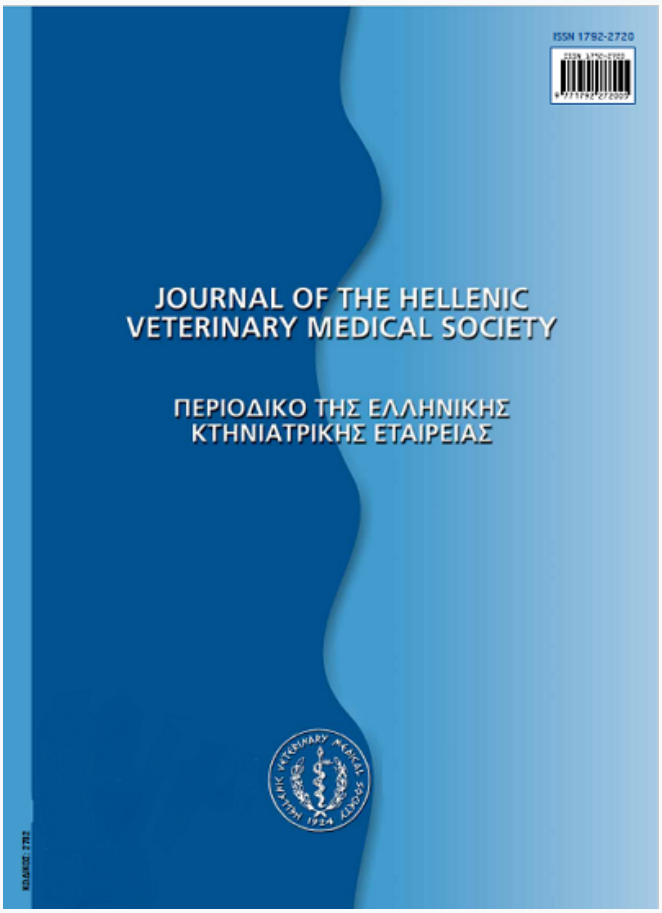
Journal of the Hellenic Veterinary Medical Society

Vol 69, No 1 (2018)



Isolation, identification and molecular characterization of Mycoplasma bovis in mastitic dairy cattle by PCR and culture methods

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doi: 10.12681/jhvms.16433

To cite this article:

IMANDAR, M., POURBAKHSH, S. A., JAMSHIDIAN, M., & ZAHRAEI SALEHI, T. (2018). Isolation, identification and molecular characterization of Mycoplasma bovis in mastitic dairy cattle by PCR and culture methods. *Journal of the Hellenic Veterinary Medical Society*, *69*(1), 815–822. https://doi.org/10.12681/jhvms.16433

J HELLENIC VET MED SOC 2018, 69(1): 815-822
ΠΕΚΕ 2018, 69(1): 815-822

Research article
Ερευνητικό άρθρο

Isolation, identification and molecular characterization of *Mycoplasma bovis* in mastitic dairy cattle by PCR and culture methods

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ABSTRACT. *Mycoplasma bovis* is well known as one of the major causative agents of mastitis in dairy cattle herds. The aim of this study was the identification of *Mycoplasma bovis* strains by PCR and traditional culture methods from a total number of 328 milk samples collected from cows with clinical mastitis symptoms from all over Iran. First step cultures in PPLO broth and agar showed 58 samples (17.69%) as positive. Out of 328 samples, 97 samples (29.57%) were positive for *Mycoplasma* genus according to the amplification of the *16SrRNA* gene performed by PCR and from them, 31 (31.97%) samples were positive by PCR on the *P48* gene. The purified *P48* positive PCR products were sequenced and results were compared to *M. bovis* reference strain *PG45* (CP002188). A phylogenic tree was created using Neighbor-joining method in MEGA6 software. The studied strain IB220 showed 100% identity with the reference strain of *M. bovis* and followed the same phylogenetic roots while studied strain IB216 showed 99.7% homology with the reference strain. Twelve selected geographical isolated strains were subjected to Gene Bank under accession numbers of KX772789 to KX772800. This is the first study of the molecular characterization of *Mycoplasma bovis* in dairy cattle with clinical mastitis from Iran.

