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Research Article

Molecular Detection of Selective Virulence Factors of *Mycoplasma bovis* Local Isolates Involved in Bovine Mastitis

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Abstract

Mycoplasma bovis is believed to be a major cause of pneumonia, mastitis, and arthritis in cattle. Mastitis is the most prevalent production-related disease in dairy herds. The present study aimed to improve the current knowledge on the virulence factors of *M. bovis*-induced mastitis in dairy cows. To this end, sampling was done from farms in Chaharmahal and Bakhtiari province. The samples were monitored for growth in a specific PPLO medium in a CO2 incubator. Simultaneously, the DNA of the samples was extracted and PCR was used to detect M. bovis strains. After identifying susceptible isolates of *M. bovis*, P48, alpha-enolase, P81, and LppB genes were investigated in *M. bovis* positive PCR samples. In total, out of 204 samples, 11 isolates suspected of *M. bovis* were obtained (5.39%) via culture method and 21 positive samples (10.29%) were obtained through PCR. 57.14 (12/21), 66.66 (14/21), and 52.38 (11/21)% of the samples were positive concerning the presence of P48, alpha-enolase and LppB genes, respectively. P81 was not detected in any of the positive samples. The results of the present study showed that the presence of *M. bovis* in dairy herds in Chaharmahal and Bakhtiari could be considered among the factors that cause mastitis in cattle. alpha-enolase gene was found to be more abundant than the other selected genes, which could play an important role in the future strategic measure to develop preventive measures against mycoplasmal mastitis.

Keywords: Mastitis, *Mycoplasma bovis*, Virulence gene, Prevalence

Introduction

Mastitis can inflict significant harm upon farm animals, primarily manifesting as diminished milk production in lactating cows during their lactation period and leading to the extermination of affected animals [1,2]. In cases of acute mastitis, substantial losses, and costs are tied to the removal and mortality of farm animals. Meanwhile, subclinical forms of mastitis, often stemming from delayed disease diagnosis, result in pronounced reductions in milk production and quality during lactation. The subclinical variant is the most prevalent type of mastitis, which poses the most significant detriment to the livestock industry. The acute form of mastitis, though less frequent, holds significance, too, with its extent of impact varying across different countries and farming setups [3,4]. Notably, acute and subclinical mastitis can also detrimentally affect the fertility of farm animals. Mastitis syndrome is inherent in all mammals, yet its prevalence is heightened in cows due to milking-induced stress. The foremost pathological changes arising from mastitis, affecting both mammary tissue and milk, encompass alterations in milk color, presence of clots, elevation in somatic cell and white blood cell counts within milk, shifts in mammary gland tissue, and the occurrence of swelling, heat, pain, and detectable stiffness upon palpation of the mammary glands. It is essential to acknowledge that many instances of subclinical mastitis are challenging to identify readily [5,6].

The etiology of mastitis can be attributed to micro-organisms and their toxins, parasites, physical trauma, or chemical irritants. Bacterial infections, notably caused by microorganisms like *Streptococcus agalactiae*,

Staphylococcus aureus, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Escherichia coli*, constitute about 95% of mastitis cases. The remaining 5% stem from other microorganisms [1,4,7].

Mycoplasma, which belong to the Mollicutes class of bacteria and lack a cell wall, are ubiquitous and can potentially infect eukaryotes. *Mycoplasma bovis* is a destructive pathogen affecting beef and dairy cows globally, significantly contributing to mastitis, pneumonia, and osteoarthritis. These maladies collectively engender substantial economic losses within the dairy farming sector. *M. bovis* infections culminate in calf mortality, weight loss in surviving calves, and diminished milk production in dairy cows [5,8]. Of particular concern is mycoplasmal mastitis, particularly the infection of teats with *M. bovis*, which results in a surge in somatic cell count (SCC) in milk (from 10^7 to 10^9 cells per mL). This type of mastitis notably curtails milk production [6,9]. Importantly, mastitis arising from *M. bovis* infection endures over an extended period and predominantly presents as a subclinical mammary gland infection [2,4]. It has been documented that *M. bovis*-induced mastitis triggers a sustained increase in SCC and pro-inflammatory cytokines in milk; however, this persistent inflammatory response is not potent enough to eliminate *M. bovis* from the mammary gland [1].

Mycoplasma infections encompass conditions such as calf pneumonia and osteoarthritis, and additionally, they can give rise to genital disorders. Managing *M. bovis* infections effectively necessitates early intervention. Presently, immunoprophylaxis and antibiotic therapy have proven ineffective against *M. bovis* [2,10]. Exploring the surface protein alpha-enolase from *M. bovis* and its binding to plasminogen becomes pertinent [5,11]. Intriguingly, antibodies against alpha-enolase from *Streptococcus iniae* exhibit protective effects in models of zebrafish and mice, hinting at α-enolase's potential as a target for *M. bovis* vaccine production. The research underscores the correlation between the presence of the bacterial surface protein α-enolase and bacterial adhesion to host cells [1,12]. Owing to the marked antigenic diversity in *M. bovis*, proteins universally conserved across strains are likely the most promising vaccine targets. One illustrative example is the surface lipoprotein P48. This highly hydrophilic protein bears conserved motifs linked to complement activation and cytokine induction, rendering it an apt starting point for probing pathogenicity mechanisms and immune shifts during infection [1,13]. Notably, ten highly protected *M. bovis* proteins, namely PdhA, PepA, Tuf, P48, P81, OppA, LppA, PepQ, O256, and DeoB, have been recommended as suitable candidates for recombinant vaccine production. Surface antigens like P48, P81, and LppB Lipoproteins hold significance in this context [1,14].

