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*Full Length Research Paper*

# Comparative study of homology of cytoplasmic membrane protein 40 KDa of *Mycoplasma agalactiae* in isolated strains in Iran

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***Mycoplasma agalactiae*** **is one of the chief factors causative contagious agalactiae in sheep and goats that involves huge geographical regions such as Iran and causes economic losses. In this disease many clinical signs may be observed for example: mastitis, agalactiae, Keratoconjuctivitis, arthritis, lameness and sometimes abortion and Pneumonia. Cytoplasmic membrane Protein 40 KDa (P40) in this bacterium is a strong immunogenic protein, major virulence factor and is specific for *M. agalactiae*. Isolated strains from Iran were considered molecularly and genetically for identification of gene concerning this protein. The P40 gene of isolated *M. agalactiae* strains in Iran was amplified with PCR technique and for further studies this fragment were cloned and sequenced. In comparative study of P40 gene sequence of isolated strains in Iran with other strains in gene bank with the use of BLAST program showed us the homology (Identities 100%) of Lorestan strain with French strains 4258, 4021 and Spanish strain $PG_2$ and as for Taleghan and Shiraz strains the most homology (Identities 99%) was achieved with French Strains 4258, 4021 and Spanish strains PG2, 6968, 5225 and Swiss strain 7375 and Italian strain 9. The Least homology (Identities 84%) for all three strains was reported with French strain 4212.**

**Key words***: Mycoplasma agalactiae*, protein 40 KDa (P40), PCR, cloning.

## INTRODUCTION

The bacterium *Mycoplasma agalactiae* belongs to Mycoplasmataceae family and is a facultative anaerobic germ and in complex media culture requires protein with serum factor and yeast extract for growing and shows egg fried colonies in media culture (Carter et al., 2004; Hasani tabatabayi et al., 2005).

This germ has no rigid cell wall and consequently is plastic and highly pleomorphic (Carter et al., 1991).

The organism is highly susceptible to heat, detergents, commonly used disinfectants, and antibiotics except cell-wall inhibitors (Gyles et al., 1986; Hasani tabatabayi et al., 2005).Contagious agalactia is an acute, subacute, or chronic disease of sheep and goats caused by *M. agalactiae* (other mycoplasmas, namely, *M. mycoides* subsp. Mycoides LC and *M. Capricolum*, are claimed to cause similar syndromes) (Carter et al., 2004). This disease involves the Mediterranean region and some parts of Europe, Asia and Africa (Bergonier et al., 1997; Zendulkova et al., 2004) and there is evidence that it exists in the United States (Carter et al., 1991) . This disease is endemic in Iran and characterized by agalactia, mastitis, arthritis, keratoconjunctivitis, and sometimes abortion and pneumoia (De La Fe et al., 2006).

Several studies in the past years have shown that pathogenic mycoplasmas are equipped with sophisticated genetic systems, which allow these agents to spontaneously change their surface antigenic make-up (Jechlinger et al., 2004).

It is implicated that these variable surface components provide the wall-less mycoplasmas with a means to avoid the host immune response and promote host colonization (Jechlinger et al., 2004).

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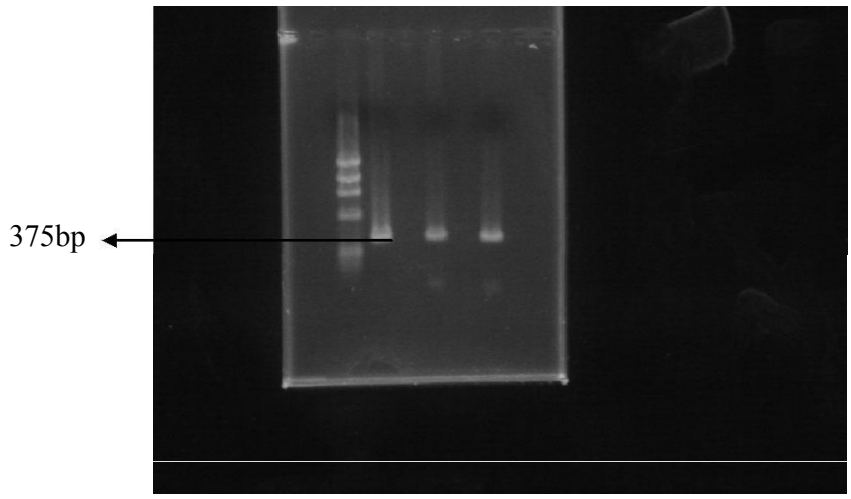


**Figure 1.** Specificity of the PCR detection assay using the primers FS1 and FS2. The formation of 375bp bands in Shiraz, Taleghan and Lorestan Strains.

