



Detection of *Mycoplasma bovis* in bulk tank milk samples by nested PCR in Mashhad, Iran

Mehran Dabiri,^a Pezhman Mirshokraei,^a Mehrnaz Rad,^b Babak Khoramian^a

^a Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

^b Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Keywords

Mycoplasma bovis, milk tank, Mashhad, nested PCR

Abstract

Mycoplasma bovis is a highly contagious major mastitis pathogen with multiple clinical presentations in dairy cows. This kind of mastitis does not respond to available antibiotics and actually there is no effective therapy for this infection, thus the best way of prevention and control is to diagnose and cull the affected cows in the herd. The objective of this study was to detect *Mycoplasma bovis* in bulk tank milk samples by nested PCR in Mashhad, Iran. One hundred and four fresh bulk tank milk samples from 52 dairy herds were collected four weeks apart. *Mycoplasma bovis* was not detected from any of them by either direct PCR on milk or after enrichment in modified Hayflick's broth. Two other mycoplasma species were detected after enrichment and one other mycoplasma

species without enrichment by mycoplasma spp. primer. Sequencing of the PCR products from two positive samples confirmed the presence of mycoplasma that were *Mycoplasma canadense* and *Mycoplasma yeatsii*.

Abbreviations

PCR: Polymer Chain Reaction
PBS: Phosphate Buffered Saline
SPP: species

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Tel: +98 51 3880 3742 Fax: +98 51 3876 3852
Web: ijvst.um.ac.ir Email: ijvst@um.ac.ir

Corresponding author:

Babak Khoramian
Department of Clinical Sciences, Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad, Azadi Sq., Mashhad, Khorasan
Razavi Province, Postal Code 9177948974, Iran
Tel: +98 51 3880 5611
Fax: +98 51 3876 3852
E-mail: Khoramian@um.ac.ir

Introduction

Mycoplasma bovis is a highly contagious major mastitis pathogen with multiple clinical presentations in dairy cows [1]. Also it is the most pathogenic agent of outbreaks of mycoplasmal mastitis in cattle [1]. The most important route of transmission inside herds is milking instruments such as milking machines, teat cups and also milker's hands [2-4]. Recent infections usually occur after the introduction of infected replacements without any sanitary precautions [2, 4, 5]. Mycoplasmal mastitis does not respond to available antibiotics and actually there is no effective therapy for this infection [1], thus the best way of prevention and control is to diagnose and cull the affected cows in the herd [2, 4]. Identification of farms with mycoplasma problem and the infected cases within a herd can be performed with culture of milk from clinical mastitis suspected cows or bulk tank milk samples. Nowadays molecular approaches such as polymerase chain reaction (PCR) with higher sensitivity, higher specificity and in shorter time periods are widely used in laboratories [6]. Mastitis due to *Mycoplasma* is reported from many regions in the world, however there is only one report of prevalence of this microorganism from Iran [7], which had been performed in a western state by applying only the culture method. Therefore the objective of this study was to determine the prevalence of *Mycoplasma bovis* in bulk tank milk samples of herds in suburb of Mashhad in northeastern Iran by nested PCR.

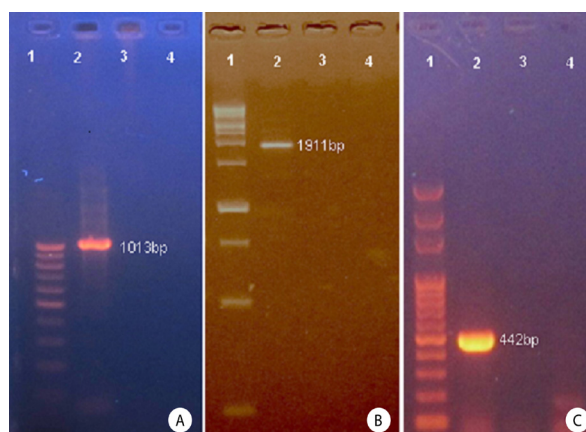


Figure 1

Gel electrophoresis of amplicons after PCR.

(A) Agarose gel electrophoresis of the 1013-bp of 16S rRNA gene of *mycoplasma* spp. Lane 1: 100 bp DNA ladder; Lane 2: positive control; Lane 3: negative control; Lane 4: sample.

(b) Agarose gel electrophoresis to show the 1911-bp PCR product of *mycoplasma bovis*. Lane 1: DNA ladder; Lane 2: positive control; Lane 3: negative control; Lane 4: sample.

