Iranian Journal of Microbiology

Volume 14 Number 6 (December 2022) 813-819

ORIGINAL ARTICLE

Cloning, expression and purification of antigenic fragments of the *Ureaplasma urealyticum UreD* protein and its value in serology

Faleh Darvishi[1], Ali Ganji[1,2], Behzad Khansarinejad[1], Shabnam Sadoogh Abbasian[1], Hamid Abtahi[1*]

[1]Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran
[2]Department of Microbiology and Immunology, School of Medicine, Arak University of Medical Sciences, Arak, Iran

Received: April 2022**, Accepted:** July 2022

ABSTRACT

Background and Objectives: The detection of *Ureaplasma urealyticum* is usually done through culture. With the change of the smallest effective factor in culture, we face the lack of growth of these bacteria, which is one of the important reasons to find a suitable alternative for the diagnosis of this bacterium. *UreD* is a protected gene in this bacterium. The aim of this was to evaluate the ability of antigenic regions of *UreD* protein to bind to patients' serum antibodies.
Materials and Methods: Antigenic regions of *UreD* protein were predicted using IEDB software with five different methods: Emini Surface Accessibility Prediction, Kolaskar and Tongaonkar Antigenicity, Chou and Fasman beta turn prediction, Karplus and Schulz flexibility scale, Ellipro –Epitope prediction based upon structural protrusion. Antigenic regions of *UreD* gene was clonned, expressed and purified. The antigenicity of this recombinant protein against the antibodies in the serum of people infected with *U. urealyticum* infections was checked in western blotting.
Results: The results showed that the antigenic regions of the *UreD* protein was producted and its antigenicity was demonstrated in western blotting. Moreover, all sera from patients infected with *U. urealyticum* reacted to the recombinant antigen.
Conclusion: Specimens from people infected with *U. urealyticum* infection was positive in Western blotting suggesting that *UreD* protein has antigenic properties. Therefore, it can be used as a suitable candidate for the design of diagnostic kits and *U. urealyticum* vaccine.

Keywords: *Ureaplasma urealyticum*; Recombinant proteins; Blotting; Western; Epitopes; Cloning; Molecular; Vaccines

INTRODUCTION

Mycoplasmas are the smallest single-celled organisms that have been isolated from plants, animals, and humans as commensals or pathogens. The most important opportunistic pathogen in the female reproductive system is *Mycoplasma urealyticum* (1). This anaerobic bacterium is optional and causes diseases such as complications before and after childbirth, pneumonia in infants, pelvic inflammation, respiratory syndrome, chorioamnionitis and epididymitis, prostatitis and acquired arthritis (2). Given the importance of diseases caused by these organisms and the serious consequences that follow, it is necessary to quickly identify and treat infected people. Therefore, the detection of these organisms is very important. Detection of these microorganisms is usually done through culture, but their isolation through

*Corresponding author: Hamid Abtahi, Ph.D, Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran.
Tel: +98-8614173526 Fax: +98-8614173526 Email: habtahi2001@yahoo.com

culture causes problems, including the long isolation time that does not allow rapid and easy detection of genital infections. Also, these organisms need specific culture media due to their difficult growth, and we can mention the high cost and instability of these media, the need for essential growth factors and the difficulty of preparing these supplements to add to the culture medium. It takes about 2 to 5 days to cultivate with *U. urealyticum*. The slightest change in culture is associated with stunted growth of these bacteria, which is one of the important reasons for finding a suitable alternative for diagnosis (3). Therefore, it is important to study and find new and repid methods. Urease is one of the most important virulence factors in *U. urealyticum* which breaks down urea into ammonia and carbon dioxide, both produce ATP and cause very serious damage to sperm. Infertility becomes irreparable. This enzyme is encoded by the *ureD* gene, an important gene family in *Mycoplasma* and *Ureaplasma* bacteria called the MBA. *UreD* is a conseved gene that makes it a suitable candidate for detection of different strains of this organism (4).