In the the present study, the lack of comprehensive investigations into *M. bovis* virulence factors implicated in mastitis, coupled with the underexplored roles of P48, alpha-enolase, P81, and LppB in mastitis cases, underscores the need for further research. This study delves into the genes associated with each factor, focusing on the milk samples collected from mastitis cases.

Materials and Methods

Ethical Approval

The procedures were applied in the method section were in accordance with the experimental local ethics committee (Code of ethics: IR.SKU.REC.1399.005).

Sampling

Two hundred and four samples were gathered from eight semi-industrial cattle farms in the Chaharmahal and Bakhtiari province, specifically in Shahrekord city. For sampling, the cow's udder was washed and each cartier was wiped separately with a disposable napkin. The first three to four milkings were then discarded, and 50 mL of milk from each cartier was poured separately into a sterile falcon tube. After labeling, the samples were transferred to the laboratory on ice within a few hours. Clinical mastitis samples were procured from cows exhibiting at least one of the subsequent characteristics: (i) Visible abnormalities in the milk, such as discoloration, clots, or pus particles; (ii) Irregular physical conditions of the udder, including acute and diffuse swelling, warmth, pain, and redness; and (iii) General bodily responses encompassing varying degrees of anorexia, toxemia, dehydration, fever, increased heart rate, cessation of rumen movements, and listlessness. Before initiating the antibiotic treatment protocol, milk samples were obtained from the affected quarters.

Subclinical mastitis samples were acquired from quarters that tested positive using the California Mastitis Test (CMT). The CMT procedure was conducted as follows: following the exclusion of the initial three milkings from each quarter, starting from the fourth milking, approximately 2-3 mL of milk was deposited into the corresponding compartment of the CMT container (Maki Kala, product ID: MAKI-D-1041). Subsequently, 2-3 mL of CMT solution (purple bromocresol-containing detergent; KerbaTEST solution, KERBL Company) was introduced into each compartment. The CMT container was agitated for 10 sec to amalgamate the milk with the CMT solution. Ultimately, the outcomes of each Cartier test were independently interpreted and recorded.

Culture in PPLO Medium

The milk samples were centrifuged at 4000 rpm for 40 min. Then the sediment was first cultured in 5 mL of PPLO Broth medium for 24 h at 37°C and in the presence

of 10% carbon dioxide. After initial enrichment, 2 mL of media containing samples were cultured in the new PPLO Broth medium including basic medium plus 20% horse serum, 0.05% thallium acetate, and penicillin G (500 U/mL) and 5 0.0% of yeast extract and incubated in a CO_2 incubator for 5-7 days at 37°C, 90% humidity and 10% CO_2. The mediums were passaged again in PPLO Broth containing supplements two more times. At the end of each stage, 200 µL of the culture were transferred to the PPLO agar medium containing 20% horse serum, 0.05% thallium acetate, and 500 IU/mL penicillin G, and then incubated for 10 days at 37 degrees, 90% humidity, and 10% CO_2. After 72 h, for 10 days, the culture mediums were examined with an inverted microscope and with 10 X magnification in terms of growth and formation of "fried egg" shaped colonies.

DNA Extraction

After the centrifuging of the milk samples at 4000 rpm for 40 min, DNA extraction was done by DNP Kit (Cinacolon, Cat. No: EX6071) according to the manufacturer's instructions. Briefly, at first, 100 µL of protease buffer was added to 500 µL of milk sediment. After adding 5 µL of proteinase K and pipetting, the mixture was incubated for 30 minutes at 55°C. The lysis buffer was placed at 37°C for 10 minutes, then 400 µL of it was added to 500 µL of milk precipitate. In the next step, 300 µL of sedimentation buffer was added to the collection, vortexed for 5 sec, and then centrifuged at 12000 rpm for 10 min. The supernatant was discarded, then 1 mL of washing buffer was added and vortexed for 3 to 5 sec, and afterward, centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellet was placed at 65°C for 5 min to dry completely. The precipitate was dissolved in 50 µL of the solvent buffer by gently shaking and placing it at 65°C for 5 min. After centrifugation at 12000 rpm for 30 sec, the supernatant containing DNA was collected. The extracted DNA was kept at -20°C until the PCR test was performed.