In *M. agalactiae* a pathogenicity island-like Locus has recently been identified that contains six distinct but related genes which encode the major immunodominant membrane proteins, the so-called Vpmas (Bergonier et al., 1997; Chopra-Dewasthaly et al., 2008; Jechlinger et al., 2004).

It was shown that these surface associated proteins vary in expression at an unusual high frequency due to site-specific DNA rearrangements (Bergonier et al., 1997; Glew et al., 2000; Jechlinger et al., 2004). These protein Variations are presumed to contribute to pathogenesis by permitting the mycoplasma to survive in different niches (tissue tropism) and/or escape from the host defence mechanisms immune evasion (De La Fe et al., 2006). *M.agalactiae* has a family of related genes (avg genes) which encode surface lipoprotein antigens that undergo phase variation and major rearrangement events within the avg genomic Locus (Flitman-Tene et al., 2003). P40 has been characterized recently, and has been shown to be involved in the adhesion of *M. agalactiae* to lamb synovial membrane cells and is one of the chief virulence factors (De La Fe et al., 2006).

P40 is a membrane protein and is expressed by all *M. agalactiae* strains except for serotype C strains, which showed nonsense mutations in their P40 genes (Fleury et al., 2002). Fab fragments of antibodies directed against recombinant purified P40 significantly inhibited adherence of *M. agalactiae* strains PG2 to Lamb joint Synovial cells LSM 192 (Fleury et al., 2002). The gene encoding P40 is specific for *M. agalactiae* species and has not been found in other mycoplasma species (Fleury et al., 2002).

## MATERIALS AND METHODS

DNA extraction was performed from three isolated strains of *M. agalactiae* in Iran content: Shiraz strain, Taleghan strain and Lorestan strain.

### A) DNA extraction

DNA extraction using Phenol/chloroform method according to protocol (Sambrook et al., 2001) with some changes was made. After extraction of DNA of three strains of *M. agalactiae* (Shiraz, Taleghan and Lorestan strains), the samples of each strain were read for determination of DNA concentration in ng/ l in Nano drop device and on the base of priority of reading indexes, ratio of 260/280 nm, 260/230 nm and optimal density (OD) were read for performing PCR, respectively (Shahhoseini, 2001).

Samples of each strain, after DNA extraction, were stored at -20°C. The Purpose of this stage was confirmation of presence of *M.agalactiae* in all received samples and confirming the lack of pollution with other organisms.

### B) PCR of samples for confirming of *M.agalactiae*

According to primers in articles (Tola et al., 1996; Tola et al., 1997) FS1, FS2 primers were selected. These primers were designed to amplify a 375 bp fragment of *M.agalactiae* chromosomal DNA.

In this reaction, Taq DNA polymerase enzyme was used and by optimizing its situation with suitable thermal program in thermocycler device, PCR was performed. After electrophoresis, the PCR product was run on Gel agarose 1.2% and all three strains showed sharp band in 375 bp, so the presence of *M. agalactiae* was confirmed (Figure 1).

According to specific primers of protein 40 KDa (P40) gene entitled 13 aF, 13 aR (Fleury et al., 2002), PCR was performed.