The aim of this study was to produce *UreD* protein and evaluate its ability to bind to antibodies in the serum of people with *Ureaplasma urealiticum* in order to provide basic information for further studies in this field.

MATERIALS AND METHODS

Materials and reagents. The included enzymes were: Polymerase enzymes, restriction enzymes, Taq polymerase, ligase, DNA polymerase, RNase, which were purchased from Thermo scientific, Fermentase, Cinacolone. The kit utilized for the product purification PCR was purchased from Roche (Germany) and the protein purification kit was purchased from Qiagen (USA).

Bacterial strains. *Escherichia coli* DH5α was used as the recombinant protein cloning host, *Escherichia coli* BL21 (DE3) pLYsS was employed as the recombinant protein expression host, and pET32a was applied as the recombinant expressive vector.

Antigenic regions. Antigenic regions of *UreD* protein were predicted using IEDB software with five different methods: Emini Surface Accessibility Prediction, Kolaskar and Tongaonkar Antigenicity, Chou and Fasman beta turn prediction, Karplus and Schulz flexibility scale, Ellipro –Epitope prediction based upon structural protrusion. Also, we used protean software to identify antigenic regions that show hydrophobic and antigenic regions and possibly surface pattern of the protein.

Construction of gene. These sequences were connected by flexible linkers (EAAAKEAAAK). Then, restriction site of 5' *BamHI* and 3' *XhoI* were considered at the beginning and end of the sequence and codon-optimized for *E. coli* expression. The whole construct was synthesized with Biomatik Company (Cambridge,ON, Canada). The synthesized DNA was inserted into the plasmid pBSK as the cloning vector. The length of the synthesized piece was 800 bp. The pBSK plasmid vector is resistant to ampicillin and 2917 bp in length.

Transformation of antigenic regions of *UreD* gene in *E. coli* DH5α. First, competent cells were implanted using calcium chloride method (5). Then, transformation was done according to heat shock protocol (6). Then competent cells were cultured in NA+ ampicillin (100 mg/ml) and were incubated at 37°C for 20 h.

Plasmid purification. One colony was cultured in NB (5 ml) at 37°C for 20h. Then, it was centrifuged 1.5 ml (4000 rpm, 5 min) and was dissolved pellet in 100µl SET buffer. Continue the steps were done according to mini-preparation protocol (6).

Gene amplification. Primers were designed according to result sequences. The forward primer (5'GATATCGGATCCATCATCACC 3') starts from the beginning of the gene and contain *BamHI* site. Reverse primer (5'GTGGTGCTCGAGGATCATAT 3') contain recognition site for *EcoRI*. PCR amplification was done according to protocol described by Abbasian et al. (6). The PCR product was analyzed with electrophoresis in 1% agarose gel in 1×TBE buffer. The PCR product was purified from agarose gel using high pure PCR product purification kit (Roche Diagnostic) according to the manufacturer guideline (7).

Cloning of *UreD* gene in bacterial expression vector. The PCR product and pET32a vector were used for cloning according to the protocol described

by Abbasian et al. (7). *E. coli* BL21 (DE3) pLYsS competent cells were prepared based on calcium chloride method and were used for transformation of plasmid (5).

Expression of *UreD* in *E. coli* BL21 (DE3) pLYsS. *E. coli* BL21 (DE3) pLYsS was transformed with pET32a-Ib-UreD plasmids according to the standard method (6) and protein was expressed according to the methods used by Abbasian (7). Protein was run in SDS-polyacrylamide gel electrophoresis [SDS-PAGE (12%)] and then gel was stained with komasiblue (7).

Purification of *UreD*. The expressed *UreD* was purified by Ni-NTA agarose resin. The quality and quantity of purified recombinant *UreD* protein was analyzed by SDS-polyacrylamide gel electrophoresis [SDS-PAGE (15%)] and Bradford methods, respectively (8).