PCR

The primer design was done using Gene Runner software for the sequences related to the genes encoding the selected virulence factors *(Table 1)*. For the molecular identification of *M. bovis*, species-specific primers [15] were applied. Briefly, PCR was performed at a total volume of 25 µL. Final concentrations in the reaction mixes were 12.5 µL of Taq DNA Polymerase Master Mix RED 2x (Ampliqon, Cat. No.: A180301), 1.5 mM MgCl2, 20 pmol of each primer, 0.5 µg of genomic DNA, and 9.5 µL of deionized Sterile water. Positive (prepared from the Mycoplasma Research Center - Razi Vaccine and Serum Institute) and negative controls (distilled water) were included in each PCR reaction. The temperature conditions were set according to the data described in *Table 2*. After the completion of the PCR reaction, the PCR products were subjected to electrophoresis on a 1.5% agarose gel next to a 100 bp DNA Ladder (Genaxxon bioscience, Cat No: M3340). Then the positive samples were sent to Bioneer Co, Korea for sequencing.

Statistical Analysis

SPSS software version 21 (IBM corporation, USA) was applied for the investigation of the relationship between the variables using the Chi-square test.

Results

Statistical Results

After using SPSS software version 21 (IBM corporation, USA) for the investigation of relationships between the variables, applying the Chi-square test, no statistically significant relationship was found between the frequencies of virulence genes ($P>0.05$). There was a statistically significant difference between the prevalence of *M. bovis* among clinical and subclinical cases ($P<0.05$) using PCR test; but in case of culture method, this relationship was not significant ($P>0.05$). Furthermore no statistically significant relationships were found between the frequencies of virulence genes among clinical and subclinical cases ($P>0.05$). If *M. bovis* was detected by PCR in the first step, the investigation of virulence genes, was done. The relationship between the frequencies of virulence genes in cases isolated using culture method was not significant ($P>0.05$). Finally, a statistically significant relationship was found between the culture method and PCR in the detection of *M. bovis* ($P<0.05$).

Identification of Suspected Isolates of *M. bovis* Using Bacterial Culture

After culturing in PPLO agar and observing the morphology of the colonies, a number of isolates were considered as suspected *M. bovis*. After observation with a light microscope, the colonies suspected to be *M. bovis* had the characteristic morphology of this bacterium, i.e. "fried egg". Colonies were denser in the center and less dense in the periphery and were different in diameter *(Fig. 1)*. In general, the diameter of hemispherical egg colonies in PPLO agar medium varies from 10 to 500 µm. After the bacterial culture, 11 suspected isolates of *M. bovis* were isolated. Using bacterial culture, the frequency of *M. bovis* in cases of clinical and subclinical mastitis were one and 10 cases, respectively *(Table 3)*.

Molecular Identification of *M. bovis* by PCR

After the electrophoresis of the PCR products using a pair of specific primers to investigate *M. bovis*, the observation of a 1626 bp band in the tested samples and the positive control indicated the presence of *M. bovis* nucleic acid *(Fig. 2)*.

From the total number of 204 examined milk samples that were simultaneously tested by bacterial culture and PCR, 21 positive samples were detected for the presence of *M. bovis* nucleic acid (10.29%), using PCR. Among 21 PCR positive samples, only eleven samples were positive using bacterial culture. Furthermore, among the *M. bovis* positive PCR products, the frequency of infection in the clinical mastitis cases was 2 and in the subclinical mastitis cases was 19 *(Table 4)*.

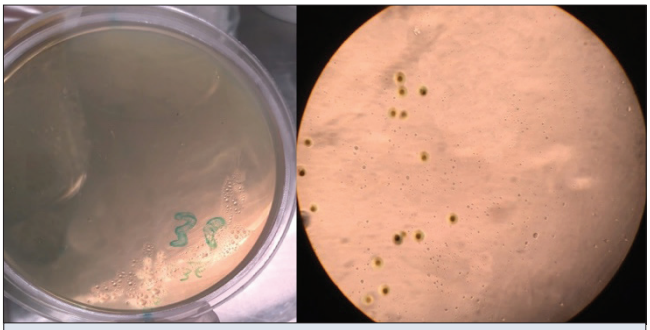

Fig 1. Suspected colonies of *M. bovis* observed on PPLO agar medium (magnification: 10X)

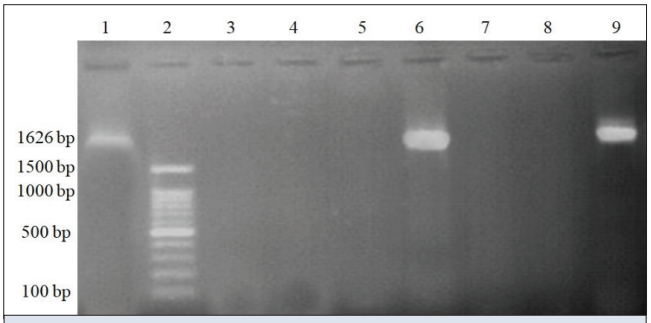

Fig 2. Electrophoresis gel of PCR product for detection of *M. bovis.* 1 and 6: Positive PCR products for M. bovis (1626 bp), 2: Ladder (100 bp), 7: Negative control, 3-5: negative PCR products, 9: Positive control (1626 bp)

