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

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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 phylogenetic 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
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 Lauer, 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 CO2. 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°C under CO2 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 bio-chemical 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°C under CO2 condition. The detection of Mycoplasma specific colonies after 5-7 days, was considered as a positive sign of the isolation of M. bovis bacteria.
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 MgCl2 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 agar 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.
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%.

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.

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 phylogenetic tree (Figure 4). The phylogenetic 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.
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 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

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<th>E value</th>
<th>Identity</th>
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Table 2. Results of antimicrobial susceptibility testing in Salmonella isolates.

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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.
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 specificity 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 phylogenetic 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 (Arcangiolì 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
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