These primers show 920 bp fragments that indicate the presence of protein 40 KDa gene. Enzyme Taq DNA Polymerase was used in this reaction. After performing many successive PCR on extracted genomic DNA from Shiraz, Taleghan and Lorestan strains, and optimization of PCR for the best amounts of reactive and regulation of thermal cycles program, PCR was performed and desired **C)**

### PCR of *M. agalactiae* strains for confirming of protein 40 kDa (P40) gene

According to specific primers of protein 40 KDa (P40) gene entitled

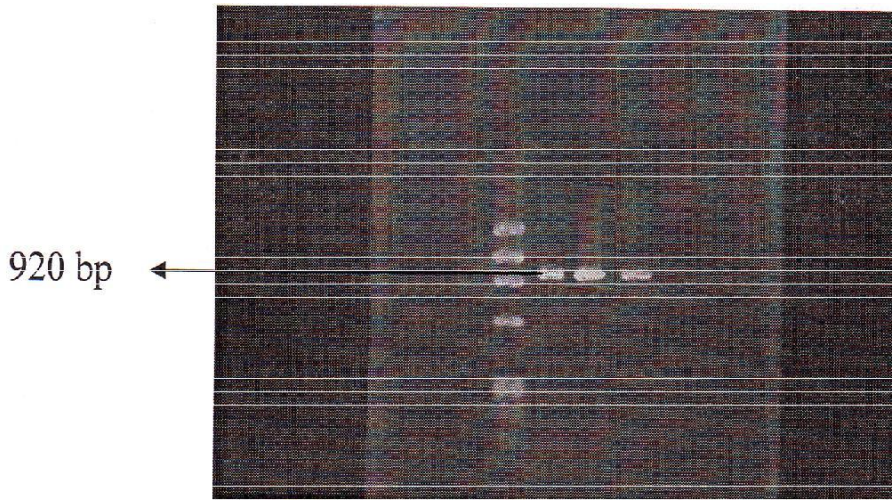


**Figure 2.** Presence of P40 in Shiraz, Taleghan and Lorestan strains using the primers 13aNot1 R and 13a Ecor1L.

13 aF, 13 aR (Fleury et al., 2002), PCR was performed. These primers show 920 bp fragments that indicate the presence of protein 40 KDa gene. Enzyme Taq DNA Polymerase was used in this reaction. After performing many successive PCR on extracted genomic DNA from Shiraz, Taleghan and Lorestan strains, and optimization of PCR for the best amounts of reactive and regulation of thermal cycles program, PCR was performed and desired sensitivity and specificity was received and optimal method was selected as final method and after performing PCR and electrophoresis of PCR product, all of the there strains showed band in 920 bp and the presence of protein 40 KDa (P40) gene was confirmed (Figure 2).

**D) Cloning**

In this stage, PCR products (P40 gene) were cloned by Vector PTZ57R/T into competent cells, *E. coli* DH5 strain. For determining sequence and more Survey and according to protocol of company kit fermentas$^{TM}$ with some changes, cloning was per-formed.

Cloning contains below stages:

**1) Ligation:** The aim of this stage was joining target DNA fragment (P40 gene) to plasmid Vector (Vector PTZ 57R/T).

In this stage, suitable ratio between amount of DNA and Vector should be used. The optimal concentration contains:

1.) DDW (Double distilled water) 6 l
2.) 5X Buffer 3l
3.) Vector 1.5 l
4.) PCR product 3 l
5.) Ligase 1.5l

Ligation product was stored in -20°C till the time of consumption.

**2) Preparation of competent cells:** The aim of this stage is preparation of competent bacteria of *E. coli* DH5α strain for entegrating plasmid vector.
At first a colony of *E. coli* DH5α strain was cultured in 2 ml of solution Transform Aid$^{TM}$ C-medium and then stored in incubator with shaking at 37°C for overnight.

In two capped tubes, each of them was poured 1500 l from solution Transform Aid$^{TM}$ C-medium and was stored in incubator at 37°C for heating.

Then 150 l from night culture of *E. coli* DH5α strain added to each of these tubes (Contains Transform Aid$^{TM}$ c-medium that had been heated) and was stored in incubator with shaking at 37°C for 20 - 30 min.