Keywords: *Mycoplasma bovis*, cattle, isolation, PCR, mastitis

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Date of initial submission: 14-2-2017
Date of revised submission: 5-5-2017
Date of acceptance: 18-6-2017

1. INTRODUCTION

Mycoplasma bovis is well known as one of the major causative agents of mastitis in dairy cattle herds. The bacteria infection can also cause abortion, infertility, arthritis, kerato-conjunctivitis, otitis media, pneumonia, and subcutaneous abscesses which lead to huge economic losses worldwide (Giacometti et al., 1999; Nicholas and Ayling, 2003). There are at least 11 other *Mycoplasma* species associated with mastitis outbreaks in cattle, but the infection induced by *M. bovis* is characterized by the increased number of severe clinical mastitis that are mostly non-responsive to treatment. *Mycoplasma* are small prokaryotes with simple encoding gene treasure which cause numerous diseases in cattle of dairy farms such as arthritis and mastitis which lead to huge economic losses worldwide (Hotzel et al., 2003). The organism is also known as the main causative agent of therapy-resistant mastitis on large dairy farms. Traditional methods of detection and isolation of *Mycoplasma* strains such as serological and culture methods are time consuming, so the more sensitive biological methods such as PCR, are widely used for the detection and molecular analysis of various bacteria such as *M. bovis* strains in every microbial laboratories worldwide (Kirk and Lauerman, 1994). *P48* is a membranous lipoprotein of slightly lower than 48kDa that is homologous to the family of the Macrophage Activator Lipoproteins (MALPs) and it is coded via a conserved sequences specific to the *M. bovis* species (Wawegama et al., 2014), so the sequence of *M. bovis* strain *PG45* under accession number of CP002188 was selected (Wise et al., 2011) to compare with the strains detected in this study. The encoding gene sequence of lipoprotein *P48* is recorded as accession numbers of DQ020481 and DQ020482 at Gene Bank (Lysnyansky et al., 2008) and it is known as one of the virulence factors of *M. bovis* species (Li et al., 2011). As the *P48* gene is well conserved in all *M. bovis* strains, primers with high sensitivity and specificity were designed (Fu et al., 2014), and used for the amplification of *P48* gene. The immunogenicity of *P48* is also well known as one of the identified immunodominant membrane protein of *M. bovis* surface antigenic variation (Behrens et al., 1994). Robino also studied the conserved surface lipoprotein *P48* as a marker for the detection of *M. bovis* strains (Robino et al., 2005). One of the housekeeping genes which are known as conserved sequences in organisms, is *uvrC* encoding gene considered as *M. bovis* marker's too (Thomas et al., 2004). The *uvrC* encodes deoxyribodipyrimidine photolyase which removes damaged DNA and play the role in the repairing system in *Mycoplasma* species (Hotzel et al., 2003). The study of the sequences of the *uvrC* encoding gene sequence may lead to understanding of the phylogenic relations of different strains of *M. bovis* species. Rossetti used a novel designed pair of primers for the amplification of the *uvrC* gene by Real-time-PCR as the bio marker of *M. bovis* strains (Rossetti et al., 2010). Because of the important role of *M. bovis* in clinical mastitis in dairy herds, this study was designed to isolate and characterize *M. bovis* strains originated from different clinical mastitis cases from all over Iran, using two traditional culture-biochemical methods in comparison to highly sensitive molecular tools such as the PCR method.

2. MATERIALS AND METHODS

Sampling was done from a huge survey population of dairy cow husbandries from all over Iran, from April 2015 to May 2016. Raw milk samples were collected from 328 cows suffering from clinical signs of mastitis. The samples were immediately placed in test tubes with transport *Mycoplasma* medium and then transferred on ice to the *Mycoplasma* reference laboratory of Razi Vaccine and Serum Research Institute, Karaj, Iran, in 24 hours and incubated at 37°C for 12-18 hours with CO_2. After the primary concentration of *Mycoplasma* bacteria in the PPLO broth, 700µl of each cultured sample was added to 5ml new sterile PPLO broth using specific antibacterial filter (PVDF) which is permeable to organisms smaller than 450nm in size such as *Mycoplasma* and viruses. Main cultures were incubated at 37° under CO_2 gas treatment for two weeks. As *M. bovis* considered a non-glucose fermentative species, the red color of the PPLO cultures was notified as a positive biochemical sign of the specific growth of *M. bovis* species. 700µl of each sample were stored at -70°C for DNA extraction. 200µl of *M. bovis* PPLO culture were used on PPLO agar and incubated at 37° under CO_2 condition. The detection of *Mycoplasma* specific colonies after 5-7 days, was considered as a positive sign of the isolation of *M. bovis* bacteria.

