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Original Article

Intraspecies Gene Variation within Putative Epitopes of Immunodominant Protein P48 of *Mycoplasma agalactiae*

Panahi [1, 2], P., Pourbakhsh [1,*], S.A., Zahraee Salehi [2], T., Esmaelizad [3], M., Madani [4,5], R.

1. Mycoplasma Reference Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
2. Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
3. Central Laboratory Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
4. Proteomics and Biochemistry Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
5. Department of Microbiology, School of specialized Veterinary Science, Science and research Branch, Islamic Azad University, Tehran, Iran

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Corresponding Author: a.pourbakhsh@rvsri.ac.ir

ABSTRACT

P48 protein of *Mycoplasma agalactiae* is used to diagnose infection and was identified as potential vaccine candidate. According to the genetic nature of mycoplasma and variable sensitivity in P48-based serological diagnosis tests, intra species variation of P48 nucleotide sequence investigated in 13 field isolates of difference province of Iran along with three vaccine strains. Samples were collected from sheep and goat and were cultured in modified PPLO broth. Two pair of primer employed to confirm genus and species of isolates and a pair of primer has developed to amplify the P48 gene. The sequencing results of PCR products were aligned and analyzed besides published sequences in GenBank. T-Cell and B-Cell epitopes and antigenicity of sequence were computationally predicted. The results have shown P48 nucleotide sequences are 99.9% identical in field isolates and vaccine strain of Iran, but analysis of GenBank published sequences have shown divergence up to 5.3% at the nucleotide level and up to 4.9% divergence in protein level of P48 sequences of Iran isolates and other available sequences in GenBank. Single nucleotide polymorphism exists in 89 positions and variable amino acid was observed at 25 residues. Phylogenetic analyses have shown that *Mycoplasma agalactiae* isolates fall into three main groups based on P48 nucleotide sequences. Immunoinformatics analysis of all available P48 nucleotide sequences have revealed that gene variation lead to differences in immunological properties, but the gene in Iranian isolates are conservative and stable. The sequence variation in epitopes can be underlying source of antigen heterogeneity as a result, affect serological tests accuracy. Due to the high level of divergence in worldwide isolates and high degree of similarity in P48 protein of Iranian isolates, designing recombinant P48 protein based on local pattern can increase the sensitivity and consistency of serological test.

Keywords: Mycoplasma, Contagious Agalactiae, P48, Variation, Antigen Heterogeneity

Variation des Gènes Intraspécifiques dans les Épitopes Putatifs de la Protéine P48 Immunodominante de Mycoplasma Agalactiae

Résumé: La protéine P48 de *Mycoplasma agalactiae* est un candidat vaccin potentiel et est utilisée dans le diagnostic de l'infection. Compte tenu de la nature génétique de Mycoplasma et de sa sensibilité variable dans les tests de diagnostic sérologique basés sur P48, la présente étude visait à étudier la variation intraspécifique de la séquence de nucléotides P48 dans 13 isolats de terrain obtenus de différentes provinces d'Iran, ainsi que de trois souches de vaccin. Après avoir recueilli des échantillons de moutons et de chèvres, ils ont été cultivés dans

paires d'amorces; en outre, une paire d'amorces a été développée pour amplifier le gène P48. Les résultats de séquençage des produits de réaction en chaîne de la polymérase ont été alignés et analysés sur la base des séquences publiées dans GenBank. Les épitopes des cellules T et B et l'antigénicité de la séquence ont été informatiquement prédits. Selon les résultats, les séquences de nucléotides P48 étaient identiques à 99,9% dans les isolats de terrain et la souche vaccinale d'Iran. Néanmoins, l'analyse des séquences publiées par GenBank a démontré des taux de divergence allant jusqu'à 5,3% et 4,9% aux niveaux de nucléotide et de protéine des séquences P48, respectivement. Un polymorphisme mononucléotidique existait dans 89 positions, et des acides aminés variables ont été observés à 25 résidus. Sur la base de l'analyse phylogénétique, les isolats de *Mycoplasma agalactiae* sont répartis en trois groupes principaux basés sur les séquences de nucléotides P48. L'analyse immuno-informatique de toutes les séquences de nucléotides P48 disponibles a révélé que la variation du gène conduisait à des différences de propriétés immunologiques. Cependant, les gènes dans les isolats iraniens étaient conservateurs et stables. La variation de séquence d'épitopes peut être la source d'hétérogénéité antigénique, affectant ainsi la précision des tests sérologiques. En raison du niveau élevé de divergence dans les isolats du monde entier et du degré élevé de similitude de la protéine P48 dans les isolats iraniens, la conception de la protéine P48 recombinante basée sur un schéma local peut augmenter la sensibilité et la cohérence du test sérologique.

