Bioinformatics analysis and expression of a truncated form of *Proteus* mirabilis Pta protein as a novel vaccine target against urinary tract infection

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ABSTRACT

Introduction: Pathogenic strains of *Proteus mirabilis* have important roles in urinary tract infection. Proteus toxic agglutinin (Pta) is amongst the most important virulence factors of P. mirabilis. This protein has a conserved sequence present in all the strains which could be evaluated as a novel vaccine target against them. The aims of the current study were the expression, purification and characterization of a truncated Pta protein of P. mirabilis strain HI4320 as well as the bioinformatics analysis of the truncated protein. Methods: The passenger domain of pta genes in P. mirabilis was evaluated by bioinformatics studies. The selected domain (residues 207-730) was amplified by PCR and cloned into pET28a expression vector. The Pta was expressed in BL21 (DE3) host and purified by Ni-NTA resin. The analyses of the purified protein were performed by SDS-PAGE and Western blotting. Results: The bioinformatics studies predicted the appropriateness of the passenger domain of Pta protein in terms of conservation, stability and cell-surface exposure. The length of PCR fragment of truncated form of pta gene was ~1500 bp. The cloning and expression of the truncated pta gene was successfully performed using pET28a-BL21 (DE3) system. Analyses of the purified Pta by SDS-PAGE and Western blotting confirmed the purification of a ~60 kDa His-tagged polypeptide. Conclusion: The high frequency of P. mirabilis infection, especially in patients with abnormalities in their urinary tracts and also the rising of antibiotic resistance among the strains of this pathogen point to the need for effective controlling measures against them. In this regard, the passenger domain of Pta could be considered as a vaccine target. The efficacy and *in-vivo* immunogenicity of this purified protein is currently under study.

KEYWORDS: Proteus mirabilis, Urinary tract infection, Pta protein, Vaccine target, Expression.

INTRODUCTION

Proteus mirabilis is a Gram-negative bacteria which is an etiological cause of urinary tract infection (UTI), especially among the elderly inhabitants in nursing homes, patients with type 2 diabetes, people with a long period of catheterization, or postoperative [1, 2]. P. mirabilis can cause different forms of UTI including cystitis, pyelonephritis, asymptomatic bacteriuria, bacteremia, urolithiasis and progression to potentially life-threatening urosepsis [2, 3]. According to the previous studies, P. mirabilis causes between 1-10% of all

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urinary tract infections and is the cause of 11 million physician visits and \$3.5 billion dollars medical costs in the United States [3, 4]. Studies have shown that 80% of nosocomial urinary tract infections are related to indwelling urinary catheters [5]. Furthermore in Europe, the fatality rate of nosocomial infections is 10% which approximately 97% of them are related to catheters [6]; while *P. mirabilis* has been shown to be an important causative agent of catheter-associated urinary tract infections (CAUTIs) and urinary stones formation [7].

The strains of *P. mirabilis* encode different virulence factors for pathogenicity. Recent studies have discovered a novel bifunctional auto-transporter (AT), proteus toxic agglutinin (PMI2341, Pta), in *P. mirabilis* strain HI4320 [8]. Pta is a surface-associated, calcium-dependent alkaline protease that its expression results in a dose-dependent cytotoxicity in cultured epithelial cells. In addition, the intoxication of host cells with Pta results in actin depolymerization, membrane damage and subsequent lysis [9]. Because of this, Pta punctures the host cell membrane and inflicts outflow of the cytosol, osmotic stress



and de-polymerization of simple protein filaments, leading to compromised structural integrity of the host cell and injuries in the bladder and kidneys. Pta also induces protease cell-cell interaction via autoaggregation [10]. This protein has two domains, each one with a distinct function. N-terminal passenger or alpha domain of this protein mediates attachment, invasion, auto-agglutination and acts as a secreted cytotoxin [9].

Vaccination could play an important role in prevention of UTIs that are caused by *P. mirabilis* strains and there are novel targets that could be evaluated as vaccine candidates against these pathogens [11, 12]. Pta can be considered as a potential vaccine target due to its sequence conservation as well as its important functions in the pathogenicity. Thus in this study, we selected a truncated fragment of Pta passenger domain (amino acid residues 207-730) based on bioinformatics analyses and expressed and purified the recombinant protein using a prokaryotic expression system as a first step to evaluate its efficacy as a possible vaccine target against UTI caused by *P. mirabilis* strains.