Table 1. Primer pairs for amplification three encoding genes of 16SrRNA, P48 and uvrC.

Kojima et al., 1997	163bp	GCTGCGGTGAATACGTTCT	16Sr RNA	M1F	Genus specific primers
		TCCCCACGTTCTCGTAGGG		M3R	
Fu et al., 2014	1341bp	GCTTCATGTGGTGATAAATACTTTA	P48	IMB-F	Species specific primers (1)
		CTATTTTTGTGTTTCTTTAGCCAAT		IMB-R	
Bashiruddin et al., 2005	1626bp	TTACGCAAGAGAATGCTTCA	uvrC	MbouvrC-L	Species specific primers (2)
		TAGGAAAGCACCCTATTGAT		MbouvrC-R	

2.1. DNA Extraction and primers design

DNA was extracted from identified biochemically *Mycoplasma* colonies using Phenol-Chloroform extraction method (Pourbakhsh, 2010). A pair of *Mycoplasma* genus specific primers was designed for the first step of screening and the discrimination from other probable bacteria. *16SrRNA* gene was selected as the target gene for detecting the genus, as described by Kojima (Kojima et al., 1997). Then two pairs of *M. bovis* species specific primers on *P48* and *uvrC* genes were designed as described by Fu and Sabramaniam, respectively (Fu et al., 2014; Sabramaniam et al., 1998) (Table 1).

2.2. PCR of *16SrRNA* gene for detection of Genus *Mycoplasma*

10ng of extracted DNA subjected as template in 100 microliter total reaction volume of 2µl of each primers (20µM, Cinnagen, Iran), 2µl of each dATP, dTTP, dGTP and dCTP (200µM Fermentase), 10µl of 10X PCR buffer, 2.5U Taq DNA polymerase enzyme (2500U - 5U/µl - Cat. No. DP1603) (Cinnagen, Iran), 1.5 mM $MgCl_2$ in an automated Thermo cycler (*FALC*, Germany) under the following programs:

a) for the *16SrRNA* encoding gene (Kojima et al., 1997), 94 °C (7.5min) of initial denaturation and then 30 cycles of denaturation at 94 °C (30s), annealing at 56 °C (30s), elongation at 72 °C (60s) and final extension in 72 °C (5min).
b) for the amplification of *P48* encoding gene (Fu et al., 2014), 94 °C (4min) of initial denaturation and then 35 cycles of denaturation at 94 °C (60s), annealing at 58 °C (60s), elongation at 72 °C (100s) and final extension in 72 °C (10min).
c) for the amplification of *uvrC* encoding gene (Bashiruddin et al., 2005), 94 °C (2min) of initial denaturation and then 35 cycles of denaturation at 94 °C (30s), annealing at 52 °C (30s), elongation at 72 °C (60s) and final extension in 72 °C (7min).

PCR products were subjected to electrophoresis on 1.5% agarose gel in TBE buffer (1X) at 100V and were visualized under UV light by *Erythro-gel* staining (*Biotium*, USA). All the positive detected *P48* PCR products presenting the specific 1341bp length band, were purified by PCR Product Purification Kit following the instructions of the manufacturer (*MBST*, Iran). The purified PCR products of the *P48* amplified target gene were sent for bidirectional sequencing (*Bioneer*, Korea). Sequencing results were compared with other *P48* sequences recorded in Gene Bank using the Bio-Edit software. Phylogenic tree were designed by the Bootstrap1000 and Neighbor-Joining Tree analysis. The statistical analysis for significance of difference between age, rate of production and herd size with the prevalence, was done using Chi-Square test.

3. RESULTS

Culture results on *PPLO a*gar growth showed fried egg appearance in 58 out of total 328 (17.69%) raw milk samples under light microscopy. Cultures showed that 270 milk samples (82.31%) were negative. We also analyzed with PCR the negative cultures of the samples. *16SrRNA* gene PCR results showed 97 out of 328 (29.57%) positive samples, including the 58 positive culture results, which indicates that the 39 samples with negative culture results were positive by PCR indicating the higher sensitivity of the method. 231 samples (70.43%) were negative according to the PCR of the *16SrRNA* gene. All of the culture positive samples were showed as positive by the PCR results, as shown in Figure 1.