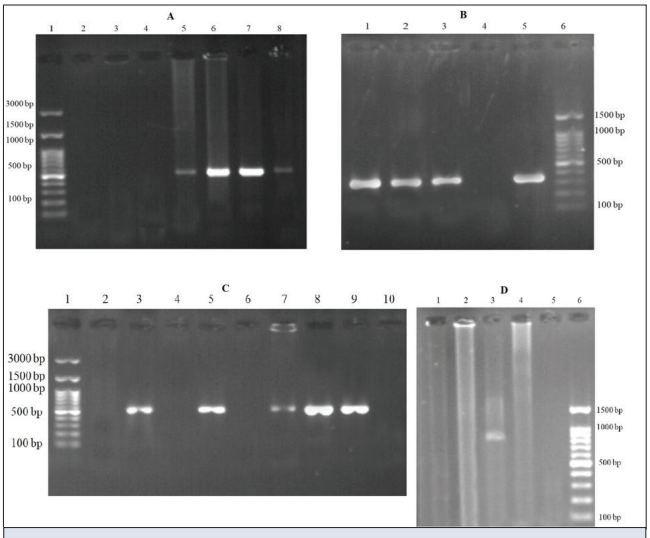

Fig 3. Electrophoresis of PCR product to detect P48, alpha-enolase, LppB and P81 genes. **A:** Detection of P48; 1: Ladder (100bp), 2: Negative control, 3, 4: Negative PCR products, 5-8: Positive PCR products for P48 gene (501 bp); **B:** Detection of alpha-enolase; 5 and 1-3: Positive PCR products for alpha-anolase (354bp), 4: Negative control, 6: Ladder (100bp); **C:** Detection of LppB ; 1: Ladder (100bp), 3,5,7-9: Positive PCR products for LppB gene (561bp), 6,2,4: Negative PCR product, 10: Negative control; **D:** Detection of P81; 1,2,4,5: Negative PCR products for P81 gene, 3: Positive PCR product for P81 (877bp), 6: 1: Ladder (100bp)

Investigation of Virulence Genes of *M. bovis* by PCR

In order to identify the selected virulence genes of *M. bovis*, PCR was performed on the DNA samples related to the cases that were found to be positive in the PCR test for the detection of *M. bovis*. After the electrophoresis of the PCR products for the detection of selected virulence genes, the bands with the sizes of 501 bp for P48, 354 bp for alpha-enolase, 877 bp for P81, and 561 bp for LppB were observed, indicating positive results for the presence of these genes. Unfortunately, in the case of the P81 gene, despite the application of several changes in the PCR protocol, only one suspected positive sample was obtained and the sample found was not confirmed by sequencing *(Fig. 3)*.

In total, out of 21 samples with positive results using the PCR test to identify *M. bovis*, after carrying out reactions related to the detection of virulence genes, 14, 11, and 12 samples were positive for the presence of the alpha-enolase, LppB, and P48, respectively *(Table 5)*.

Sequencing

In order to verify the products obtained in PCR reactions, sequencing was performed. 50 µL of the amplified fragments were sent to Gene Fanavaran Company for sequencing. The degree of homology (identity) of the sequence extracted from reading the PCR products with the sequences recorded in the gene bank and the expected amplicon was a good indication of the adequacy of PCR *(Fig. 4, Fig. 5, Fig. 6)*.

Table 1. *Sequence of primers used in this study*

Primer Name	Primer Sequence	Length	Tm (˚C)
MboF	TTACGCAAGAGAATGCTTCA	20	54
MboR	TAGGAAAGCACCCTATTGAT	20	54
P48F	TCAGTGCCATTGGTTGCTGCT	21	61
P48R	TGCTGCAGGTTCGCCAGTGC	20	65
AEF	ACCGGTGTTGCAAATGTGCCT	21	61
AER	TGGCAGTGGCAATGTGTGAGC	21	63
P81F	GGTAAGGGCGACGAGGACGA	20	65
P81R	CGGTTCCAACTTCTTCAGCGCCT	23	66
LppF	TGCTGCTAAATGTGGGGACGGT	22	64
LppR	CCTTTGCCAGGTTGTGCTGCT	21	63

Table 2. The thermal conditions for the amplification of the uvrC and virulence genes	
Gene	**PCR Cycling**
uvrC	94°C for 5 min followed by 35 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 60 s, followed by a final extension at 72°C for 10 min
P48	94°C for 5 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 35 s, followed by a final extension at 72°C for 10 min
alpha-enolase	94°C for 5 min followed by 30 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min
P81	94°C for 5 min followed by 30 cycles at 94°C for 45s, 67°C for 45 s and 72°C for 45 s, followed by a final extension at 72°C for 5 min
LppB	94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, followed by a final extension at 72°C for 10 min