Collection of serum samples from patients with *U. urealyticum*. To collect positive serum samples for *U. urealyticum*, we tested the sera of 50 high-risk individuals with ELISA kit according to the kit protocol.

Antigenicity of recombinant antigenic regions of *UreD* gene. For assay of antigenicity of recombinant antigenic regions of *UreD* gene, we used western blot analyses. The western blot analyses was done according to the protocol described by Abbasian. In western blot assay, normal human sera and patients sera were used as negative and positive control, respectively (9).

RESULTS

Determination of antigenic region. Results of Emini Surface Accessibility Prediction was presented that areas with the highest antigenic properties were identified based on the availability of epitopes at the protein level (Fig. 1). Results of Kolaskar and Tongaonkar Antigenicity was used to predict and evaluate epitopes based on antigenicity (Fig. 1). Results of Chou and Fasman beta turn prediction was presented that identifies betaterns that have antigenic properties. Results of Karplus and Schulz flexibility scale was presented that high flexibility epitopes were identified by it. Results of Ellipro–Epitope prediction based upon structural protrusion was presented that Predicts existing space epitopes. Also, the results of the Protean software are shown in Fig. 2.

Finally, tree antigenic regions that were agreed upon by all the mentioned software were selected and shown in Table 1.

Purification of the pBSK plasmid containing the synthesized gene. After transformation of the recombinant vector into *E. coli* DH5α, plasmid was extracted from the obtained colonies and the result of purification of this plasmid can be seen in the Fig. 3.

Gene amplification. The amplified fragment had the expected size of 800 bp compared to 100 bp DNA ladder (Fig. 3).

Cloning of *UreD* gene in bacterial expression vector. The recombinant plasmid (pET32a- *UreD*) was sequenced. The result was confirmed by being compared with databases and employing basic local alignment search tool (BLAST) software. To confirm the transformation of pET32a- UreD into *E. coli* BL21 (DE3) pLYsS, PCR reaction and enzymatic digestion were performed (*BamHI*, *HindIII*) (Figs. 4 and 5).

Expression and purification of *UreD*. Protein production in various tested induction conditions was analyzed with SDS-PAGE. The greatest yield was achieved by applying 1.5× NB and 1 mM IPTG, 4 h incubation of the obtained medium under 200 rpm, shaken at 37ºC, was the most convenient condition. The produced 22 kDa protein after 2 h and 4 h of the induction are indicated with corresponding arrows in Fig. 6.

SDS-PAGE was used to analyze the quality of purified proteins. Because of 6His.tag sequence in the amino acid end of the recombinant *UreD* was purify

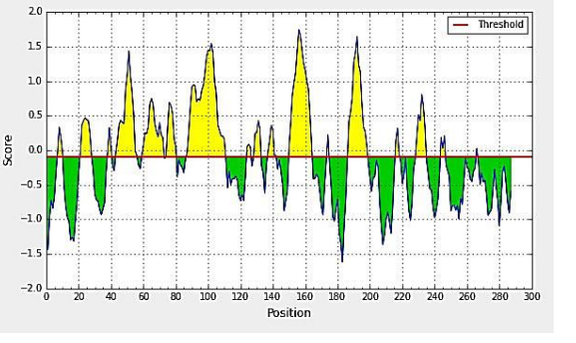

Fig. 1. Areas with the highest antigenicity of *UreD* protein based on surface epitope availability.