MATERIALS and METHODS

Determining the physicochemical properties of Pta

The protein sequence of Pta (accession number AM942759.1) from *P. mirabilis* HI3244 strain was obtained from the National Centre for Biotechnology information (http://www.ncbi.nlm.nih.gov). Then, physicochemical properties of the truncated Pta protein were computed using ProtParam online tool (http://us.expasy.org/tools/protparam.html).

Secondary and tertiary structure prediction

Prediction of secondary structures of the truncated Pta protein was performed by GOR4 secondary structure prediction method (https://npsa-prabi.ibcp.fr/cgibin/npsa) that determines the arrangement of the sequences as α -helices, β -sheets and coils structures. Then, the putative three-dimensional structure (3D) of the selected domains of Pta protein (residues 207-730) was modeled using the **I-TASSER** (http://zhanglab.ccmb.med. umich.edu/I-TASSER) Quality I-TASSER approach is based on the multiple threading alignments which generates 3D models along with their confidence score (C-score). Energy minimization for the 3D models was done using Chiron: Rapid Protein Energy Minimization Server (http://accelrys.com/products/discovery-Discovery studio program (http://troll.med.unc.edu/chiron) was used to visualize the modeled 3D structures. The recognition of the potential errors in initial tertiary structures and comparison of the models were performed by ProSA-web and PROCHECK program. The determined Z-score in ProSA-web is depicted in a plot, which includes the Z-score of experimentally determined structures deposited in PDB and then the residue-by-residue stereochemical quality of models is verified by Ramachandran plot (http://mordred.bioc.cam.ac.uk/ _rapper/rampage.php) [14, 15].

Bacterial strains and growth conditions

Proteus mirabilis HI4320 standard strain, isolated from a human urinary tract was obtained from microbial culture collection of Pasteur Institute of Iran, Tehran, Iran and was cultured in 5 ml of Luria-Bertani (LB) broth medium for 24 h at 37°C. Bacterial identification and confirmation of this isolate was performed by routine culture method and biochemical tests, as described previously [16].

DNA extraction and PCR amplification

Bacterial genomic DNA was extracted using DNA extraction kit (Roche, Germany). The quality and purity of the extracted DNA was investigated by agarose gel electrophoresis and NanoDrop spectrophotometer. Then, PCR amplification of pta gene was performed by primers designed for conserved 5' and end the (Pta-For: gene CATGCCATGGCCGCGCAAGTGATTAATAAT and Pta-Rev: $CCC\underline{AAGCTT}GGCTAAAGTCGTATTTTCTTTC).$ These primers were designed based on the putative passenger domain (residues 207-730) of Pta. PCR reactions were performed by Eppendorf thermocycler and carried out in a master mix containing 500 ng of DNA template, 1 x reaction buffer, 200 µM of dNTPs (10 mM), 2 mM of Mgcl2 (50 mM), 1 μM of primers (10 pmol), and 1U of pfu DNA polymerase (Fermentas, Lithuania). The PCR program was performed with an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for a 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1% agarose gel under UV light.

Cloning of pta gene into the cloning vector

For cloning, enzymatic digestion with NcoI and HindIII was performed on the pET28a vector and amplified gene. Then, T4 DNA ligase (Fermentas, Lithuania) was used for the ligation of digested PCR product and pET28a. Competent BL21/plysS (Invitrogen, USA) were transformed by the resulting plasmids following the manufacturer's instructions. The LB plates containing 30 μ g/ μ l kanamycin were used to select the transformed colonies. The colonies were subjected to plasmid extraction and PCR by the custom primers. Then, a miniprep from a positive clone was confirmed by double digestion with NcoI and HindIII enzymes and finally the chosen recombinant plasmids were subjected to sequencing (Genfanavaran, Iran).

Induction and expression of Pta protein

The expression host *E. coli* BL21/plysS was used for expression of Pta. Briefly, recombinant *E. coli* BL21/plysS cells were grown overnight in LB medium containing kanamycin (50 µg/ml) at 37°C. Then, 500 ml of LB broth medium was inoculated with 5 ml of the overnight culture of *E. coli* BL21/plysS. The growth of bacterial culture was observed by an optical density (OD) at 600 nm of approximately 0.5. The Pta protein expression was achieved using isopropyl-beta-thio galactopyranoside (IPTG) inducer to a final concentration 0.5 mM and incubation in a shaking incubator at 37°C for 4 h [16].