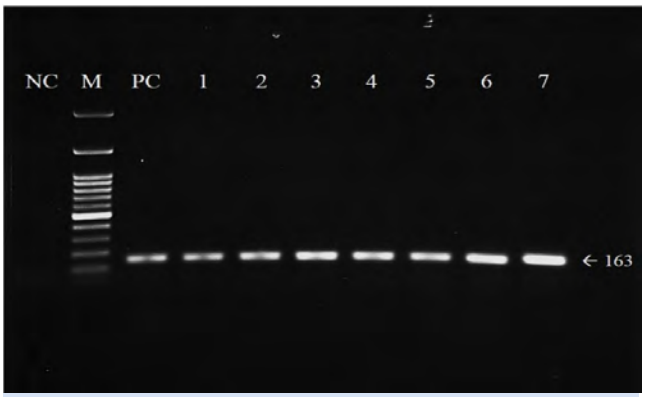

Fig 1. PCR results of the amplification of the *16SrRNA* gene.
M: Marker 100bp, PC: Positive Control (*M. bovis* ATCC 25523/ PG45), NC: Negative Control, 1-7: *Mycoplasma* detected samples.

Species specific PCR on *P48* gene was performed on samples which were proved as *Mycoplasma* by PCR of the *16SrRNA* gene. Results showed 31 out of 97 strains as *M. bovis* species and 66 samples were negative, belonging to other *Mycoplasma* species, Figure 2. The prevalence of clinical mastitis caused by *M. bovis* in this study was calculated as 31.97%.

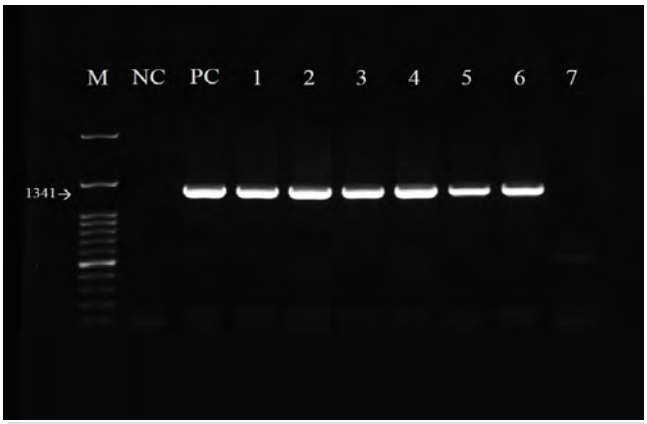

Fig 2. PCR results using specific Primers *P48*.
M: Marker 100bp, NC: Negative Control (*M. agalactiae* NCTC 10123), PC: Positive Control (*M. bovis* ATCC 25523/ PG45), 1-6: 1341 bp length PCR products of M. bovis species.

Another PCR with species specific designed primers on the *uvrC* gene, was performed on randomly selected samples which proved as *M. bovis* by *P48* specific primers as mentioned above. Results showed a 1626 bp PCR product as expected, Figure 3.

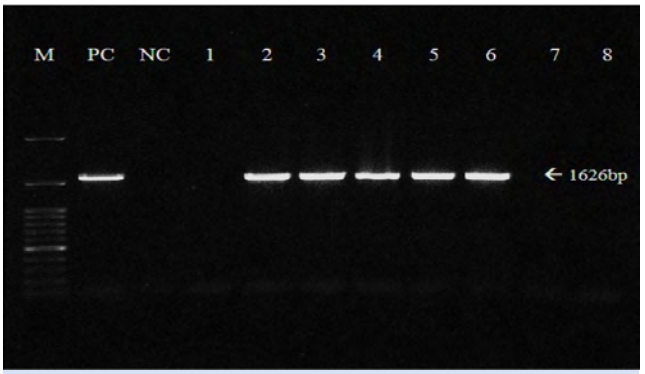

Fig 3. PCR results using specific Primers uvrC.
M: Marker 100bp, PC: Positive Control (*M. bovis* ATCC 25523/ PG45), NC: Negative Control, 2-6: Suspected samples, 1, 7, 8 are negative samples.