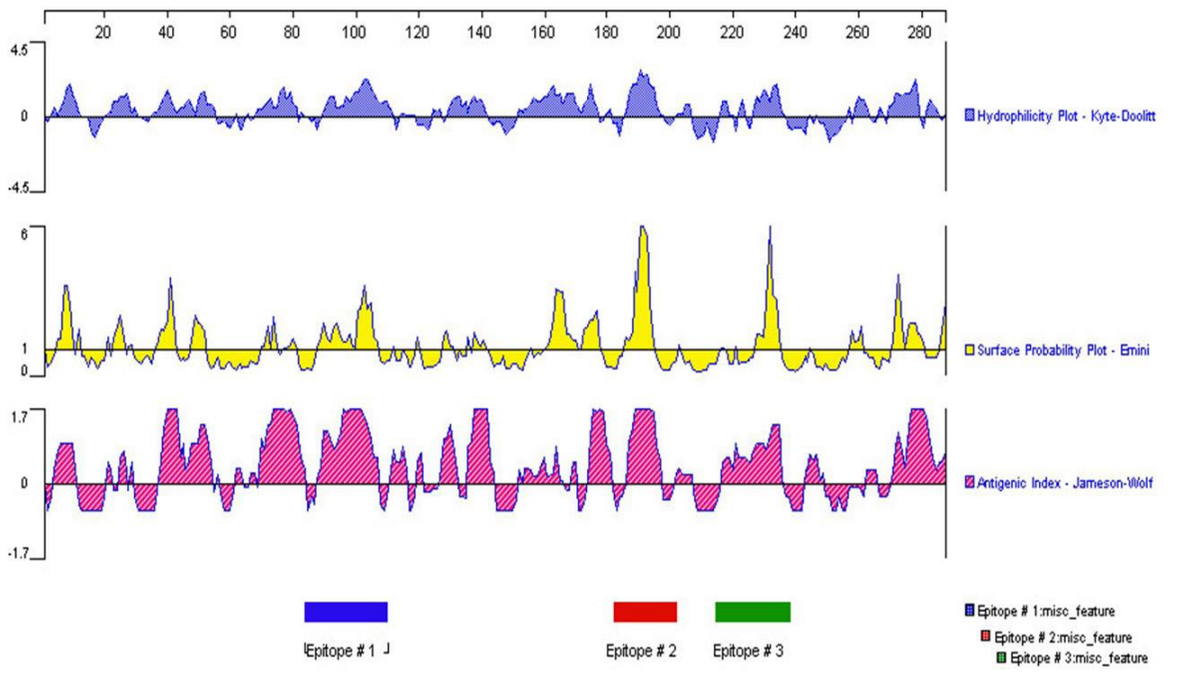

Fig. 2. Epitope 1: hydrophobic regions - epitope 2: superficial regions - epitope 3 :regions antigenicity.

Table 1. Table included: Selected tree antigenic regions, Linker sequence and the final nucleotide sequence synthesized with the linker.

Amino acid number	Protein sequence	Linker sequence	The final nucleotide sequence synthesized with the linker
84-110	IITTQSSAKAYKAVDGKTSEQHTNITL	EAAAKEAAAK	Attattaccacccagagcagcgcgaaagcgtataaagc
183-202	DNLKFQPRKNDESAFGIMDG		Ggtggatggcaaaaccagcgaacagcataccaacatta
215-238	EVVEEDVIKIRDLVKEKYPDMDMI		cCctggaagcggcggcgaaagaagcggcggcgaaag
			ataAcctgaaatttcagccgcgcaaaaacgatgaaagcg
			cgtttGgcattatggatggcgaagcggcggcgaaagaag
			cggcggCgaaagaagtggtggaagaagatgtgattaaaa
			ttcgcgatctggtgaaagaaaaatatccggatatggatatgatt

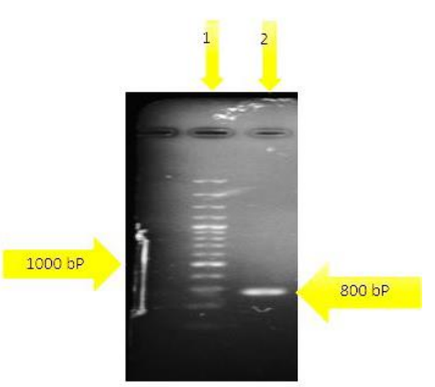

Fig. 3. *UreD* gene amplification by PCR. Lane 1: Molecular weight marker (100 bp ladder); Lane 2: amplified: *UreD*.