Table 3. Frequency of M. bovis in cases of mastitis using bacterial culture			
Mastitis Form	**Total Frequency**	**Frequency of *M. bovis* Positive Cases**	**Percentage of *M. bovis* Frequency**
Clinical	61	1	1.63
Subclinical	143	10	6.99
Total	204	11	5.39

Table 4. Frequency of M. bovis in cases of mastitis using PCR			
Mastitis Form	**Total Frequency**	**Frequency of *M. bovis* Positive Cases**	**Percentage of *M. bovis* Frequency**
Clinical	61	2	3.27
Subclinical	143	19	13.28
Total	204	21	10.29

Table 5. Frequency of M. bovis virulence genes using PCR				
Virulence Genes	**Frequency Among Clinical Cases**	**Frequency Among Subclinical Cases**	**Total Frequency of Positive Cases**	**Percentage of Positive Results**
Alpha-enolase	2	12	14	66.66
LppB	2	9	11	52.38
P48	2	10	12	57.14

Discussion

The economic losses inflicted by mastitis are not solely attributed to afflicted cows but also encompass diminished milk production [16]. Additional expenses encompass medication costs, costs tied to the period of milk abstinence, veterinary charges, supplementary labor expenses, compromised milk quality, supplementary diagnostic supply expenses, and a decrease in the quality and quantity of a cow's life. Hence, addressing this disease necessitates an approach encompassing epidemiology, prevention, and treatment [17]. In the current study, 204 milk samples were procured from semi-industrial farms in Shahrekord, cultured on PPLO Agar medium, isolating 11 suspected *M. bovis* isolates. Subsequently, a PCR test revealed the presence of *M. bovis* nucleic acid in 21 samples (10.29%). The PCR test proved more sensitive to *M. bovis* than the bacterial culture method [18]. This highlights the significance of incorporating molecular tests, such as PCR, in conjunction with phenotypic assessments like bacteriological culture to diagnose the presence of *Mycoplasma* in mastitis precisely.

This outcome is congruent with prior research, which asserts that 30% of mastitis milk may not yield bacterial growth in culture media. However, PCR remains adept at identifying bacteria even when growth is hindered or terminated [18].

Though the culture method is meticulous due to the gradual growth of the *Mycoplasma* genus, distinguishing between various species based on colony examination is intricate [19]. While bacterial culture remains a definitive and precise technique, its application in detecting *Mycoplasma* in milk can be impeded by inherent or induced inhibitors, necessitating many additives contingent upon the *Mycoplasma* species [14]. The PCR method's heightened sensitivity and specificity in discerning *Mycoplasma* species has been proven. PCR's sensitivity and specificity for detection of mastitis milk samples has been reported at 96.2% and 99.1%, respectively [12]. Furthermore, PCR sensitivity in detecting *M. bovis* in milk has been documented in a number of previous studies [20,21].

Internationally, various studies have utilized the uvrC gene as a diagnostic target for *M. bovis*. Thomas et al.[21] identified the uvrC gene as an amplification target for *M. bovis* strains. Later, Rosetti et al.[22] devised a novel Real-time PCR method for uvrC gene detection, facilitating the direct identification of *M. bovis* from milk and tissue samples sans DNA extraction. Similarly, Imandar et al.[15] identified *M. bovis* in cows with clinical mastitis using culture and PCR based on the uvrC gene. Those findings underscore the prevalence of *M. bovis* in Iranian dairy herds, potentially emerging as a principal cause of clinical mastitis in the country.

In congruence with the abovementioned research, the present study employed the uvrC gene to detect *M. bovis* species. The study's outcomes validate the PCR method's high sensitivity in detecting *M. bovis*, echoing the findings of earlier investigations. Notably, the prevalence of *M. bovis* varies across different regions in Iran. Moshkelani et al.[23] conducted a study utilizing a PCR test based on 16SrRNA gene fragment amplification to assess the