12 sequenced strains of this study as shown in the table 2 were aligned and analyzed by Bio-Edit software and were compared to *Mycoplasma bovis PG45* MU clone A2 complete genome, recorded at Gene Bank under accession number CP002188. PCR products of *PG45* amplification test were selected for sequencing and results were compared using online multi nucleotide sequence BLAST software. Twelve strains isolated from different geographical areas were compared. Results showed the maximum and minimum identity of 100% and 99% among these geographical obtained strains as shown in table 2. From the 12 selected strains only two strains (Query_160084 and 160085) had 99% identity.

Strain IB220 showed the maximum identity with the reference *M. bovis* sequence and the other strains showed a minimum identity of 99% (Table 3).

Finally, the above twelve strains were further analyzed for phylogenic tree (Figure 4). The phylogenic tree that was based on the molecular analysis and BLAST comparison results also confirmed the 100% identity of the strain IB220 with the reference strain of *M. bovis* in Gene Bank following the same phylogenetic roots, while the studied strain of IB216 has the identity of 99.7% with the reference strain. Alignment results showed 99.7% similarity between IB220 and IB216 samples and 99.8% similarity to other strains of the study as well. All the 12 geographical isolated strains from different parts of the country that were sequenced in this study were subjected to Gene Bank under accession numbers of KX772789 to KX772800.

Table 2. Results of antimicrobial susceptibility testing in Salmonella isolates.

	Max score	Total score	Query cover	E value	Identity	Accession
IB252	2091	2091	100%	0.0	100%	Query_160091
IB240	2091	2091	100%	0.0	100%	Query_160089
IB239	2091	2091	100%	0.0	100%	Query_160088
IB230	2091	2091	100%	0.0	100%	Query_160087
IB228	2091	2091	100%	0.0	100%	Query_160086
IB215	2091	2091	100%	0.0	100%	Query_160083
IB40	2091	2091	100%	0.0	100%	Query_160082
IB20	2091	2091	100%	0.0	100%	Query_160081
IB13	2091	2091	100%	0.0	100%	Query_160080
IB216	2085	2085	100%	0.0	99%	Query_160084
IB241	1890	1890	90%	0.0	100%	Query_160090
IB220	1801	1801	86%	0.0	99%	Query_160085

Table 3. BLAST comparison analysis of 12 selected strains with the reference strain of PG45 ATCC 25523. IB220 showed 100% identity with the reference strain.

	Max score	Total score	Query cover	E value	Identity	Accession
IB20	2252	2849	0%	0.0	99%	Query_41471
IB228	2248	2846	0%	0.0	99%	Query_41476
IB252	2242	2840	0%	0.0	99%	Query_41481
IB230	2242	2840	0%	0.0	99%	Query_41477
IB240	2231	2829	0%	0.0	99%	Query_41479
IB239	2231	2829	0%	0.0	99%	Query_41478
IB216	2217	2809	0%	0.0	99%	Query_41474
IB40	2213	2810	0%	0.0	99%	Query_41472
IB215	2207	2805	0%	0.0	99%	Query_41473
IB13	2085	2683	0%	0.0	99%	Query_41470
IB241	1884	2473	0%	0.0	99%	Query_41480
IB220	1807	2367	0%	0.0	100%	Query_41475

In order to study the relation between the age and the prevalence of the infection, samples were classified in five different groups including 65 samples (2 positive, 63 negative) at age 24-36 months, 56 samples at age 36-48 months (4 positive, 52 negative), 76 samples at age 48-60 months (8 positive, 68 negative), 42 samples (12 positive, 30 negative) at age 60-72 months and 89 samples at age more than 72 months (5 positive and 84 negative). In our study, there was statistical significance in the incidence of *M. bovis* mastitis among the different age groups ($P<0.05$).