**3) Transformation:** The aim of this stage is entering the Vector plasmid contain target DNA fragment (P40 gene) into competent cells of bacteria.
This stage consists of:

1.) 1.5 ml of competent cells of *E. coli* DH5α strain was poured into microtube and was centrifuged in high speed for a minute.
2.) The supernatant was poured off and the cellular deposit dissolved in 300 l of TTS solution (even volume of T-solution A and T-solution B) and was put into the ice for 5 min.
3.) Centrifuge in high speed for a minute.
4.) The Supernatant was poured off and the cellular deposit dissolved in 120 l of TTS solution and was incubated into the ice for 5 min.
5.) 2.5 l of ligation product was poured into a microtube and was put into the ice for 2 min.
6.) 50 l of suspension cells was added to this microtube (ligation product) and was put into the ice for 5 min.
7.) In Sterile conditions, this cellular suspension was cultured on the LB-agar medium which contain Ampicillin (already was heated in incubator at 37°C).
8.) Finally it was put in incubator at 37°C for 24 h.

**E) Making matrix plate for culturing and screening**

The aim of this stage is isolation and selection of bacteria which contains recombinant plasmid Vector. After growing of bacteria on LB-agar medium which contains Ampicillin, in this stage, matrix plate was made from these colonies. For this purpose, on the LB-agar medium contains Ampicillin, after dividing the plates into cells and numbering the cells in sterile conditions, Separate colonies were made (from being grown colonies in previous stage) and then were put in incubator at 37°C for 24 h (Figure 3) .

**Figure 3.** Matrix plate was made of Shiraz, Taleghan and Lorestan strains, respectively.

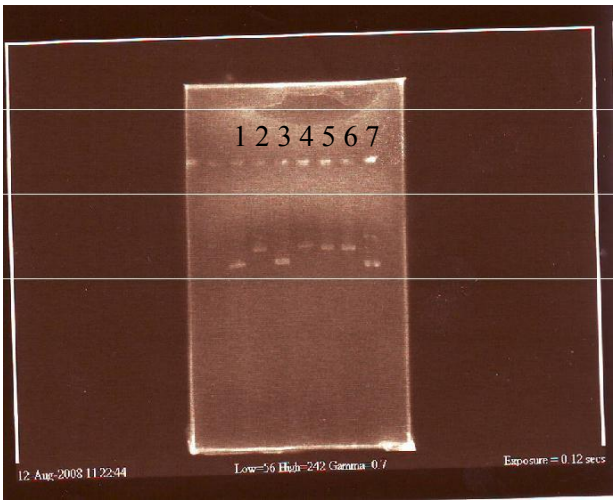


**Figure 4.** Negative control plasmid (Lane 7), inserted samples of P40 gene into PTZ57R Vector (Lanes: 2, 4, 5, 6) and not being inserted samples (Lanes 1, 3).

**F) Assessment of recombinant single colonies by PCR (confirmation of P40 gene cloning)**

The aim of this stage is to confirm the insertion of plasmid vector which contains target DNA fragment (P40 gene) into competent bacteria. For this purpose, PCR was performed on being grown recombinant single colonies in matrix plate (which resulted from last stage).

Material and methods for PCR in this stage for example, Primers and polymerase enzyme are the same with being performed PCR in stage C and matrix plate was used as DNA template. After electrophoresis of PCR product in gel agarose, 2% from those one getting sharp bands in 920 bp in gel agarose can be selected and their matrix were used for extraction of plasmid.

**G) Extraction of plasmid**

Extraction of plasmid was performed by large scale method (Sambrook et al., 2001) with a little change. The numbers that showed sharp bands in gel agarose, their matrix plate were selected and cultured in LB broth medium. At first in sterile conditions in tubes $5^{CC}$ of LB broth medium with 5 l of Ampicillin solution (50 mg/ml) were added and stored in incubator at 37°C and shaken overnight.

After a night, the content of tubes was darker which indicated the growing of bacteria. After that, tubes content were transferred to an erlen meyer with $500^{CC}$ volume and $100^{CC}$ of LB broth medium with 100 l Ampicillin solution were into it already. This complex was incubated at 37°C with shaker about 4 - 5 h and then 500 l of chloramphenicol solution was added (to prevent the growing of bacteria and therefore plasmids amplified into bacteria) and this complex should be incubated at 37°C with shaker for overnight.