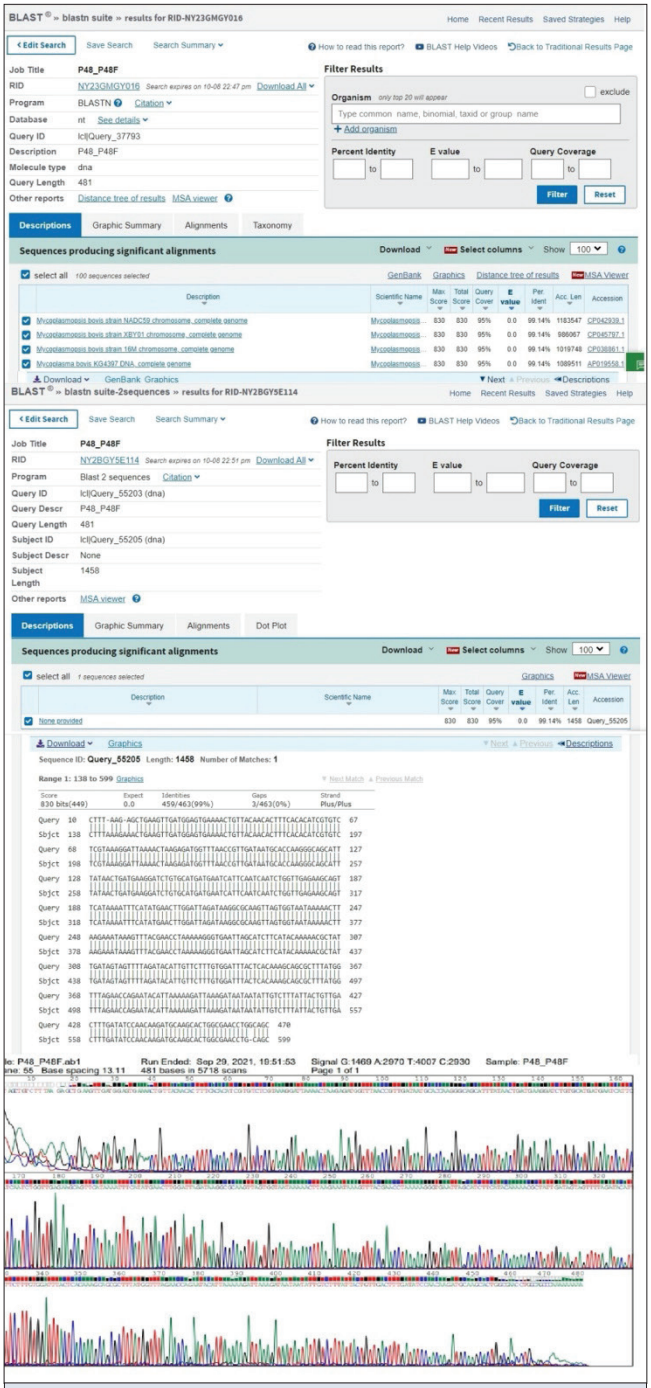

Fig 4. Alignment of the sequence extracted from the reading of the P48 gene PCR product with the sequences recorded in the gene bank and the expected amplicon

prevalence of subclinical mastitis in dairy herds in the Chaharmahal and Bakhtiari provinces. The prevalence of mycoplasmal mastitis was 48.67%.

A study was conducted in Shahrekord by Sharifzadeh et al.[24] using both culture and PCR methods to assess the abundance of *Mycoplasma* in industrial and semi-industrial dairy milk tanks. They found that the contamination of these tanks with *M. bovis* was 3.3%. The difference in the prevalence of mastitis due to *M.*

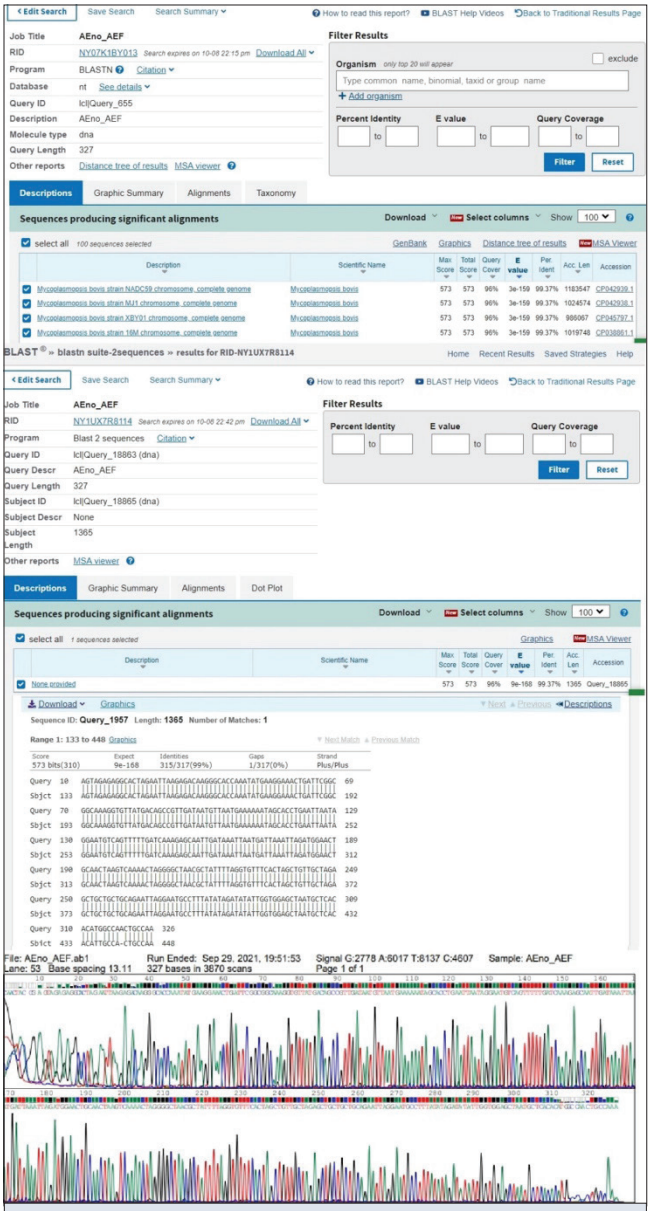

Fig 5. Alignment of the sequence extracted from reading the PCR product of the alpha-enolase gene with the sequences recorded in the gene bank and the expected amplicon