(C) Agarose gel electrophoresis to show the 442-bp nested PCR product of *mycoplasma bovis*. Lane 1: 100 bp DNA ladder; Lane 2: positive control; Lane 3: negative control; Lane 4: sample.

Results

Mycoplasma bovis was not detected from any bulk milk samples by either direct PCR on milk or after enrichment in modified Hayflick's broth (Figure 1). Two other mycoplasma species were detected after enrichment and another mycoplasma species without enrichment by mycoplasma spp. primer (Table 2). Sequencing of the PCR products from two positive samples confirmed the presence

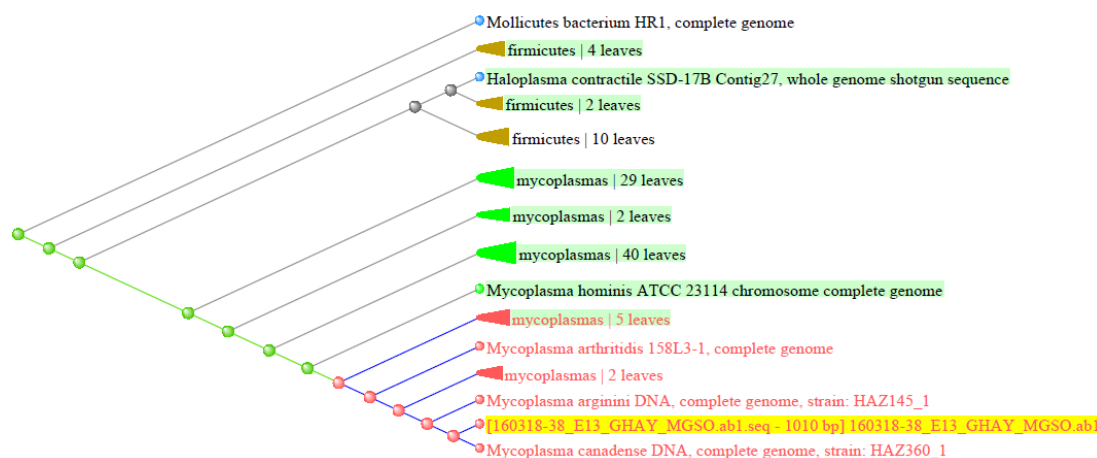


Figure 2

Alignment of the sequence of PCR product of mycoplasma bovis 16s rRNA.

of mycoplasma that were *Mycoplasma canadense* and *Mycoplasma yeatsii* (Figure 2).

Discussion

This cross-sectional survey of bulk milk tank from representative dairy herds in Mashhad, Iran, was specifically aimed at determining *Mycoplasma bovis* and the possible role of other causative organisms. The findings indicated that no evidence of *Mycoplasma bovis* was found using nested PCR. Several PCR assays have been described in the scientific literature for the detection of *Mycoplasma*

contrast with another study performed in Iran that reported high prevalence of mycoplasma mastitis [7]. This could be related to the improved mastitis control programs in farms during recent years or geographical differences in the two regions being studied with more than 1000 km distance between them. Investigations in other countries showed the between-herd prevalence of *Mycoplasma bovis* in bulk milk ranging from 0 to 8 percent in New Zealand [14], USA [15], Greece [16] and Belgium [17]. Detection of mycoplasma in bulk tank milk has been shown to be repeatable, but in our study one of the farms was negative in first sampling.

Table 1

The oligonucleotide primers used in this study

Primers	Sequence (5'-3')	Annealing temperature	Size of amplified product (bp)	Reference
16S rRNA_F	5-GCTGGCTGTGTGCCTAATACA-3'	56°C	1013 bp	19
16S rRNA_R	5-TGCACCATCTGTCACTCTGTAAACCTC-3'			
PpMB920-2	5'- TTTTAGCTCTTTTTGAACAAAt-3'	48°C	1911 bp	11
PpMB920-1	5'-GGCTCTCATTAAAGAATGTC-3'			
PpSM5-1	5'-CCAGCTCACCCCTTATACATGAGCGC-3'	54°C	442 bp	11
PpSM5-2	5'-TGA CTCACCATT TAGACCGACTAT TTCAC-3'			

bovis [8-12]. The nested *Mycoplasma bovis* PCR was selected for use in the survey as it was reported to have a detection limit of 5 cfu/ml of milk, was analytically specific, and had a better diagnostic sensitivity than culture [11]. In addition, the PCR assay using the primary primers [PpMB920-1 and -2] was reported to differentiate all true-positive and true-negative isolates in a blinded ring trial of different PCR systems and laboratories [13]. However, in this study for confirmation of results, we used another primer set for detection of mycoplasma spp. The results of this study showed that *Mycoplasma bovis* was not detected from any of 52 farms and other *Mycoplasma* spp. were detected in three of 104 bulk tank milk samples. This is in