Induction evaluation by SDS-PAGE and Western blotting

The bacterial pellets were suspended in loading buffer, heated for 5 min at 95°C and 30 μ l of each sample was subjected to a concentration of 12-15% polyacrylamide gels. For Western Blot, the crude lysates or the purified protein samples separated by SDS-PAGE, transferred into the nitrocellulose membrane (Schleicher and Schuell, Germany) using a liquid transfer system (Bio-Rad, USA). Membranes were blocked with skimmed milk in PBST. The membranes were incubated with the conjugated His-tag antibody (Abcam, USA) and DAB-H2O2 was used for detection of expressed protein [17].

Purification of recombinant protein Pta

The protein purification was achieved with nickelnitrilotriacetic acid (Ni²⁺-NTA) affinity chromatography under denaturing conditions. Briefly, the pellet from overnight culture was re-suspended in lysis buffer (urea 8M, NaH2PO4 0.1M, NaCl 0.3M, pH 8.0) and this suspension was sonicated and



centrifuged for 25 min at 13'000 rpm. Then, the supernatant was collected and added to chromatography column containing Ni-NTA gel matrix (Qiagen, Hilden, Germany). The recombinant proteins bound to agarose were removed by elution buffer (urea 8M, NaH2PO4 0.1M, NaCl 0.3M, Imidazole 500mM, pH 8.0) and analyzed by SDS-PAGE and Western Blotting. The impurities and remnant urea in the eluted proteins were removed by dialysis (10-15 kDa cut-off). Finally, concentration of the purified protein was estimated by Bradford assay at 595 nm wavelength.

RESULTS

Analysis of Pta protein structures

The primary structure of truncated Pta protein from P. mirabilis was studied by bioinformatics tools that indicated its expected Mw as 57.5 kDa and its theoretical isoelectric point (pI) as 5.11, revealing the acidity nature of the protein. The instability index was computed to be 16.15 that classified Pta as a stable protein. The half-life of the protein was greater than 10 h in E. coli, in-vivo system. The aliphatic index of Pta was 71.07. The Grand average of hydropathicity (GRAVY) was equal to -0.542. The negative and positive charged residues were 61 and 46, respectively and the extension coefficient was 95230. Then, the secondary structure of the truncated Pta was studied which determined the arrangements of amino acid sequences into structures such as α -helices, β -sheets and coils. These results showed that the residues of the truncated form of Pta were made up of 134 strands, 100 alpha-helices and 290 coils (Fig. 1).

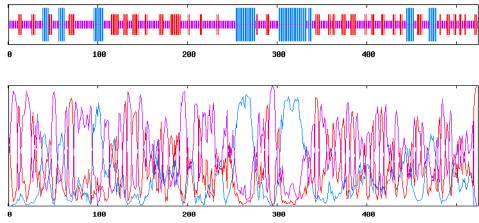
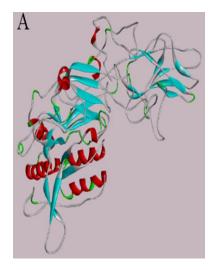
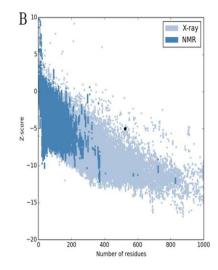


Fig. 1. Analysis of the secondary structure of truncated form of Pta protein , evaluated by GOR4 server that predicted the arrangements of the amino acids as α -helices, β -sheets and coils structures at specified positions. Extended strand: purple, coil: red, helix: blue.

The tertiary structure of truncated Pta protein was modeled using I-TASSER server. The server generated several 3D models which among them the best model of this protein according to the C-score (C-score = -2.33) was selected for further analysis. The 3D structure of truncated Pta protein is shown in Fig. 2A. The evaluation of the selected model using

the ProSA (Protein structure analysis) web showed that the obtained Z-score from ProSA (Z=-5) was within the range of scores found for native proteins (Fig. 2B). Then, the stereochemical quality of this structure was evaluated by Ramachandran plot and the results indicated that only 4% of the residues were in dis-allowed region (Fig. 2C).





