

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

Choubini E<sup>1</sup>, Asadi Karam MR<sup>2\*</sup>, Khorshidi A<sup>1#</sup>, Habibi M<sup>2</sup>, Ghasemi A<sup>1</sup>, Bouzari S<sup>2</sup>

<sup>1</sup> Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran.

<sup>2</sup> Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran.

## 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

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 bi-functional 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

\*Corresponding Author: Mohammad Reza Asadi Karam, Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran. No. 69, Pasteur Ave., Tehran 13169-43551, Iran.

Email: m\_asadi12@yahoo.com

Tel/Fax: (+98) 2166953311/ (+98) 2166492619

#Co- Corresponding Author: Ahmad Khorshidi, Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran

Email: Khorshidimalahmadi@gmail.com

Tel/Fax: (+98) 3155540021

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/cgi-bin/npsa>) that determines the arrangement of the sequences as  $\alpha$ -helices,  $\beta$ -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 server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>) [13]. 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-studio/>). Discovery studio 4.5 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](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 3' end of the gene (Pta-For: CATGCCATGGCCGCGCAAGTGATTAATAAT and Pta-Rev: CCCAAGCTTGGCTAAAGTCGTATTTCTTTC). 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  $\mu$ M of dNTPs (10 mM), 2 mM of MgCl<sub>2</sub> (50 mM), 1  $\mu$ M 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 *Nco*I and *Hind*III 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  $\mu$ g/ $\mu$ 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 *Nco*I and *Hind*III 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  $\mu$ g/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  $\mu$ 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-H<sub>2</sub>O<sub>2</sub> was used for detection of expressed protein [17].

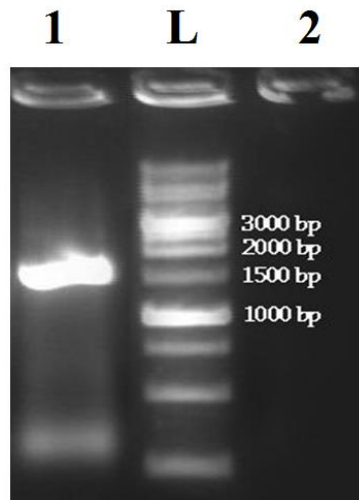
### Purification of recombinant protein Pta

The protein purification was achieved with nickel-nitrilotriacetic acid (Ni<sup>2+</sup>-NTA) affinity chromatography under denaturing conditions. Briefly, the pellet from overnight culture was re-suspended in lysis buffer (urea 8M, NaH<sub>2</sub>PO<sub>4</sub> 0.1M, NaCl 0.3M, pH 8.0) and this suspension was sonicated and



### PCR amplification of truncated *pta* of *P. mirabilis*

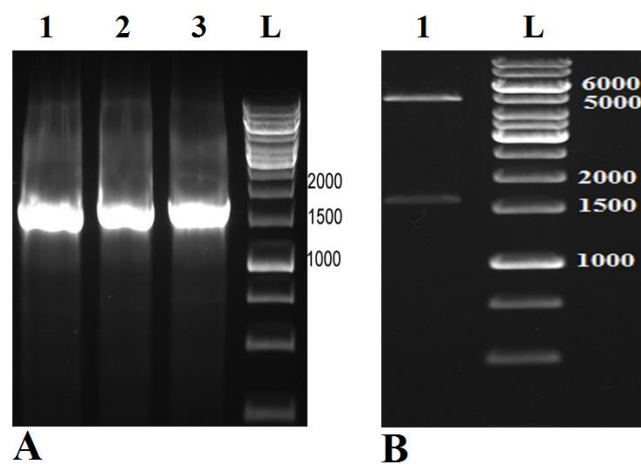
The PCR condition was optimized for amplification *pta* gene was present in the *P. mirabilis* strain HI4320 and the length of the truncated *pta* amplicon was confirmed to be ~ 1500 bp (expected: 1569 bp; Fig. 3).



**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 *Nco*I and *Hind*III 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.

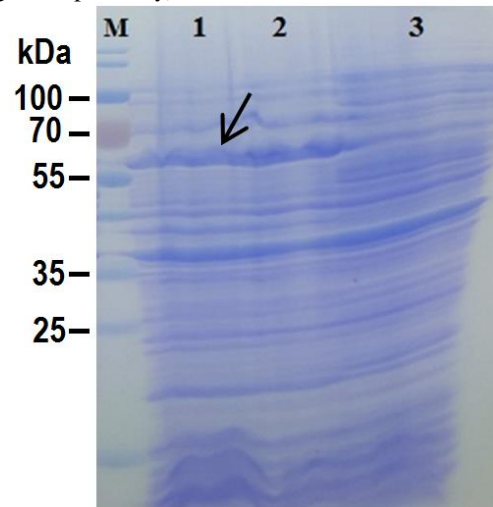


**Fig. 4.** Confirmation of the proper cloning of *pta* in pET28a. The cloning of *pta* gene in pET28a vector was performed by A) PCR amplification of cloned *pta* gene (Lines 1-3: positive clones; L: 1 kbp DNA ladder) and B) double digestion of pET28a-*pta* by *Nco*I and *Hind*III enzymes (Line 1: digested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

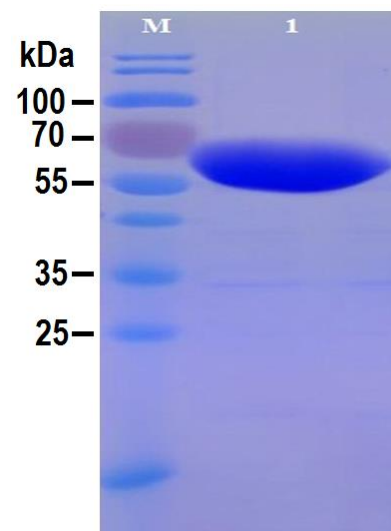
### Expression and purification of recombinant Pta protein

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

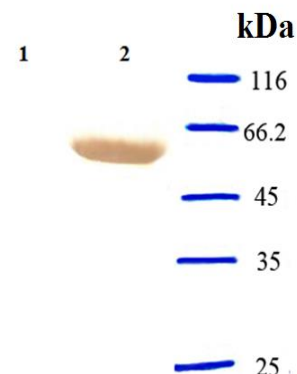
purified protein confirmed the size (~60 kDa) and in-frame expression of the truncated his-tagged recombinant Pta (Fig. 6 and Fig. 7, respectively).



**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: pre-stained protein marker (Fermentas, Lithuania).



**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),  $\beta$ -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 full-length 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.

## ACKNOWLEDGEMENT

The study was supported by Pasteur Institute of Iran (Grant No: 923) and Kashan University of Medical Sciences (Grant No: 94167).

## 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. Papazafropoulou 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, Walky 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.xMM16199 [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 C $\alpha$  geometry: phi,psi and C $\beta$  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, Himpel 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. 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.