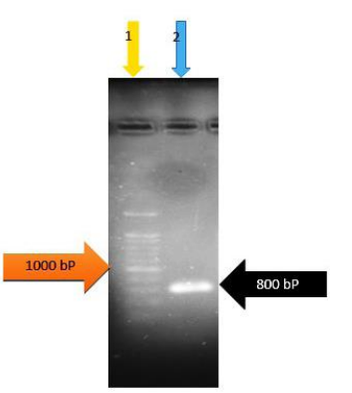

Fig. 4. PCR production of *UreD* gene Lane 1: Molecular weight marker (100 bp ladder); Lane 2: PCR produc.

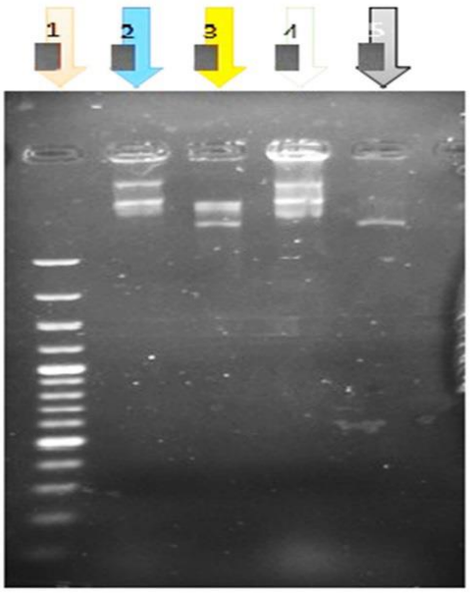

Fig. 5. Result of enzymatic digestion electrophoresis: (1) Marker (2) and (4) undijested pET32a (3) pET32a+ *UreD* (5) dijested pET32a.

by Ni-NTA kit. Molecular weight of recombinant Ib-AMP4 is 2 kDa, that's because added extra peptide sequences by vector pET-32a, about 20 kDa was added to size of recombinant *UreD* and it's molecular weight was observed 22 KDa on gel (Fig. 6). Construction purified protein was 0.2 mg/ml.

The results of the western blot test can be seen in the following figures. Based on the results, in all serum samples of patients with *U. urealyticum* infections, bands related to antigen-antibody reaction were observed in PVDF papers. At the same time, no band was observed in the antibody reaction with the antigen in the serum samples of healthy individuals (Figs. 7 and 8).

DISCUSSION

Ureaplasmosis is an opportunistic pathogen that is usually easily treated with appropriate antibiotics in non-pregnant patients. But the biggest side effect is pregnancy consequences and neonatal complications, which are associated with the risk of taking antibiotics (10). *Ureaplasma* is naturally resistant to the beta-lactams family, because they lack walls and peptidoglycans. Due to the lack of folic acid synthesis, sulfonamides are ineffective against them (11).
Due to the dangerous side effects of infections caused by this bacterium and increasing antibiotic

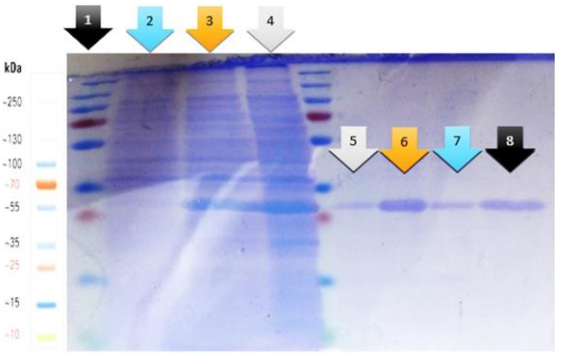

Fig. 6. Expression of recombinant antigenic fragments of *UreD* protein and its purification (1). Protein marker, (2) before induction (3, 4) after induction (5-8) purified protein.