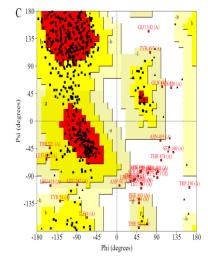


Fig. 2. Analysis of the tertiary structure of truncated form of Pta protein. A) The 3D structure of the truncated form, predicated by I-TASSER server. The red, blue and grey areas represent helices, beta-sheets and coils, respectively. B) The Z-score of the model after refinement process was -5 which is within the range of native protein conformations. The Z-score of this protein is shown in large black spot. Z-score plot consists of the Z-scores of all experimentally protein chains in PDB determined by NMR spectroscopy (dark blue) and X-ray crystallography (light blue). C) The Ramachandran plot was depicted in PROCHECK server and showed that about 4% of residues were in dis-allowed region.



PCR amplification of truncated pta of P. mirabilis

The PCR condition was optimized for amplification pta gene was present in the P. mirabilis strain HI4320 and the length of the truncated pta amplicon was confirmed to be ~ 1500 bp (expected: 1569 bp; Fig. 3).

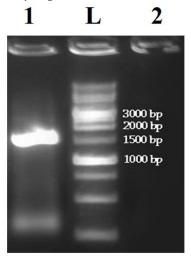


Fig. 3. PCR amplification of truncated form of pta gene. Line 1: amplified product of *pta* gene; Line 2: Negative control; L: 1 kbp DNA ladder.

Cloning of the selected fragment of pta gene in pET28a vector

Confirmation of cloning of the genes by PCR amplification and digestion with *NcoI* and *HindIII* restriction enzymes showed that there was successful insertion of the target fragments into pET28a expression vector (Fig. 4A and B, respectively). Nucleotide sequencing also demonstrated that no changes had occurred in the sequence of the targeted gene as compared to the *pta* gene that was used for designing of the primers.

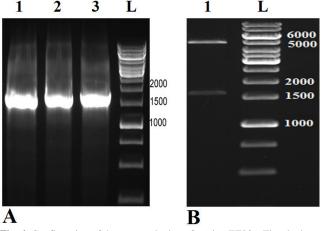


Fig. 4. Confirmation of the proper cloning of pta in pET28a. The cloning of pta gene in pET28a vector was performed by A) PCR amplification of cloned pta gene (Lines 1-3: positive clones; L: 1 kbp DNA ladder) and B) double digestion of pET28a-pta by *NcoI* and *HindIII* enzymes (Line 1: digested cloned pta and L: 1 kbp DNA ladder).

Expression and purification of recombinant Pta protein

Expression of *pta* gene cloned in pET28a was optimized by parameters such as different concentrations of IPTG and incubation times. Optimum expression was attained with 0.5 mM IPTG and incubation time of 4 h, leading to a purified protein with a relatively high concentration (2 mg/ml). The SDS-PAGE result of the overexpression of Pta protein is shown in Fig. 5. Analyses by SDS-PAGE and Western blotting of the

purified protein confirmed the size (~60 kDa) and in-frame expression of the truncated his-tagged recombinant Pta (Fig. 6 and Fig. 7, respectively).

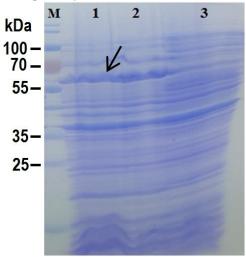


Fig. 5. Analysis of the pta expression in crude lysate of *E. coli* host by SDS-PAGE. Line 1: pET28a-pta induced by IPTG 0.5 mM; Lane 2: pET28a-pta induced by IPTG 1 mM; Line 3: Un-induced construct; M: prestained protein marker (Fermentas, Lituania).

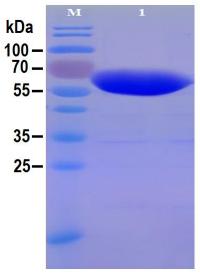


Fig. 6. Analysis of purified protein Pta by SDS-PAGE. Line 1: purified protein Pta (The size of the purified protein is approximately 60 kDa); M: pre-stained protein marker.

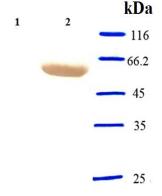


Fig. 7. Analysis of purified protein Pta by Western Blot with anti-His conjugated antibody. Line 1: Uninduced construct; Line 2: purified protein Pta.