The content of erlen meyer was transferred to two falcon tubes with volume of $50^{CC}$ and the rest stages with the following order was done:

1.) Falcon tubes were centrifuged in 4000 rpm at 4°C for 15 min.
2.) The deposit with $20^{CC}$ of STE solution dissolved well.
3.) This complex was centrifuged in 4000 rpm at 4°C for 15 min.
4.) The supernatant was removed and the deposit with $3^{cc}$ of (solution I) were mixed well and then $4^{cc}$ of (solution II) was added to them and was moved upside down slowly. Then they were incubated into ice for 10 min.
5.) $3^{CC}$ of (solution III) was added and was incubated in laboratory temperature for 10 min.
6.) Centrifuge in 4000 rpm at 4°C for 15 min.
7.) The supernatant was filtered in the other tube and equal volume of it, Isopropanol was added and was put in laboratory temperature for 10 min.
8.) Centrifuge in 5000 rpm at 4°C for 15 min.
9.) The supernatant was removed and 1cc of ethanol 70° was added to its deposit and dissolved them and they were transferred to microtube.
10.) Centrifuge in 5000 rpm at 4°C for 10 min.
11.) The supernatant was removed and the deposit was dried at 60°C.
12.) The deposit was dissolved in 100 l of double distilled water (DDW).

**H) Electrophoresis of extracted plasmid for confirmation of cloning**

The extracted plasmid (containing P40 gene) was electrophoresis in gel agarose and control plasmid vector PTZ57R and without plasmid vector PTZ57R was used instead of marker. The samples that were inserted and the site of their bands were the same with control plasmid which were used for sequencing (Figure 4).

## RESULTS

Two cloned samples from each strain by vector PTZ57R/T into competent cells (*E. coli*, DH5 strain) that had shown sharp bands in gel agarose were transferred to MWG Company from Germany for sequencing of P40 gene. In comparative study of nucleotide sequence of P40 gene in isolated strains of *M. agalactiae* in Iran according to Meg Align program, the most homology were achieved between Shiraz strain with Losresten strain (99.7%) and the least homology were achieved be-

tween Shiraz strain and Taleghan strain (74.6%).

In comparative study of nucleotide sequence of P40 gene of Shiraz, Taleghan and Lorestan strains with sequence of this gene in other strains presenting in gene bank with the use of BLAST program showed us the most homology (Identities 100%) of Lorestan strain with French strains 4258, 4021 and Spanish strain $PG_2$ and the least homology (Identities 84%) was achieved with French strain 4212.

The most homology (Identities 99% ) of Taleghan strain was achieved with French strains 4258 , 4021 and Spanish strains $PG_2$ , 6968 , 5225 and Swiss strain 7375 and Italian strain 9 and the least homology (Identities 84%) was achieved with French strain 4212 .

The most homology (Identities 99% ) of Shiraz strain was reported with French strains 4258 , 4021 and Spanish strains PG2, 6968 , 5225 and Swiss strain 7375 and Italian strain 9 and the least homology (Identities 84% ) was shown with French strain 4212 . According to BLAST program , the most homology of three strains in Iran have been shown with French strains 4258 , 4021 and Spanish strain PG2 and the least homology (Identities 84%) was reported with French strain 4212.

## DISCUSSION

For fast and specific detection and making sure of being *M. agalactiae* in isolated strains in Iran according to reference (Tola et al., 1997), related primers were used that were conformity with S. Tola et al. results but with regard to some reactive measures and regulation of PCR cycles program was a little bit different.

This difference was distinct especially in annealing temperature which is an important factor in improvement of PCR that according to melting temperature of primers (Tm) the best result was achieved at 52°C.

In the part related to confirmation of P40 gene in isolated strains in Iran, (according to Fleury et al., 2002) related primers were used and the presence of this gene was confirmed. The results that have been showed in recent study are conformity with B. Fleury et al. results but with regard to some reactive measures and PCR cycles program are different that the best temperature for annealing (after successive tests) was achieved at 61.5°C.