Mots-clés: Mycoplasme, Agalactie Contagieuse, P48, Variation, Hétérogénéité Antigénique

INTRODUCTION

Mycoplasma is smallest self-replicating pathogen with minimal genome. *Mycoplasma agalactiae* is the main and classical agent of Contagious Agalactia (CA), a highly contagious disease that affects goats and sheep. CA is characterized by mastitis, arthritis, kerato conjunctivitis, and occasionally abortion. Primary diagnosis of the diseases is based upon clinical signs. The clinically diagnosis of CA should be confirmed by lab tests. The culture, serology and molecular diagnosis are laboratory method to detect and confirmation of *M. agalactiae*. Current diagnostics methods of *M. agalactiae* suffer from many drawbacks (Fusco et al., 2007). Detection of antibodies in serum by enzyme-linked immunosorbent assay (ELISA) provides rapid diagnosis but serological methods which rely on whole cell preparations as antigens are vulnerable to potential cross reactivity (Stear, 2005; Alberti et al., 2008). Developing better diagnostic tests are necessary for control, eradication and vaccine evaluation of *M. agalactiae* the use of a recombinant protein in the ELISA is expected to greatly improve the assay. Identification of immunogenic protein can help diagnostics and vaccines development. So far a number of surface protein of *M. agalactiae* has been shown to stimulate the host humoral response, including lipoproteins P80 (Tola et al., 2001), P48 (Rosati et al., 2000) and the variable antigens (Vpma) family (Glew et al., 2002). Vpma undergo high frequency phase and size variation and do not permit easy serological identification of mycoplasma isolates and significantly hinder stable, specific, and strongly immunogenic antigens for *M. agalactiae* (Rosengarten and Yogev, 1996). Antigenic variation is a strategy by which a broad diversity of microbial pathogens are able to escape the immune system (Palmer et al., 2016). Antigenic variation is created via two general types of mechanisms, genetic and epigenetic. Genetic events (mutation and recombination) change the DNA sequence of an antigen encoding gene or its regulatory element. By contrast, epigenetic mechanisms affect the expression of a gene without altering its primary nucleotide sequence (Deitsch et al., 2009). Mycoplasmas have evolved a number of genetic mechanisms directed towards phase and size variation of major surface components (Rosengarten et al., 1994; Bergonier et al., 1996; Nyvold et al., 1997). Previous studies have shown antigenic variation in mycoplasma species results from genomic rearrangements by DNA inversion events (Citti et al., 2010). Antigenic variation by size and phase variation mechanism occurs in some

M. agalactiae lipoproteins (Bergonier et al., 1996; Glew et al., 2002; Citti et al., 2010). Antigenic variation can be the result of mutation in surface protein gene. During evolution the Mycoplasmas have lost mismatch repair pathways and many DNA repair proteins that increase the mutation rate (Carvalho et al., 2005). The strategy of mutation is to generate population diversity by high-frequency, random mutation so that at least one variant may survive in a particular context. Previous studies reported variable nucleotide sequence is underlying source of protein heterogeneity in *M. hominis* P120 gene (Nyvold et al., 1997). P120 has a typical prokaryotic lipoprotein signal peptide and does not have repetitive sequences but possesses variable regions, which undergo spontaneous mutations and cause antigenic variation of P120 (Nyvold et al., 1997). Major surface antigen P48 of *M. agalactiae* is belonging to the basic membrane protein family (Bmp). Several studies have been conducted on the antigenic and immunogenic properties of P48 of *M. agalactiae* and revealed that the stable and immunodominant antigen P48 could be a good alternative for detection and identification of *M. agalactiae* (Rosati et al., 2000; Chessa et al., 2009). Although P48 have shown immune response in a high percentage of cases but sensitivity of serological test based on recombinant P48 (r-P48) have reported varies between 60 and 100 percent of cases (Poumarat et al., 2012). The reason of sensitivity variation in the P48-based serum assay may lie in antigenic variation. Although some research has been carried out on expression of recombinant protein P48 (Rosati et al., 2000; Chessa et al., 2009), but no study has investigated the intra species variation of *M. agalactiae* P48 gene. In this paper we report identification of P48 gene and investigate sequence variation, bioinformatics and immunoinformatics characterization in *M. agalactiae* P48 protein as important aspects of developing diagnostic tests or protective recombinant vaccine.