This can be related to new epidemic of mycoplasma mastitis in the farm or cows which have already recovered from an initial infection and have stopped or lowered shedding mycoplasma to an undetectable limit. In addition, bulk-tank milk samples generally represent only healthy, untreated cattle within a herd, since milk from unhealthy or treated cattle is kept separately. In our study, enrichment in Hayflick media improved isolation of mycoplasma from bulk tank milk samples. Similarly, Gonzales and colleagues showed the use of pre-enrichment in Hayflick's broth yielding 6% more isolates of *Mycoplasma* from bovine milk [1]. In addition, large herds (500 animals or more) were more likely to have positive *Mycoplasma* cul-

Table 2

Nested PCR and modified Hayflick's medium culture results for detection of *mycoplasma* in 104 bulk tank milk samples

	<i>Mycoplasma</i> Spp.		<i>Mycoplasma bovis</i> (nested PCR)	
	First sampling	Second sampling	First sampling	Second sampling
PCR on milk				
(without enrichment)	0/52	1/52	0/52	0/52
With enrichment	1/52	2/52	0/52	0/52

tures than medium-sized [100–499] or small herds (<100) [7].

Materials and Methods

The central region of Mashhad has a total of 57 dairy herds which included 16625 dairy cows. To ensure accurate screening, two fresh bulk tank milk samples from 52 herds were collected four weeks apart. Samples were obtained 1 to 2 hours after milking. The milk in the bulk tank was mixed for 5 to 10 minutes. Approximately 15 ml of bulk milk was taken from the top of the tank using a sterile syringe and pipette. The sample was poured in a 15 ml screw cap sterile conical tube and transported to the laboratory on the ice.

A method previously described by Pinnow et al. [11] was used to prepare the bulk tank milk samples. Briefly, milk samples were vortexed to homogeneity. 1 ml from each sample was transferred to a 1.5 ml microfuge tube, and 500 µl of sterile PBS was added. After a short vortex, the samples were centrifuged at 14000×g for 20 minutes at room temperature. The supernatant was removed and the pellet was resuspended in 1 ml of PBS and centrifuged with the same conditions but for 10 minutes. Then, the pellet was subjected to DNA extraction (GeneAll[®], South Korea). The samples were pre-enriched in Hayflick's broth as described by Baas et al. [18]. 16S rDNA gene of *Mycoplasma* genus was detected by PCR method using specific oligonucleotide primers shown in Table 1 [11, 19]. Amplification was performed in a final volume of 25 ml containing 10 µl of Taq DNA polymerase 2x master mix red containing; 2 mM MgCl₂, Tris-HCl pH = 8.5, [NH₄]₂S₀₄, 4 mM MgCl₂, 0.2% tween 20, 0.4 mM dNTPs, 0.2 units/µl ampliqon Taq DNA polymerase inert red dye and stabilizer (Ampliqon[®], Denmark), 0.5 mg/mL of each primer and 3 µl template DNA. The PCR conditions consisted of a pre-denaturation step at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72° C. A final extension step was performed at 72° C for 5 min. Amplified products were analyzed by electrophoresis on 1% agarose gel. DNA bands were visualized by staining with ethidium bromide and photographed under UV illumination. The conditions for the nested PCR reaction were followed according to Pinnow et al. [2001]. In the external PCR reaction primers PpMB920-1 and PpMB920-2, and for nested reaction primers PpSM5-1 and PpSM5-2 (Table 1) were used. Each tube had a total volume of 25 µl, which contained the same concentration of reagents as the genus specific PCR reaction. The PCR conditions consisted of a pre-denaturation step at 94°C for 10 min, followed by 35 cycles of 30 s (for external reaction), 45 s (for internal reaction) at 94°C, 60 s at 48 °C (for external reaction), 54°C (for internal reaction), and 150 s (for external reaction), 120 s (for internal reaction), at 72°C. A final extension step was performed at 72°C for 5 min. The PCR product from the external reaction was diluted 1:100. Five microliters of this dilution was used as the template for the nested reaction. Positive and negative controls were always included. Amplified products were analyzed by electrophoresis on 1% agarose gel. DNA bands were visualized by staining with ethidium bromide and photographed under UV illumination.

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Author Contributions

Conceived and designed the experiments: B.K., P.M. Performed the experiments: M.D. Consulted for microbiology experiments: M.R. Wrote the paper: M.D., B.K.

Conflict of Interest

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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