Original Article

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High Yield Expression and Modified Purification of Novel Recombinant Truncated Protein FimH.MrpH against Urinary Tract Infections by *Escherichia coli* and *Proteus mirabilis*

Biotechnology Section

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ABSTRACT

Introduction: Urinary Tract Infections (UTIs) rank third among the list of most common bacterial infections. FimH and MrpH are the most important antigens of *Escherichia coli* and *Proteus mirabilis* isolates, respectively.

Aim: To design a novel truncated fusion protein of immunogenic virulence factors and to assess expression efficiency of fusion protein made.

Materials and Methods: N-terminal domains of MrpH (first 405 nucleotides) and FimH (first 495 nucleotides) that have functional and antigenic properties were selected to make a fusion protein. In silico and bioinformatics studies were designed to determin physico-chemical parameters. The Fusion gene was amplified by overlap PCR. After sequencing, it was inserted and expressed into pET28a vector. The expressed protein was purified by Ni-NTA column according to a modified protocol.

Results: According to in silico studies, fusion *tMrpH.tFimH* was predicted as high quality model. About 900 bp long fragment was amplified by overlap PCR. Enzyme digestion and sequencing confirmed the cloning of the fusion gene in pET28a vector. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) showed approximately 30 kDa band and Western blot confirmed the presence of fusion protein. A modified purification method has been tested which resulted in higher yield with high level of purity.

Conclusion: In order to have effective vaccine against UTI, we must study a wide variety of strategies. This study provides the new protocol for efficient purification of tMrpH.tFimH protein in which the quantity and purification of protein is high. This recombinant protein could be introduced as vaccine candidate in the future study.

Keywords: Epitope, Fusion gene, Histidine tag, Immunogen, Vectors, Western blotting

INTRODUCTION

UTI rank third among the list of most common bacterial infections, with nearly 4.5 million UTI cases diagnosed annually [1]. UTI results in nearly 7 million office visits, with additional 1 million visits to emergency rooms which lead to over 100,000 hospitalisations every year in the USA [2].

Uropathogenic *Escherichia coli* (UPEC) are the most common aetiological agent responsible for uncomplicated UTI [3]. UPEC strains causes 80% of community acquired and 30% of nosocomial UTIs [4]. In urinary tract, UPEC strains mediate adhesion and colonisation by the expression of several types of fimbrial and non fimbrial adhesins [4]. Type I pili which can mediate mannose-sensitive agglutination are expressed by the majority of UPEC strains derived from patient with UTIs.

FimH is the adhesin protein responsible for binding to mannosylated glycoprotein ligands and is located at the distal tip of the hetero polymeric type I pilus rod [4,5]. Amino acids located at the N-terminal half of the FimH adhesin (residues 1-156 of the mature protein) are involved in receptor recognition [6].

P. mirabilis not only causes UTIs, but also increases the risk of urinary stones, which complicates further the problems associated with the urinary tract [7]. The MR/P (Mannose-Resistant/*Proteus*-like) fimbria is an important factor in bladder colonisation of *P. mirabilis*. MrpH, the tip adhesin of MR/P fimbria, is composed of two domains too: An N-terminal receptor-binding domain and a C-terminal pilin domain [8].

Irrational drug therapy in UTIs has led to the emergence of antibiotic resistance amongst bacteria isolates. So, the prevention of UTIs is much more important in current scenario and therefore the concept of a vaccine has been pursued [9].

Looking at the significance of this problem, designing strategies for development of effective vaccines against UTIs has become a public health concern [10].

Due to the potential risks associated with live attenuated vaccines [11], we are exploring the possibility of the use of recombinant fusion proteins (composed of important virulence factors like adhesion fimbriae) in the development of a vaccine against UTI.

In our laboratory, previously we studied fusion protein MrpH.FimH as vaccine candidate [12]. In that study the two antigens were used in full form (whole gene), which contained unwanted domains that made difficulties with the protein expression. Therefore, we needed to design truncated form that makes the protein with smaller sizes, better expression level and with the same immunogenic domains. In this study with the help of in silico studies, novel truncated recombinant protein was designed as vaccine candidate, consisting of truncated FimH (tFimH) of UPEC, truncated MrpH (tMrpH) of *P. mirabilis* as the most important virulence factors and in parallel with this aim, we optimised the modified purification method to obtain high yield of proteins.