MATERIAL AND METHODS

Samples collection and M. agalactiae isolation. A total of 125 field sampleswere freshly collected or previously cultured and stored at -70 °C, in addition three vaccine strains were investigated. All field samples were taken from sheep and goat with typical clinical sign of CA during 2011-2015. The samples were collected from various lesions (eye swab, joint fluid or milk secretion) and transported on ice to the Mycoplasma Reference Laboratory Razi vaccine and serum research institute (RVSRI), Karaj, Iran. The specimens were filtered into fresh PPLO broth prepared as previously describe with some modification (Khan et al., 2005). The broth supplemented with, glucose (5g/L), cysteine (0.14 g/L), thallium acetate (250 mg/L), 12.5 mLof 0.2% phenol red, 1% nicotinamide adenine dinucleotide 14mL/L, Heat inactivated horse serum 100mL/L, and 500 U/mLpenicillin. Inoculated broth were incubated at 37 °C and checked daily up to 21 day. The broths were examined daily for signs of growth (indicated by a fine cloudiness or opalescence) or changes in pH indicated by a color change. If there is no or little growth was seen after 21 days, then another subculture weredone by a 10% (v/v) inoculums of broth into fresh broth. Samples with sign of no growth were discarded after forth subculture. Subculture were made from positive broth culture onto PPLO agar media and plate were examined by 40X magnification for typical fried egg colonies after 48h incubation at 37 °C. Mycoplasmas in late logarithmic phase of growth were

Table 1. Primer sets for polymerase chain reaction

Target	Primmer Name	Sequence	Length (bp)	Reference
16srRNA	M1F	5'-GCT GCG GTG AAT ACG TTCT-3'	163	Kojima et al., 1997
	M3R	5'-TCC CCA CGT TCT CGT AGGG-3'		
P80	FS1	5'-AAG GTG CTT GAG AAA TGGC-3'	375	Tola et al., 1997a
	FS2	5'-TTG GCA GAA GAA AGT CCA ATCA-3'		
P48	P48F	5'-GCATCTTGTGGTGACAAGTAC-3'	1264	-
	P48R	5'-CTTGTTTCAGAAGCCAATCAG -3'		

used for experiments or stored in PPLO broth with 10% glycerol at -70 °C.

Table 2. Isolates source and characteristics

No.	Accession NO.	Isolation date	Isolation Area	Host	Source/lesion
1	MF280274	1966	Iran/Taleghan(Vaccine Strain)	Sheep	Milk
2	MF280275	1966	Iran/Fars (Vaccine Strain)	Sheep	Milk
3	MF280276	1966	Iran/Lorestan(Vaccine Strain)	Goat	Milk
4	MF280287	2011	Iran/Kurdistan1	Sheep	Milk
5	MF280289	2011	Iran/Kurdistan2	Sheep	Milk
6	MF280286	2011	Iran/Kurdistan3	Goat	Milk
7	MF280288	2011	Iran/Kurdistan4	Goat	Milk
8	MF280285	2011	Iran/Ilam	Sheep	Milk
9	MF280281	2011	Iran/Khuzestan	Sheep	Eye
10	MF280277	2011	Iran/Semnan	Goat	Milk
11	MF280282	2012	Iran/Golestan1	Goat	Eye
12	MF280283	2012	Iran/Golestan2	Goat	Milk
13	MF280284	2012	Iran/Golestan3	Goat	Milk
14	MF280280	2012	Iran/Kerman	Goat	Milk
15	MF280278	2015	Iran/East Azerbaijan1	Sheep	Milk
16	MF280279	2015	Iran/East Azerbaijan2	Sheep	Milk
17	CU179680	1952	Spain	Goat	not available
18	FP671138	1991	Spain	Sheep	Joint fluid
19	AJ132423	1999	Italy /Sardinia	not available	not available
20	EU000536	2007	India	not available	not available
21	EU000537	2007	India	not available	not available
22	EU000538	2007	India	not available	not available
23	EU000539	2007	India	not available	not available
24	JX289837	2011	India	not available	not available