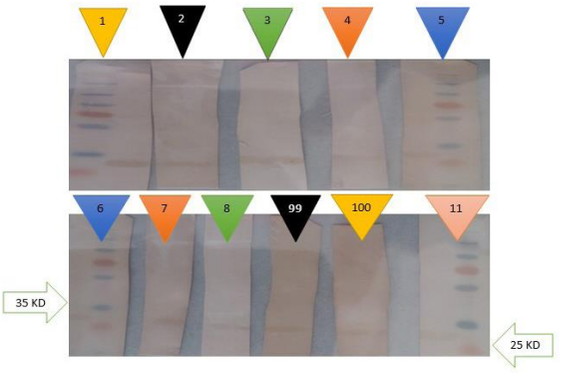

Fig. 7. Western blot results (1,6,7,12): Protein marker, (2-5, 8-11): positive sample with a band of people with *U. urealyticum* infections.

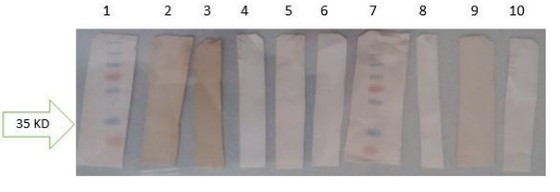

Fig. 8. Western blot results: No band was observed on the reaction of recombinant protein with the serum of healthy individuals.

resistance, rapid and timely diagnosis is of great importance and due to the difficulty and length of the diagnostic process. The use of recombinant proteins is very efficient due to the small size of the fragment and the ability to easily clone and the ability to analyze different parts of a protein separately to produce an ELISA diagnostic kit and vaccine. Since the sensitivity and specificity of the ELISA kit is due to the exposure of the epitopes of that protein to B cells,

using part of the protein is less cross-referenced than the whole protein in the kit design. Also, in the production of protein vaccines, the smaller the protein and the higher the antigenic properties, the better the results. As a result, using small portions of protein instead of total protein in diagnostic basins such as Western blotting and ELISA provide an acceptable answer.

One of the genes that can be a good candidate for designing diagnostic kits is *ureD* gene. Neypolles et al. found that the *UreD* gene belongs to the group of urease genes in the bacterium *U. urealyticum*, is a conserved gene and causes the production of urease enzyme which is one of the most important virulence factors in *Ureaplasma*. In another study, Mackenzie et al. examined the importance of the enzyme *U. urealyticum* using a study of monoclonal antibodies (MAbs) against the enzyme urease (12).

In 1989, Mr. Blanchard and colleagues demonstrated a sequence of *U. urealyticum* using cloning, DNA hybridization, and western blotting techniques which has a homology with the fragment of Auraz Providence story, which was cloned in *E. coli*. Experiments show that the urease gene produced from *Ureaplasma* has a homology among other bacteria such as *Helicobacter pylori* and providence the IC61 probe and urease genes have been shown to be conserved sequences among prokaryotes. Negative urease samples were confirmed by Western blotting for negative reporting which were connected by a flexible linker (13, 14). We surveyed these areas using the latest bioinformatics software and we selected the best areas in terms of epitope and antigenicity. After examination by western blotting technique for the ability to detect *ureD* protein by B lymphocytes, with the least dilution of protein and primary and secondary antibodies in the shortest possible incubation time of 30 minutes, we were able to obtain the best results. The positive and negative samples obtained from the Biofarm kit from the United States were well overlapped with the western blotting technique.

CONCLUSION

In this study, no reaction was observed between antibodies in the serum of healthy individuals with high-risk behaviors with this protein this indicates the specificity of the antigenic regions of the recombinant *ureD* protein can be used for diagnostic tests such as ELISA. Also, given that the recombinant protein produced by the patient's antibodies is detected, it is possible that the recombinant protein produced could be used as a vaccine to prevent infections caused by of *U. urealyticum*.