bovis in the current study (10.29%) compared to previous studies in Chaharmahal and Bakhtiari may be attributed to various factors, including the sampling location, farming system type, hygiene levels on each farm, and sampling conditions. Moreover, differences in the specific target gene amplified for diagnosing *M. bovis* could also contribute to the variations observed.

Given that hygienic practices during milking are more rigorously followed in industrial farms compared to semi-industrial ones in this province and that pathogen dilution is higher in milk tank samples, it was anticipated that the prevalence of *M. bovis* in the study conducted by Sharifzadeh et al.[24] would be lower than in the current study. At the time of sampling, we realized that,

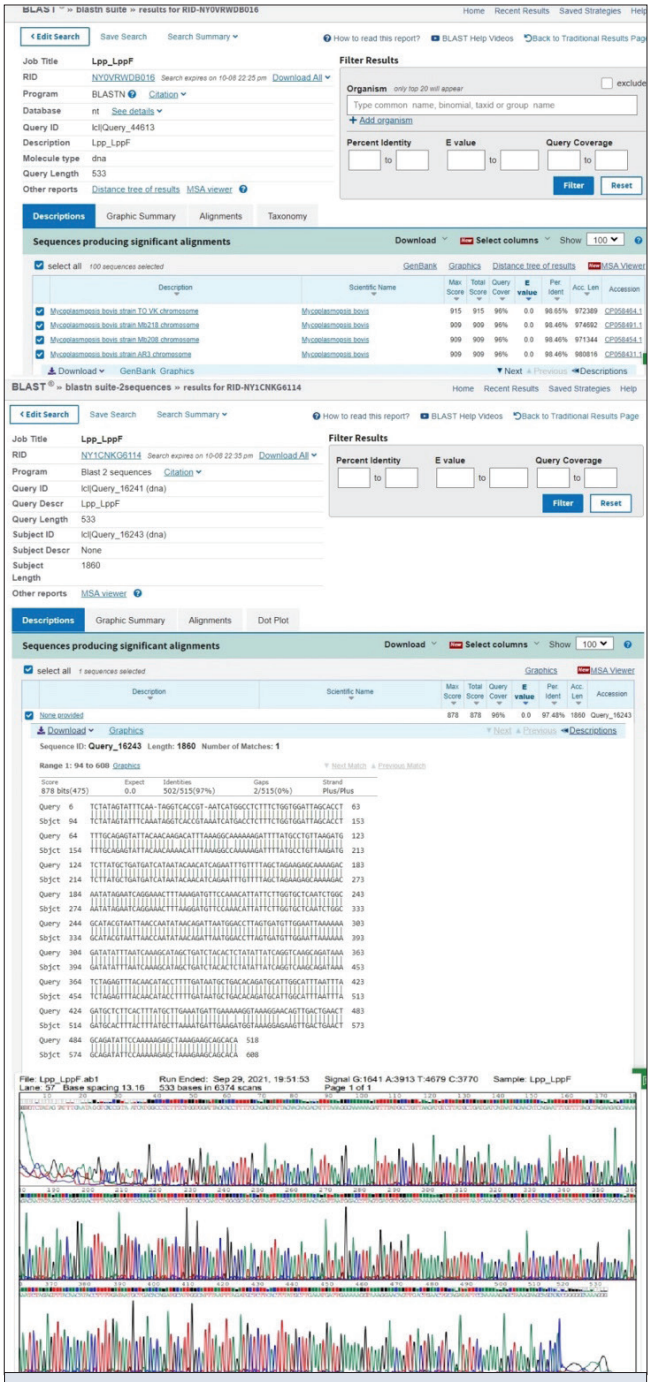

Fig 6. Alignment of the sequence extracted from the reading of the PCR product of the LppB gene with the sequences recorded in the gene bank and the expected amplicon

unfortunately, none of the necessary milking hygiene standards are observed in the sampling farms. The higher prevalence rate reported by Moshkelani et al.[23], compared to the current study, could be attributed to various factors. Notably, their study identified mycoplasmal mastitis in 50 dairy cows in general without limiting it to a specific species. Other factors that may have affected the prevalence include differences in management practices, choosing an adequate sample size, sampling times, and conditions.