DNA Extraction and PCR amplification. DNA extraction have performed on PPLO culture positive sample by phenol/chloroform method (Abtin et al., 2013). The genus and species of the samples were detected and confirmed by primers M (Kojima et al., 1997) and FS (Tola et al., 1997a) in separate reaction. All primer set used in this study are listed on Table 1. Confirmed *M. agalactiae* samples (Table 2) were subjected to P48 gene PCR amplification. Primer selection was performed on the P48 gene (GenBank nucleotide sequence accession number CU179680) by using Oligo7 software. Each PCR reaction contained the following dNTPs 200µM, 10 X PCR buffers 5µl, Enzyme Taqpolymerase 1 unit, $MgCl_2$ 1.5 mM, forward primer 10 pmol, reverse primer 10 pmol, DNA 100 ng and double distilled water to 50 µl total volume. The reaction was carried out in a thermal cycler (Mastercycler gradient, Eppendorf) with the following program: Initial denaturation for 3 min at 93 °C; 35 cycles of denaturation at 93 °C for 1 min, annealing at 54 °C for 30 s, and extension at 72 °C for 80 s; and final extension at 72 °C for 5 min. Amplicons were visualized by UV transilluminator after electrophoresis on cyber green (1%) gel agarosein 1×Tris–acetic acid–EDTA (TAE) buffer.

Table 3. Residues substitution in *M. agalactiae* P48 gene

Substitution	Frequency
Asparagine-Aspartic acid	0.5%
Aspartic acid-Glutamic acid	0.35%
Proline-Serine	0.26%
Alanine-Serine	0. 17%
Alanine-Asparagin	0. 17%
Glutamic acid – Alanine	0.08%
Threonine- Alanine	0.08%

DNA sequencing andNucleotide sequence alignment. PCR products were sequenced in both directions by Sanger method (Macrogen, Seoul, Korea). Nucleotide sequences were analyzed by DNASTAR package tools (Madison, WI, USA) using the Clustalw algorithm. Nucleotide sequences of P48 gene were retrieved from GenBank (http://ncbi.nlm.nih.gov/). The sequencing results of samples were compared to previously published P48 nucleotide. The phylogenetic tree was conducted by MegAlign software. Protein stability index were calculated by online tool (http://web.expasy.org/protparam/).

Epitopes prediction. The obtained nucleotide sequences of P48 insilico translated by ExPASy translate tool (http://web.expasy.org/ translate). B-Cell and T-Cell epitopes were predicted using the bioinformatics

software listed in Table 4. The sequences of predicted epitope were subjected to antigenicity prediction using an online antigen prediction server, VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/ VaxiJen/VaxiJen. html).

RESULTS

In this study, sixteen *M. agalactiae* were recovered, including three local vaccine strains (Taleghan, Lorestan and Fars) and thirteen field isolates from six provinces of Iran (Kerman, East Azarbaijan, Khuzestan, Semnan, Kurdestan, Golestan and Ilam). Isolate sources and characteristics are listed in table 2. The P48 gene was presented in all isolated and vaccine strains (Figure 1).

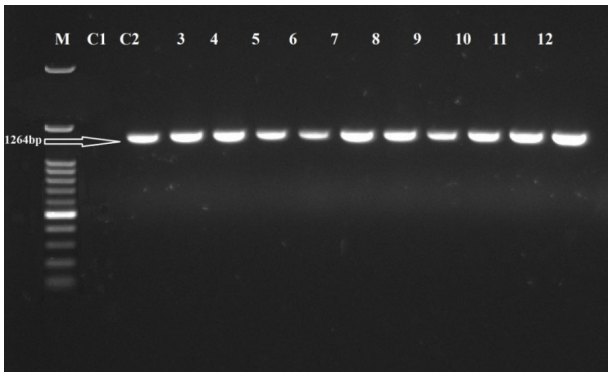

Figure 1. P48-PCR: PCR electrophoresis analysis in %1 gel agarose. M: Marker (100bp DNA ladder). Lane C1: Negative control (uncultured PPLO broth), Lane C2: Positive control (1264bp band, P48 gene of *Mycoplasma agalactiae*). Lane 3 to 12 *Mycoplasma agalactiae* isolates.