DISCUSSION

P. mirabilis is the most common pathogen of the urinary tract that is more common in complicated urinary tract infections, particularly in catheter-associated UTI (CAUTI) that causes about 10-44% of long-term CAUTIs [18, 2]. Evidence shows that urease, hemolysin, and Pta are considered as the most important virulence factors of this uropathogen which has been involved in the urinary tract injuries in the suffering patients [19]. P. mirabilis has been increasingly shown resistance to numerous classes of antibiotics in recent years which will complicate the treatment of these infections in the future [20]. For example, resistance to trimethoprim-sulfamethoxazole (SXT), β-lactams, aminoglycosides, fosfomycin, nitrofurantoin, tetracycline and sulfonamides have been reported in this regard [21, 20]. In addition, the increase in antibiotic resistance among P. mirabilis strains which cause UTIs calls for evaluating new antigens as vaccine candidates against these pathogens.

One of the important criteria for selection of an ideal vaccine target against *P. mirabilis* UTI would be its conservation among clinical *P. mirabilis* isolates. Other studies and our previous findings (unpublished data) have indicated that Pta is greatly conserved among these isolates [22]. Conservation of sequences among different *P. mirabilis* isolates is another criterion for selection of a vaccine antigen. Here, we compared the pta sequence of *P. mirabilis* strain used in this study with the submitted pta sequences in GenBank and observed the conserved residues among pta sequences in different *P. mirabilis* strains. Furthermore, the important role of Pta in pathogenicity of *P. mirabilis* in the urinary tract could be another reason for selecting this antigen as a suitable vaccine target against UTIs [10].

Due to the difficulties of using huge polypeptides such as a fulllength Pta protein with 1084 amino acids (~ 120 kDa Mw) for immunization purposes such as technical limitations as well as higher costs of production and purification, we focused on a truncated form of Pta as a vaccine target in this study. According to the previous investigations, the passenger domain of Pta plays the most important role in pathogenicity of P. mirabilis strains and significant conservation among Pta in these isolates are demonstrable [19]. Thus, after bioinformatics evaluations, we designed a truncated fragment of Pta passenger domain (amino acid residues 207-730) which implied a conserved, stable and cell-surface-exposed fragment which was expressed and purified consequently. On the other hand, we showed that the truncated Pta was expressed robustly in pET28a-BL21 [23, 24] and could be purified with high quality and concentration (2 mg/ml). By comparing the yield of the purified truncated form of Pta in this study with its full length yield, achieved by Alamuri et al. [8] (1 mg/ml), we could find the advantage of truncation of Pta protein in the yield of purification as compared to the full length of Pta.