ACKNOWLEDGEMENTS

This research was supported by the financial assistance from Molecular and Medicine Research Center of Arak University of Medical Sciences, Arak, Iran. The authors would like to thank the Deputy of Research and Technology of Arak University of Medical Sciences.

REFERENCES

1. Stellrecht KA, Woron AM, Mishrik NG, Venezia RA. Comparison of multiplex PCR assay with culture for detection of genital mycoplasmas. *J Clin Microbiol* 2004; 42: 1528-1533.
2. Ljubin-Sternak S, Mestrovic T. Chlamydia trachonmatis and Genital Mycoplasmias: Pathogens with an Impact on Human Reproductive Health. *J Pathog* 2014; 2014: 183167.
3. Akhi MT, Ebrahimi E. The Role of Ureaplasma urealyticum in male non-gonococcal urethritis in Tabriz. *Rawal Med J* 2009; 34: 65-67.
4. Waites KB, Xiao L, Paralanov V, Viscardi RM, Glass JI. Molecular Methods for the Detection of Mycoplasma and Ureaplasma infections in humans: a paper from the 2011 William Beaumont Hospital symposium on molecular pathology. *J Mol Diagn* 2012; 14: 437-450.
5. Abtahi H, Salmanian AH, Rafati S, Behzadian Nejad G, Mohammad Hassan Z. High level expression of recombinant ribosomal protein (L7/L12) from Brucella abortus and its reaction with infected human sera. *Iran Biomed J* 2004; 8: 13-18.
6. Mirjamali N A-S, Soufian S, Molaee N, Abbasian SS, Abtahi H. Cloning and expression of the enzymatic region of *Streptococcal hyaluronidase*. *Iran J Basic Med Sci* 2014; 17: 667-672.
7. Hasanzadeh L, Ghaznavi-Rad E, Soufian S, Farjadi V, Abtahi H. Expression and antigenic evaluation of VacA antigenic fragment of *Helicobacter Pylori*. *Iran J Basic Med Sci* 2013; 16: 835-840.
8. Khaki M, Ganji A, Abtahi H, Mosayebi G, Baazm M, Sadoogh Abbasian S, et al. Computer simulation and additive-based refolding process of cysteine-rich proteins: VEGF-A as a model. *Int J Pept Res Ther* 2018;

24: 555-562.
9. Mahmoudi S, Abtahi H, Bahador A, Mosayebi G, Salmanian AH, Teymuri M. Optimizing of nutrients for high level expression of recombinant streptokinase using pET32a expression system. *Maedica (Bucur)* 2012; 7: 241-246.
10. Waites KB, Katz B, Schelonka RL. Mycoplasmas and ureaplasmas as neonatal pathogens. *Clin Microbiol Rev* 2005; 18: 757-789.
11. Capoccia R, Greub G, Baud D. Ureaplasma urealyticum, Mycoplasma hominis and adverse pregnancy outcomes. *Curr Opin Infect Dis* 2013; 26: 231-240.
12. Huang C, Zhu HL, Xu KR, Wang SY, Fan LQ, Zhu WB. Mycoplasma and ureaplasma infection and male infertility: a systematic review and meta-analysis. *Andrology* 2015; 3: 809-816.
13. Vancutsem E, Soetens O, Breugelmans M, Foulon W, Naessens A. Modified real-time PCR for detecting, differentiating, and quantifying *Ureaplasma urealyticum* and *Ureaplasma parvum*. *J Mol Diagn* 2011; 13: 206-212.
14. Niazi Amraii H, Abtahi H, Jafari P, Mohajeriani HR, Fakhroleslam MR, Akbari N. In vitro study of potentially probiotic lactic acid bacteria strains isolated from traditional dairy products. *Jundishapur J Microbiol* 2014; 7(6): e10168.