The nucleotide sequences obtained from field isolates and vaccine strains of Iran *M. agalactiae* P48 gene showed 94.9% to 99% similarity to the published sequences of other countries. Interestingly, a novel specific nucleotide substitution cytosine to thymine silent mutation at nucleotide position 420 was identified in all Iran isolates. This substitution was identified only in Iranian sample (vaccine strains and all field isolates). The substitution was conserved in all field isolates of Iran demonstrating that the gene product is conserved in Iran epidemic strains (Figure 2). There was a nucleotide substitution at position 1217 in Kurdistan 1 and Kurdistan 2 isolate, results in serine to phenylalanine residue change (Figure 3). This single nucleotide polymorphism (SNP) was the only variation has identified in P48 gene of Kurdistan province sheep *M. agalactiae* isolates, although variation was not observed in goat isolate from the same province. Two above mentioned change were specifically seen in Iran`s isolate, making the difference between isolates of Iran and other parts of the world. Many nucleotide substitutions in P48 sequences were at synonymous sites and result in no change of the aminoacid in 71.6 present of SNPs. The substitutions cytosine-thymine was relatively high as 0.94% and substitutions Guanine-Adenine (0.609%) and Cytosine-Adenine (0.15%) were also observed within P48 nucleotide sequences. Adenine-Thymine, Thymine-cytosine and guanine-cytosine mutation were 0.13%, 0.12% and 0.03%, respectively. Overall residue changes in P48 were calculated 1.65%. The residue substitution have shown in Table 3. Phylogenetic analyses of P48 have shown that *M. agalactiae* isolates fall into three main phylogenetic groups (Figure 4). The P48 nucleotide sequences of Iran were located in group B (Figure 4). The analysis of multiple alignment and phylogenetic tree shows the Iranian isolate and some isolate from different geographical regions of world including gene accession number CU179680.1 (or PG2 isolated from Spain), AJ132423.1 (isolated from Italy) and EU000539.1 (isolated from India) fall in a group with 99% homology. Isolates Sequence divergence ranged 0.1 to 5.3 percent and divergence within phylogenetic group was 4.1 to 5.3 percent. The residue divergences in all available isolates were up to 4.9 percent. Aspartic acids to Glutamic acid substitution at two residues in group B isolate were resulted in higher stability of the gene product. Stability index of P48 in group B phylogenetic were calculated 21.57 that shown improved 0.7 and 2.4 unit in compare to group A and C, respectively. Overall, result of sequences analysis have shown high variation exist in P48 DNA sequences. Iran isolates encoded amino acid sequenceof P48 differed from EU000536, EU000537,

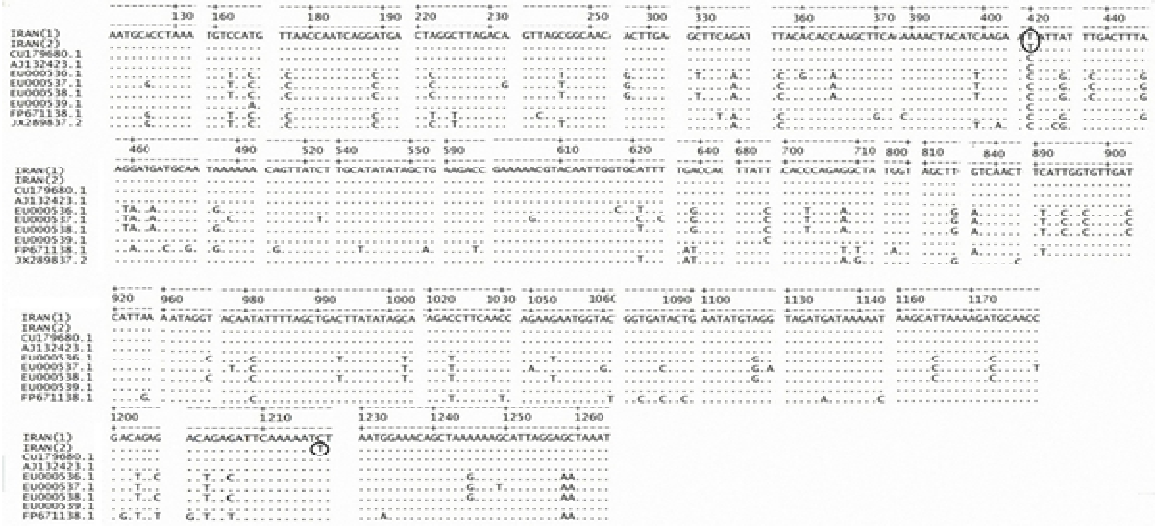

Figure 2. *Mycoplasma agalactiae* P48 nucleotide polymorphism, Iran (2): Kurdistan 1 and 2, Iran (1): other isolates (from Kerman, East Azarbaijan 1 and 2, Khuzestan, Semnan, Kurdestan 3 and 4, Golestan 1, 2, and 4, and Ilam) and vaccine strains (from Fars, Taleghan, and Lorstan) (Alignment was performed using ClustalW method; circles shows specific mutation of the Iranian isolate.)