In conclusion, the passenger domain of Pta protein in *P. mirabilis* strain was studied by bioinformatics analyses as a vaccine target and was successfully expressed in pET28a-BL21 expression system which could be purified with high yield. To prove the effectiveness of this epitope-based vaccine candidate, complementary *in-vivo* and *in-vitro* experiments are required which are currently underway.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- 1. Matthews SJ, Lancaster JW. Urinary tract infections in the elderly population. Am J Geriatr Pharmacother. 2011;9(5):286-309. doi:10.1016/j.amjopharm.2011.07.002.
- 2. Papazafiropoulou A, Daniil I, Sotiropoulos A, Balampani E, Kokolaki A, Bousboulas S et al. Prevalence of asymptomatic bacteriuria in type 2 diabetic subjects with and without microalbuminuria. BMC research notes. 2010;3(1):169.
- 3. Karlowsky JA, Lagacé-Wiens PR, Simner PJ, DeCorby MR, Adam HJ, Walkty A et al. Antimicrobial resistance in urinary tract pathogens in Canada from 2007 to 2009: CANWARD surveillance study. Antimicrobial agents and chemotherapy. 2011:AAC. 00066-11.
- 4. Nielubowicz GR, Mobley HL. Host-pathogen interactions in urinary tract infection. Nature Reviews Urology. 2010;7(8):430-41.
- 5. Stickler D, Feneley R. The encrustation and blockage of long-term indwelling bladder catheters: a way forward in prevention and control. Spinal Cord. 2010;48(11):784-90.
- 6. Stickler D. Clinical complications of urinary catheters caused by crystalline biofilms: something needs to be done. Journal of internal medicine. 2014;276(2):120-9.
- 7. Norsworthy AN, Pearson MM. From Catheter to Kidney Stone: The Uropathogenic Lifestyle of Proteus mirabilis. Trends in Microbiology. 2016.
- 8. Alamuri P, Mobley HL. A novel autotransporter of uropathogenic Proteus mirabilis is both a cytotoxin and an agglutinin. Mol Microbiol. 2008;68(4):997-1017. doi:10.1111/j.1365-2958.2008.06199.xMMI6199 [pii].
- 9. Alamuri P, Mobley HL. A novel autotransporter of uropathogenic Proteus mirabilis is both a cytotoxin and an agglutinin. Molecular microbiology. 2008;68(4):997-1017.
- 10. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature reviews microbiology. 2015;13(5):269-84.
- 11. Billips BK, Yaggie RE, Cashy JP, Schaeffer AJ, Klumpp DJ. A live-attenuated vaccine for the treatment of urinary tract infection by uropathogenic Escherichia coli. J Infect Dis. 2009;200(2):263-72. doi:10.1086/599839.
- 12. Scavone P, Sosa V, Pellegrino R, Galvalisi U, Zunino P. Mucosal vaccination of mice with recombinant Proteus mirabilis structural fimbrial proteins. Microbes Infect. 2004;6(9):853-60. doi:10.1016/j.micinf.2004.04.006S1286-4579(04)00154-6 [pii].
- 13. Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics. 2008;9:40. doi:10.1186/1471-2105-9-401471-2105-9-40 [pii].
- 14. Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 2007;35(Web Server issue):W407-10. doi:gkm290 [pii]10.1093/nar/gkm290.
- 15. Lovell SC, Davis IW, Arendall WB, 3rd, de Bakker PI, Word JM, Prisant MG et al. Structure validation by Calpha geometry: phi,psi and Cbeta deviation. Proteins. 2003;50(3):437-50. doi:10.1002/prot.10286.
- 16. Asadi KM, Oloomi M, Habibi M, Bouzari S. Cloning of fimH and fliC and expression of the fusion protein FimH/FliC from Uropathogenic Escherichia coli (UPEC) isolated in Iran. Iran J Microbiol. 2012;4(2):55-62. 17. Habibi M, Asadi Karam MR, Shokrgozar MA, Oloomi M, Jafari A, Bouzari S. Intranasal immunization with fusion protein MrpH.FimH and MPL adjuvant confers protection against urinary tract infections caused by uropathogenic Escherichia coli and Proteus mirabilis. Mol Immunol. 2015;64(2):285-94. doi:10.1016/j.molimm.2014.12.008S0161-5890(14)00347-2 [pii].
- 18. Hung E, Darouiche R, Trautner B. Proteus bacteriuria is associated with significant morbidity in spinal cord injury. Spinal Cord. 2007;45(9):616-20. 19. Alamuri P, Eaton KA, Himpsl SD, Smith SN, Mobley HL. Vaccination with proteus toxic agglutinin, a hemolysin-independent cytotoxin in vivo,



protects against Proteus mirabilis urinary tract infection. Infection and immunity. 2009;77(2):632-41.

- 20. Schito GC, Naber KG, Botto H, Palou J, Mazzei T, Gualco L et al. The ARESC study: an international survey on the antimicrobial resistance of pathogens involved in uncomplicated urinary tract infections. International journal of antimicrobial agents. 2009;34(5):407-13.
- 21. Ma KL, Wang CX. Analysis of the spectrum and antibiotic resistance of uropathogens in vitro: Results based on a retrospective study from a tertiary hospital. American journal of infection control. 2013;41(7):601-6.
- 22. Cestari SE, Ludovico MS, Martins FH, da Rocha SPD, Elias WP, Pelayo JS. Molecular detection of HpmA and HlyA hemolysin of uropathogenic Proteus mirabilis. Current microbiology. 2013;67(6):703-7.
 23. Francis DM, Page R. Strategies to optimize protein expression in E.
- 23. Francis DM, Page R. Strategies to optimize protein expression in E. coli. Curr Protoc Protein Sci. 2010; Chapter 5: Unit 5 24 1-9. doi:10.1002/0471140864.ps0524s61.
- 24. Sorensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in Escherichia coli. J Biotechnol. 2005;115(2):113-28. doi:S0168-1656(04)00456-0 [pii]10.1016/j.jbiotec.2004.08.004.