MATERIALS AND METHODS

This observational study was conducted to construct novel fusion protein in Pasteur institute of Iran during May 2016 to March 2017.

Fusion protein modeling: sequences of t*fimH* gene of uropathogenic *Escherichia coli* and t*mrpH* gene of *P.mirabilis* were obtained from NCBI data base (Genbank accession nos. JX847135.1 and KJ130024.1 respectively) [13]. For modeling of fusion protein, I-Tasser server a hierarchical modeling approach based on multiple threading alignment was used, which generates 3 dimensional models along with their Confidence score (C-score). The more

C-score shows the better quality. The quality and reliability of modeled structure were validated and evaluated using RAMPAGE [14] and ProSa web [15]. Several parameters of the primary structure including instability index, estimated half-life, aliphatic index, Grand Average of Hydropathicity (GRAVY), theoretical isoelectric point (pI), molecular weight and amino acid composition were determined by the ExPASyProtParam online tool [16].

Bacterial strains: UPEC CFT073 strain and *P. mirabilis* HI4320 strain were obtained from Pasteur institute of Iran. The bacterial strains were cultured at 37°C and after 18-24 hour were confirmed by biochemical tests e.g., TSI, SIM, Simon citrate, MR-VP, Phenylalanine deaminase, Lysine decarboxylase.

Primary PCR amplification of t*mrpH*, t*fim*H genes: DNA was extracted by the Phenol and Chloroform method. PCR amplification of truncated *fimH* gene of UPEC and truncated *mrpH* gene of *P. mirabilis* was performed as described previously [17]. Primers [Table/Fig-1] were designed so as to introduce a *Nco*I site at the 5´-terminus and a *Hind*III site at 3'-terminus of the recombinant gene.

PCR conditions were as follows: An initial denaturation for five minute at 94°C, followed by step 1 including: one minute at 94°C, 1 minute at 45°C and one minute at 72°C (10 cycles), and then step 2 including: one minute at 94°C, one minute at 55°C, and one minute at 72°C (20 cycles), with a final step at 72°C for five minute.

Construction of fusion gene *MrpH.FimH*: Amplification of t*fimH* and t*mrpH* genes was performed by their specific primers [Table/Fig-1]. Construction of the t*mrpH.tfimH* fusion gene was performed using overlap PCR as described previously [17]. The amplified fusion gene was digested by *Nco*I and *Hind*III and then cloned in pTZ57R vector, and the selected recombinant plasmid was subjected to sequencing (MWG service).

Cloning and expression of fusion protein MrpH.FimH: Cloned gene in pTZ57R vector was used as the source of DNA. PCR was performed using the *Pfu* DNA polymerase (Fermentas, USA). The PCR products were gel-purified, digested and cloned into expression vector pET28a (Novagen, USA) under the T7 promoter with histidine Tag to generate protein with His6 at the C-terminal of the protein. The ligated plasmid was transformed into competent *E. coli* BL21 (DE3). Cloning was confirmed by gel electrophoresis of vector, colony PCR, enzyme double digestion and sequencing respectively. *E. coli* BL21 (DE3) cells contains t*mrpH.tfimH*/pET28a were grown overnight in Luria Bertani (LB) broth containing kanamycin (50 µg/mL) at 37°C. Then, expression of the cloned gene was induced by different concentrations of inducer Isopropyl β-D-1-Thiogalactopyranoside (IPTG) (final concentration 0.1-1 mM). After incubation for different times (2, 3, 4, 7, 12 and 24h), cells were harvested by centrifugation (3000×g for 10 minutes) at 4°C and stored at -70°C till further use.