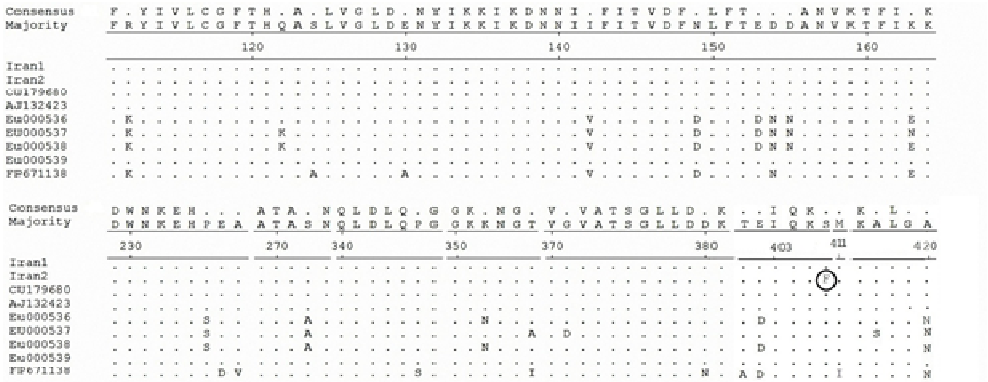

Figure 3. *Mycoplasma agalactiae* P48 protein alignment by ClustalW; Iran (2): Kurdistan 1 and Kurdistan 2, Iran (1): other isolates (from Kerman, East Azarbaijan 1 and 2, Khuzestan, Semnan, Kurdestan 3 and 4, Golestan 1, 2, and 4, and Ilam) and vaccine strains (from Fars, Taleghan and Lorstan) of Iran (Residue changes of Kurdistan 1 and Kurdistan 2 isolates were highlighted with a circle.)

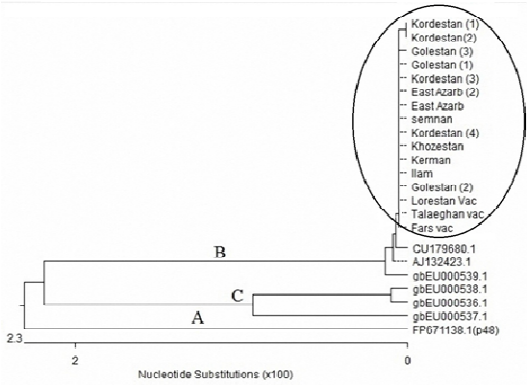

Figure 4. Phylogenetic tree of P48 gene of *Mycoplasma agalactiae* isolates (The P48 gene of the Iranian isolate was sequenced and aligned along with GenBank sequence, and phylogenetic tree was constructed by ClustalW method. The P48 nucleotide sequences of the Iranian isolates (black circle) were located in group B.)

Table 4. T-Cell and B-cell epitope prediction of P48

Server	Link	**Method**	Start position	Peptide	Percentile rank	VaxiJen score
IEDB (MHCI)	http://tools.iedb.org/mhci	Netmhcpan (BoLA-N:00101)	131	SGFRYIVL	0.2	0.9329
			276	SGTPQATTAI	0.4	*
			230	FGGIPWPAVSDFI	0.5	0.5515*
			294	SYPVAGSL	0.5	-0.0502
			139	CGFTHQASL	0.7	0.2136
						0.6262*
IEDB (MHCII)	http://tools.iedb.org/mhcii	NetMHCIIpan (HLA-DRB1*01:18)	326	FTSVMKLIGQAVYNI	1.7	-
			137	VLCGFTHQASLVGLD	4.48	0.23997
			285	INSVVKATASYPVAG	3.17	0.5482*
			201	KQAAYIAGRALADYF	3.64	0.6009*
			41	ISTLAHITSRKGLKL	8.34	0.0809
						0.7541*
SVMTriP	http://sysbio.unl.edu/SVMTriP/	Linear epitopes	183	FIKKIGEGHLVPVIFDTKQA	1.000	0.1429
			297	VAGSLSTDTAKEIKKLADKD	0.850	0.4545*
			338	SVMKLIGQAVYNILADLYSK	0.840	0.0318
Bcepred	http://www.imtech.res.in/raghava/bcepred/bcepred_submission.html	Continuous epitopes	99	DKAQVSGNKN	>1.90	1.3424*
			150	LDENYIKKIKDNNI	>1.90	0.4594
			214	FSQVYKDQPEKRTIG	>1.90	*
			249	IDWNKEHPEAKTKSLNET	>1.90	0.6192
			352	DLYSKGENQLDLQP	>1.90	*
						0.9577*
						1.0473