Study analysis showed the prevalence of 38.71% infection in high rate of production cows, 38.71% in cows with moderate rate of production and 22.58 % in cows with low rate of milk production. This study did not show statistically significant between the prevalence and rate of production ($P>0.05$). The majority of positive samples were in the herds with the average size of over than 800 cows. There was significant relation between herd size with the incidence of infection ($P<0.05$). Results also showed that 142 out of total 328 milk samples were taken from

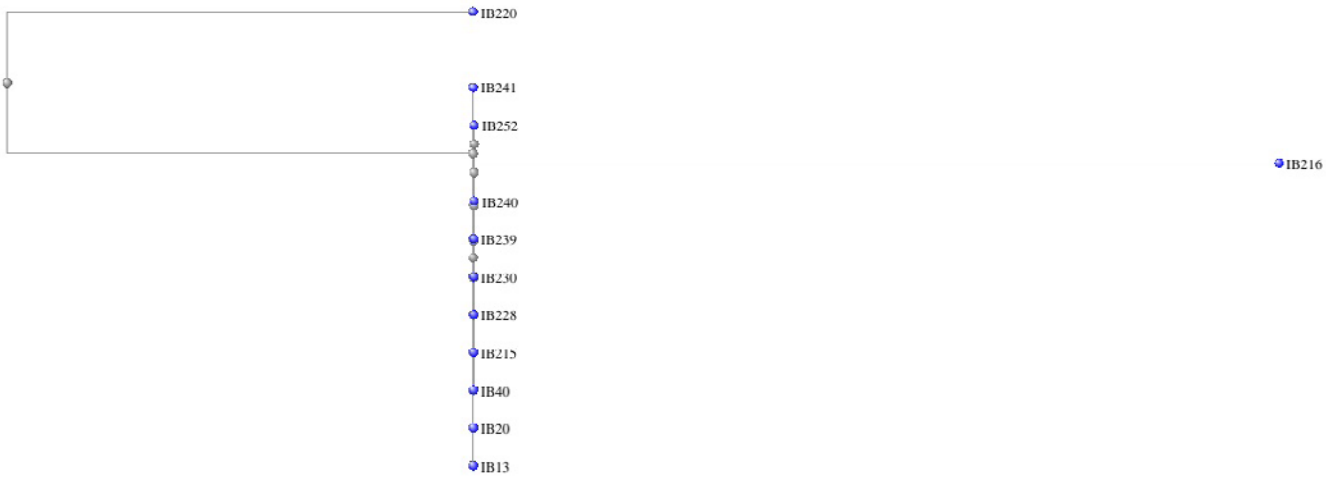

Fig. 4. The Phylogenic Tree drawn by Tree was drawn using Neighbor-joining method in MEGA6 software.

cows with the clinical recorded history of 1 to 10 times acute mastitis.

4. DISCUSSION

Mycoplasma bovis is widely known to be the most important etiological agent of various bovine diseases leading to huge economic losses (Pfützner and Sachse, 1996). In current study *Mycoplasma bovis* from mastitic milk of cattle farms in Iran were isolated and identified using PCR and culture methods. As clinical and pathological signs are not characteristic for *M. bovis* infection, laboratory diagnosis is necessary. Traditional methods for detection and isolation of *Mycoplasma* strains are time consuming, so more sensitive methods such as PCR, are widely used in every microbial labs. In this study culture results of milk samples showed 58 out of total 328 (17.69%) *M. bovis* bacteria with fried egg appearance under light microscopy. In a conducted study, comparing obtained isolates with existing isolates in Gene Bank based on the gene sequence *16SrRNA* proved 100 percent similarity between isolates (Cai et al., 2005).

Except for the 58 samples identified as positive by cultures, 39 samples from the negative cultures were found positive in the *16SrRNA* encoding gene PCR. All of the positive samples detected by culture were led to positive PCR results. The comparison of the two methods of PCR and the cultures for the detection of mycoplasma species in milk samples, showed that sensitivity and specifity of PCR are 96.2% and 99.1%, respectively. Also, the sensitivity and specificity of PCR on bulk tank milk compared with cultures of the same samples are 100% and 99.8%, respectively (Baird et al., 1999). In the study by Pinnow and colleagues, the sensitivity of Nested-PCR for the detection of *M. bovis* in milk kept for 2 years, was 100%, while the sensitivity of culture was 27%; so this approach was introduced as an appropriate method to identify and isolate *M. bovis* in clinical samples (Pinnow et al., 2001).