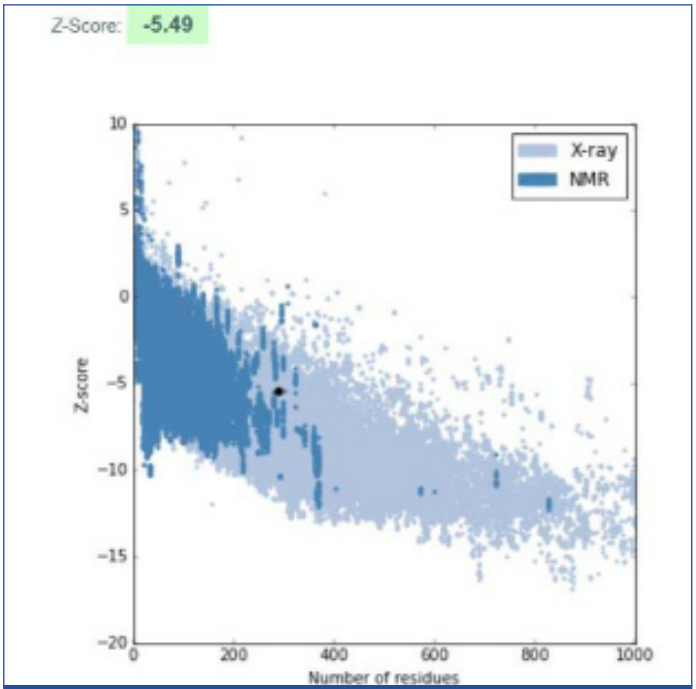
SDS-PAGE analysis: The expressed protein was subjected to 12% SDS-PAGE and gel was analysed for protein expression.

Western blot: For Western blot, the samples in SDS-PAGE gel were transferred into the nitrocellulose membrane (Schleicher and Schuell, Germany) using a liquid transfer system (Bio-Rad, USA). Nitrocellulose membrane was blocked with skimmed milk in PBST (PBS 1% + 0.05% Tween 20) and then washed several times by PBST. The membrane was incubated with the conjugated His–tag antibody (Abcam, USA) for 1.5 hour at room temperature. Finally, the blot was developed using substrate {Diaminobenzidine (DAB)-H_2O_2 solution}.

Modified protein purification: Expressed protein was purified using Ni-NTA Column (Qiagen) according to the manufacturer's instructions combined with the Reichelt P et al., procedure [18] with some modifications. Briefly, cell pellet obtained from 1 L culture was suspended in 50 mL lysis buffer, pH 7.2 (50 mMTris-base, 1 mM PMSF, 500 mMNaCl, 10 mM imidazole, Lysozyme 0.1 mg/mL, DNase 0.01 mg/mL and 8 M urea) and incubated for two hour

Target gene	Primer sequences	Size of PCR product (bp)	Annealing temperature (°C)
trunc*fimH*	F: 5'-GGT ATC GCA CCA CCA TTC GCC TGT AAA ACC-3' R-5'-CCC AAG CTT CAC CACCAC ATC ATT ATT G-3'	495	55
trunc*mrpH*	F: 5'-CAT GCC ATG GGC ATG GCC TCT ATT TTT TC-3' R: 5'-GGT TTT ACA GGC GAA TGG TGG TGC GAT ACC-3'	405	55
Fusion *mrpH.fimH*	F: 5'-GGT ATC GCA CCA CCA TTC GCC TGT AAA ACC-3' R: 5'-GGT TTT ACA GGC GAA TGG TGG TGC GAT ACC-3'	900	55

[Table/Fig-1]: Primers used to amplify trunc*fimH*, trunc*mrpH*, *fusion mrpH.fimH* genes. F: Forward, R: Reverse; bp: Base Pairs



[Table/Fig-2]: ProSa-web shows Z-score value of *tMrpH.tFimH* and composition similarity of recombinant protein to native proteins.

Estimated half-life	Instability index*	Aliphatic index	GRAVY**	soelectric point (pI)	Molecular weight
1 hour	30.01	82.68	-0.102 (hydrophil)	7.74	31377.30 Da

[Table/Fig-3]: Some of chemo- physical parameters of fusion protein tMrpH.tFimH according to ExPASyProtParam online tool.
*: According to ExPASyProtParam online tool criteria this fusion protein is classified as stable; **: Grand average of hydropathicity (GRAVY), smaller value shows protein is more hydrophilic.

