISA.

Because of the lack of cell wall in *M. bovis*, certain groups of antibiotics do not effective [2]. These antibiotics are used to treat for the secondary bacterial infections but often ineffective to treat Mycoplasma infections [4]. Because of the difficulties of the treatment with antibiotics, vaccine is important for *M. bovis* infections. One experimental vaccine study in calf reported that a single dose of vaccine prepared from saponised *M. bovis* cell can provide effective control against mycoplasma induced calf pneumonia. Calves tested for 6 months after immunisation had high level of humoral immunity [4]. No vaccine is currently used against *M. bovis* infection in Turkey.

Considering these results, the ELISA was found to be more useful method than PCR to detect *M. bovis* infection because of the persistence of *M. bovis* antibodies especially in chronic infections and the results also induce a strong need for the effective vaccine development for Mycoplasma infections in Turkey.

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