It was indicated that the identification of *M. bovis* strains in cattle herds and the evaluation of their pathogenicity and antigenic variation is important (Behrens et al., 1994). *P48* is a membranous lipoprotein of 48kDa weight that is homologous to the family of the Macrophage Activator Lipoproteins (MALPs) and is coded via a conserved sequence specific to the *M. bovis* species (Wawegama et al., 2014). As the *P48* gene is a conserved sequence in all *M. bovis* strains, designed primers by Fu (Fu et al., 2014), were used for amplification the *P48* gene in this study. Robino also studied the conservative surface lipoprotein *P48* as a marker for the detection of *M. bovis* strains (Robino et al., 2005). Study of *P48* sequence may lead to understand the phylogenic relations of different strains of *M. bovis* species. Specific PCR of *P48* gene was done on samples identified as *Mycoplasma* by PCR on *16SrRNA* gene. Results revealed that 31 out of 97 *Mycoplasma* detected samples were belonged to *M. bovis* species (31.97%) and 66 samples (68.04%) were belonged to

other *Mycoplasma* species. The results of the study demonstrated that the cattle farms from Iran were infected with *M. bovis*. The pollution is very different compare to the rest of the world as the presence of bacteria in milk samples in this study was lower than its presence in Italy (Radaelli et al., 2011) and higher than other countries such as France (Arcangioli et al., 2011). Sequenced strains of this study were aligned and analyzed by Bio-Edit software and were compared to other *P48* sequences of *M. bovis* recorded at Gene Bank (NCBI). The results of alignment analysis indicated that the majority of the *M. bovis* strains, which was isolated from Iranian cows, had 99-100% homology to the reference strain of *M. bovis*. In previous studies, other gene was used for evaluating phylogenetic relationship among the isolated *M. bovis* strains. So far, there was no study based on *P48* gene examining the phylogenetic relation between *M. bovis* strains, but other studies have been conducted on the basis of other genes. In Egypt, assigning of *Vsp* genes sequence for molecular analysis of the isolated *M. bovis* strains showed that some isolates have homology with reference strain PG45 and some other were divided into different groups (Eissa et al., 2012). In Germany, the phenotypic changes of *M. bovis* isolated from culture methods and specific PCR was shown that the isolated strains are different from *PG45* and there is dissimilarity between them; 5 isolates had identical DNA profiles but three other isolates performed different patterns. The results showed that protein patterns within species *M. bovis* strains can be very useful for the comparison (Hala and Hotzel, 2013).

According to studies, cows in all ages may be affected by this type of mastitis but fresh cows mostly show severe symptoms (Radostits et al., 2000). In this study, most of positive cases were in 60-72 months age group with 38.8% and 48-60 months with 25.8%, respectively. The results of this study are inconsistent with the other studies. Fu showed that the highest rate of the prevalence of infection were seen in cows in age more than 1 year old with the prevalence of 10.5% infection following by a rate of infection up to 9.61% in cows at age 3 with no reliable relations between the age of prevalence and rate of pregnancy in cattle (Fu et al., 2014). The most positive cases were related to cattle with herd size 800-1500 (45.16%) and cattle with 1500 herd size and higher (32.25%), respectively. This is consistent with findings in other studies. Research in United States showed that an increase of mycoplasma mastitis is associated with an increase in herd size. It had been shown that risk of *M. bovis* mastitis and possibility of a positive sample in a big herd with more than 350 cattle, is 15 times higher than herds with fewer than 350 cattle and the same it is in their milk tanks (Park, 2014). In other study in dairy herds in Northern Italy that was conducted on a sample of clinical mastitis in dairy cows, the results showed that the occurrence of mastitis is common in larger herds (Radaelli et al., 2011).

5. CONCLUSIONS

In this study results showed the highest prevalence of positive samples in cows with average and high production rates. The highest prevalence belongs to 4-6 years old group, high production cows with the average size of over than 800 cows. This is the first study on the isolation, identification and molecular characterization of *Mycoplasma bovis* infection in dairy cattle with clinical mastitis from Iran. Results suggested *M. bovis* as one of the main causative agents of dairy cow mastitis in Iran.

6. ACKNOWLEDGMENTS

We would like to thank the Mycoplasma Reference Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran that this study was managed and performed there, we would also thank the Department of Microbiology, Science and Research Branch, Islamic Azad University, Tehran, Iran, for their kind regards of technical support.

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