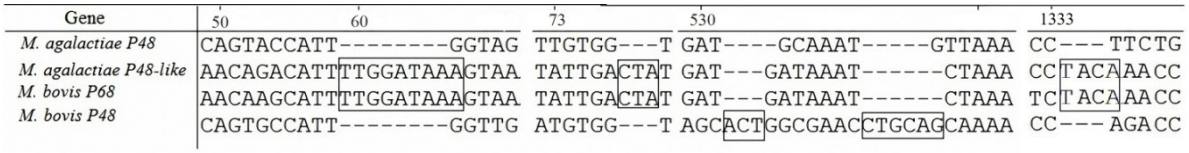

Figure 5. Insertion\deletion mutation in *Mycoplasma agalactiae* P48 and P48-like, as well as M. bovis P48 and P68 genes (The corresponding insertion\deletion sequences are boxed. The *M. agalactiae* P48 gene length is 1398 bp, containing 66 bp of leader peptide sequence, with four tryptophan TGA codons that are the characteristics of Mycoplasma species. However, in standard codon table, TGA refers to the stop codons. P48 encodes a 423-amino acid peptide with a calculated molecular mass of 48.77 kDa and the predicted isoelectric point of 8.64 [http://web.expasy.org/compute_pi/].)

EU000538 and FP671138 at 12, 14, 13 and 16 residues, respectively. SNPs were indentified at 89 positions and variable amino acids were observed at 25 residues. SNPs of *M. agalactiae* P48 gene are shown in figures 2, 3 and proteins alignment are shown in figure 3. P48 protein residue of Iran *M. agalactiae* isolate were showed 94 to 100% homology to *M. agalactiae* P48 protein of other countries isolate. The homology of P48 gene of *M. agalactiae* with *M. bovis* P48 were calculated 74% to 80% and similarity to P48-like gene of *M. agalactiae* and P68 gene of *M. bovis* were also 74% to 80%. The blast results have shown significant homology to Sugar ABC transporter of mycoplasma. Alignments of P48 and homologous gene (P48-like, P48 *M. bovis* and P68 *M. bovis*) sequence by Clustal W method have revealed frame shift Insertion\Deletion (Indel) mutationin five positions (Figure 5). The *M. agalactiae* P48 gene length is 1398 bp, contain 66bp of leader peptide sequence, with four tryptophan TGA codons that are characteristic of mycoplasma species but in standard codon table TGA refer to the stop codons. P48 encoding a 423 amino acid peptide with a calculated molecular mass of 48.77 kDa and the predicted 8.64 isoelectric point (http://web.expasy.org/

compute_pi/). The number of computationally predicted continues and liner epitope were 54 and 22, respectively. Best ranked B-cell and T-cell epitope and their variable residue and antigenicity have shown on table 4. Antigenicity score calculated by vaxiJen tool (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html).

DISCUSSION

In the present study antigenic heterogeneity and epitope variations of *M. agalactiae* P48 have been investigated by nucleotide sequencing and immunoinformatics tools. Mycoplasmas have Immunomodulating activity on the host immune system. This role attributed to their lipoproteins, which are the most abundant surface proteins (Razin et al., 1998). Since all mycoplasma lack a cell wall, the surface of their membrane acts as the primary interface in the interaction with the host and the environment (Nouvel et al., 2010). Several surface proteins involved in evasion of the host immune response or pathogenecity, such as Vpma (Glew et al., 2002), P40 (Oravcová et al., 2009) and P80 (Tola et al., 2001) have been described in *M. agalactiae* (Rosati et al., 1999; Oravcová et al., 2009). However, because of their intrinsic variable nature of Vpma, these proteins are unsuited to use as markers of infection or for development of related immunogenic products (Bergonier et al., 1996; Glew et al., 2002). Previous studies have shown that fewer than ten membrane proteins of *M. agalactiae*, with molecular masses between 18 and 80 KDa, are responsible for immune responses in sheep (Tola et al., 1997b). Some of these proteins are exposed on the cell surface (Tola et al., 1997b) and could be utilized to generate a vaccine against *M. agalactiae* (Chessa et al., 2009; Nicholas et al., 2009). P48 major surface membrane lipoprotein of *M. agalcatie* were described as a stable, specific and serologically recognized antigen. Recombinant P48 have been used in ELISA test (Rosati et al., 2000; Poumarat et al., 2012). Genes are existed in some species of Mycoplasma. The P48 gene of *M. bovis* (Bovine pathogen) and malp gene of *M. fermentans* (Human pathogen) are ortholog of this gene. P48-like gene of *M. agalactiae* and P68 of *M. bovis* are hemolog genes of P48 of *M. agalactiae*. In the present study, five frame shift indel mutation were observed in P48 and their hemologs, such frame shift indelsmutations, act as important drivers of protein divergence and contribute to ongoing genome reduction (Williams and Wernegreen, 2013). Frame shift inducing indels indicating selective pressure to maintain the reading frame (Williams and Wernegreen, 2013). BLASTs of P48 have shown similarity with ATP-binding sugar transporter. ABC transporters represent at superfamilies of active membrane transport proteins with a highly conserved ATPase domain that binds, hydrolyzes ATP and supplying energy for the uptake of nutrients and for the extrusion metabolic wastes. This system is necessary for growth and survival. The limited variation in protein sequence of P48 confirms the vital and conservative attribute of this gene. A large number of insertion sequences (IS) have been described in mollicutes which are responsible for genetic instability. Transposition of IS are contribute to the genetic variability of bacteria. No insertion sequence has been identified within P48 gene in present study. These finding confirm genetic stability of P48 gene. P48 gene nucleotide sequencing result of Iran isolate have been indicating that the epidemic isolates and vaccine strains has the same sequence and differs from the reference strain PG2 in one nucleotide. Two specific Iranian variations found at position 420 and 1217 could utilize as genetic marker. Aspartic acid to glutamic acid substitutions at two positions in phylogenetic group B could have resulted in increased protein stability, as mentioned in result section. The more stable protein has advantage to utilizing in diagnostics and vaccines products. According to the results of the present study fourteen isolates of sixteen sample collected from different regions of Iran are 100% identical and two sample have one nucleotide differences, demonstrated that a common ancestor is responsible for *M. agalactiae* infections in Iran. These results are also in agreement with previous study that have reported only