Another study conducted in Ardabil examined 80 milk samples from dairy cows using culture in the Hyflick medium and immunoperoxidase testing. They found 39 samples (48.75%) of clinical mastitis cases were positive for *M. bovis* [25]. The methods employed for detecting *M. bovis* in study may have been effective in reporting a relatively high prevalence of *M. bovis*. Similarly, a study by Taleb Khan Grossi et al.[26] revealed an infection rate of 15.38% of mycoplasmal mastitis in dairy herds around Mashhad which is lower than prevalence rate reported by Moshkelani et al.[23], presumably due to a larger sample size and less incidence of antibiotic resistance in that year. Additionally, variation in dairy cattle breeding systems, milking hygiene within the herd and the timing and location of research can all lead to different results being obtained.

In the present study, the frequency of the alpha-enolase gene in the samples was higher than that of other selected genes, at 66.66%. Song et al.[27] introduced alpha-enolase as a virulence factor associated with *M. bovis* adhesion. Zhao et al.[28] further stated that specific highly conserved bacterial proteins, such as alpha-enolase, which are involved in metabolic regulation or cellular stress responses, also act as adhesives. *M. bovis*-encoded enolase is considered a pathogen-related factor that plays a role in metabolic pathways. Prokaryotic alpha-enolase may contribute to pathophysiological processes [28]. Additionally, surface-associated enolase is an adhesion-related factor in *M. bovis*, promoting adhesion by binding to plasminogen. Enolase could be a significant protein contributing to the virulence of *M. bovis* [29]. Although according to the available information, there are no reports comparing the difference in the prevalence of this virulence factor with other *M. bovis* virulence factors, the results of the current study revealed a higher frequency of the alpha-enolase gene among the selected virulence factors, indicating the particular functional importance of this protein.

The P48 protein is an immunodominant lipoprotein situated within the membrane. The P48 protein of *M. bovis* shares homology with the protein found in *M. agalactiae*. It serves as a valuable indicator of *M. bovis* infection and is an alternate candidate for the formulation of specific serological tests targeted at *M. bovis* [30]. Robino et al.[31] reported the detectability of the P48 protein in all the tested isolates. Fu et al.[32] developed a direct competitive ELISA for the detection of *Mycoplasma bovis* infection based on P48 protein.

Prysliak et al.[33] evaluated immune responses to ten proteins from *M. bovis*, including P48 and P81. These proteins demonstrated high conservation levels, exhibiting 98% to 100% similarities between the PG45, HB0801, Hubei-1, and CQ-W70 strains.

Consequently, these proteins emerge as promising targets for potential vaccines. Within the scope of this study, an exploration was undertaken to ascertain the frequency of select highly protected proteins, namely P48, P81, and LppB, in instances of bovine mastitis. Notably, P48 emerged as the second most prevalent virulence factor, with an incidence rate of 57.14%. Although there exists a lack of studies on the occurrence of this gene and other has chosen *M. bovis* genes, indications point toward the substantial significance of P48 [34]. LppB, an antigen shared among mycoplasmas, surfaced as a standard and distinct element [35]. In this study, the occurrence of this gene in samples collected from bovine mastitis cases registered at 52.38%, suggesting a possible pivotal role played by this gene in the pathogenicity of infections attributed to *M. bovis* in dairy cows. Encoded by the P81 virulence gene of *M. bovis*, the P81 protein stands as a membrane lipoprotein and represents one of the bacterial surface antigens. This specific protein has found utility in developing serological and PCR-based diagnostic methodologies for *M. bovis* [36]. A previous investigation spotlighted the efficacy of 9 recombinant proteins, including LppB and P81, when formulated with an adjuvant, in stimulating an effective Th17 response against *M. bovis*. Noteworthy results emerged from Zhang et al.[37] study, underscoring the inhibitory antibody titers found within the anti-P81 serum. Despite implementing several adjustments in the PCR method, the targeted gene could not be conclusively identified in this current study. However, it remains uncertain whether this gene is present in *Mycoplasma* strains within the study area, and ascertaining the abundance of this gene within the study population necessitates further research. Righter et al.[16] scrutinized 19 *M. bovis* isolates, discovering that using probes designed for *M. bovis* detection resulted in nucleotide disparities among 19 negative field samples. These disparities hindered probe hybridization, primarily due to critical discrepancies within the probe binding site. Across these isolates, the P81 gene encoding the membrane lipoprotein exhibited absence, truncation, or sequence elongation. This collection of findings signifies variations within the P81 gene that hinder primer or probe binding [36,37]. The study's results above partly clarify the current study's challenge in identifying *M. bovis* P81.

This current study unveiled the highest frequency of virulence genes within alpha-enolase and the lowest frequency within LppB. Notably, differences in the gene prevalence did not reach statistical significance, thereby underscoring the necessity for future research involving native isolates to definitively pinpoint the primary virulence factors.

Declarations

Availability of Data and Materials: The data that support the findings of this study are openly available.

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Author Contributions: Azam Moktari and Masoud Ghorbanpoor Najaf Abadi designed the study and performed analysis, Negar Ghazvineh performed the tests. Ali Kadivar collected the samples and performed the test in the field. Azam Mokhtari and Somayeh Shahrokh Shahraki wrote the manuscript. All authors read and revised the manuscript.

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