After cloning of target gene for confirming of cloning, the usual method is plasmid extraction and excision of it with enzyme that this extraction by large scale method requires 2 - 3 stages of successive bacteria culture (each stage for 24 h) and then labor of extraction of plasmid should be done. While in comparison with this method, by PCR on grown separate colonies (directly by using the method in part of F), survey of presence of recombinant plasmid vector (containing target gene) is possible simply and definitely.

Also by using this method after ligation, positive separate colonies were studied. The results that are achieved of sequencing of P40 in isolated strains in Iran are conformity with the results of Fleury et al. (2002) (presence and specificity of this gene in *M. agalactiae* strains that have been studied).

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### REFERENCES

Bergonier D, Berthelot X , Poumarat F(1997). Contagious agalactiae of small ruminants: current knowledge concerning epidemiology, diagnosis and control. Rev.Sci. Tech. off. Int .Epizoot. 16: 848-873.

Carter GR, Chengappa MM (1991). Essentials of Veterinary Bacteriology and Mycology. Lea and Febiger; Fourth edition, pp:237-243.

Carter GR, wise Darla J (2004). Essentials of Veterinary Bacteriology and Mycology. Sixth edition. Iowa state Press. pp. 171-175.

Chopra-Dewasthaly R,citti C, Glew MD, Zimmermann M, Rosengarten R, Jechlinger w (2008). phase-Locked mutants of *Mycoplasma agalactiae*: defining the Molecular switch of high-frequency VPma antigenic Variation. Mol Microbiol. 67(6): 1196-210.

De La Fe C, Assuncao P, Rosales RS, Antunes T, Poveda JB(2006). Characterisation of Protein and antigen Variability among *Mycoplasma Mycoides* subsp. Mycoides (LC) and *Mycoplasma agalactiae* field strains by SDS-PAGE and immunoblotting. The vet. J. 171. pp. 532-538.

Fleury B, Bergonier D, Berthelot X, Peterhans E, Frey J, Vilei EM (2002). characterization of P40, a cytoadhesin of *Mycoplasma agalactiae*. Infect. Immun. 70: 5612-5621.

Flitman-Tene R, Mudahi-orenstein S, Levisohn S, Yogev D(2003). Variable Lipoprotein genes of *Mycoplasma agalactiae* are activated in vivo by promoter addition via site-specific DNA Inversions. Infect.Immun. Vol 71, No.7, pp:3821-3830.

Glew MD, Papazisi L, Poumarat F, Bergonier D, Rosngarten R, Citti C (2000). Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant Variable surface proteins in *Mycoplasma agalactiae*. Infect. Immun. 68(8): 4539-4548.

Gyles CL, Thoen CO (1986). Pathogenesis of Bacterial infections in animals. Iowa state university Press/Ames., First edition, pp. 205-215.

Hasani tabatabayi A, Firouzi R (2005). Disease of animals due to bacteria. Tehran university Press. pp. 469-480.

Jechlinger w, chopra- Dewasthaly R, Glew M, citti C, Rosengarten R (2004). Mulecular basis of *Mycoplasma agalactiae* Pathogenicity. Berl Munch Tierarztl Wochenschr. 117(11-12): 472-479.

Sambrook J, Russell D (2001). Molecular cloning: A Laboratory Manual. Vols 1-3., Third edition, ISBN 978-087969576-7.

Shah hoseini MH(2001). polymerase chain reaction. Pars Iran press. First edition.

Tola S, Angioi A, Rocchigiani AM, Idini G, Manunta D, Galleri G, Leori G (1997). Detection of *Mycoplasma agalactiae* in sheep milk samples by polymerase chain reaction. Vet. Microbiol. 54: 17-22.

Tola S, Idini G, Manunta D, Galleri G, Angioi A, Rocchigiani AM, Leori G (1996). Rapid and specific detection of *Mycoplasma agalactiae* by Polymerase chain reaction. Vet. Microbiol. 51: 77-84.

Zendulkova D, Madanat A, Lany P, Pospisil Z, Ball HJ (2004). Detection of *Mycoplasma agalactiae* antigen in sheep and goats by Monoclonal Antibody-Based Sandwich ELISA. ACTA VET. Brono 2004, 73: 461-464.