one strain of *M. agalactiae* is responsible for infections in Italy (Tola et al., 1997b), although our analysis sequence data of gene bank have demonstrated that variation can occurred in vast geographical country such as India. Indian isolated P48 nucleotide sequences fall in to two phylogenectic group (Figure 4). Investigation of aminoacid residue variations among P48 gene of *M. agalactiae* isolates has shown numerous T-Cell and B-Cell epitope residue alteration. The residue substitution in top ranked MHCI, MHCII, liner and continuous epitopes were 80%, 40%, 30% and 60%, respectively. In the present study the intraspecies P48 variation were studied for the first time. These variations may be underlying source of P48 protein heterogeneity and antigen variation. Although, many changes in a protein coding region can change the amino acid sequence of a protein without affecting its function, but sometimes the single synonymous codon substitutions can influence protein folding and emergence of new antigenic variants (Komar et al., 1999). These antigenic variants may driven by immune selection. Sequence variation might have an influence on antibody-antigen interaction and receptor binding avidity. Single amino acid substitutions in an Ab binding region can change the specific binding of a certain functional epitope that a single critical replacement fixed in, may have a strong effect on its antigenicity by affecting many epitopes within that site (Wang et al., 2002). Previous study have demonstrated that sequence differences at the epitope level between isolates can impact CD8+ T cell priming and the degree of cross-reactivity with other epitope variants (Ziegler et al., 2014). It has been demonstrated single point mutation is mechanism of escape for retroviruses variants (Leslie et al., 2004). Although variation in P48 is relatively low, this limited variation as a source of antigen heterogeneity are important for developing vaccine and diagnostic tests, hence protein antigenicity can be significantly reduced by alteration of single critical residues without destroying biological activity (Alexander et al., 1992). The results were in agreement of the previous study demonstrated that antigenic variability of *M. agalactiae* beside the surface switching mechanisms relies upon epitope differences, partially related to the isolates geographic origin (Bergonier et al., 1996). We have computationally demonstrated that naturally occurring polymorphisms of *M. agalactiae* P48 gene alter the antigenic properties of gene product. According to the result, P48 variation could contributes to immune evasion through antigenic variation. The inconsistency of immunological test could elucidate from gene differences. Hence, the antigenicity of the P48 might be modified by mutations. The results emphasize the need for monitoring in all geographical regions. Variable sequence raised the question whether the different sequence and epitopes of P48 are related to the *M. agalactiae* virulence. Any single residue substitution could contribute to the change of protein characteristics. The impacts of sequence differences in epitopes need further testing to be fully characterized. The overall evidence obtained in this study has shown that *M. agalactiae* P48 gene SNPs could be a source of antigen heterogeneity, but have not represented dynamic population in Iran. The results have demonstrated that P48 gene of *M. agalactiae* of Iran isolates are conservative and stable. Therefore the gene product is a good alternative for developing vaccine and serologic test based on recombinant protein.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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