at Room Temperature (RT) on the rotator to lyse properly. Lysing was completed by freezing/thawing using liquid nitrogen and sonication. The lysate was clarified by centrifugation (12000×g for 20 minutes at 4°C). Both (supernatant and pellet) fractions of cell lysate were analysed by 12% SDS-PAGE. The solubilised proteins in supernatant were purified under hybrid conditions (denaturing and renaturing conditions) with some modifications such as using urea, imidazole and 0.1% Triton X114 together in washing buffers. The solution was loaded onto the pre-equilibrated column with 3 mL Ni^{+2}-NTA agarose resin for 0.5 hour to allow the His6-tagged protein to bind with Ni^{+2} in the column. The unbound and weakly bounds of nonspecific proteins were washed by washing buffer 1, pH:7.2 (8 M urea, 50 mMTris-base, 500 mMNaCl, 0.1% Triton X114 and 10 mM Imidazole) and washing buffer 2, pH:7.2 (8 M urea, 50 mMTris-

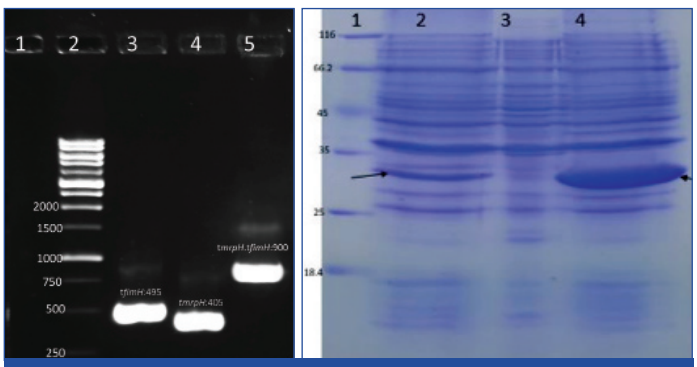
base, 500 mMNaCl, 0.1%Triton X114 and 20 mM Imidazole). The specific bounded proteins were eluted by elution buffer, pH: 7.2 (8 M urea, 50 mMTris-base, 500 mMNaCl and 500 mM Imidazole). For removing urea and imidazole, protein solution was put into dialysis tubing with an appropriate Molecular Weight cut-off (14000 MWCO) and dialyzed against dialysis buffer (25 mM TrisHCl, 100 mM NaCl, 7 to 0 M urea) and at the last step against 1X PBS (pH 7.2), finally analysed by 12% (w/v) SDS-PAGE. Purified proteins were cleaned from lipopolysaccharide contamination by Triton X-114 (Sigma) [18] and the Limulus Amebocyte assay was used to determine the level of LPS contamination. The concentration of protein was measured by NanoDrop and Bradford assay. Protein also was purified according to conventional method (Qiagene Ni-NTA procedure) and compared the results of both methods.

RESULTS

Fusion protein modeling: Sequence and composition of recombinant protein obtained by the ExPASyProtParam online tool. Recombinant fusion protein was modeled using I-Tasser server. According to I-tasser criteria and its C-score (+ 0.22), tMrpH.tFimH had the high quality structure. Validation of model was performed using Protein structure analysis (ProSa) web and Rampage. The Ramachandran plot of modeled structure revealed that, residues of model fell within the allowed regions (>98%). Additionally, validation of 3-D structure with ProSa-web revealed that Z-score value of tMrpH.tFimH (-5.49) was closer to the range of native conformations of crystal structures [Table/Fig-2], indicating the quality of this model. The physiochemical parameters of the tMrpH.tFimH fusion protein have been shown in [Table/Fig-3].

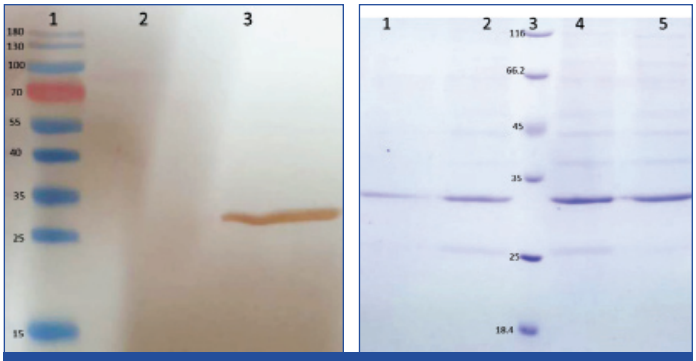
PCR amplification: DNA of standard strains were used for amplification of t*fimH* and t*mrpH* genes, which showed 495 and 405bp segments respectively [Table/Fig-4]. Fusion gene t*mrpH.tfimH* was constructed by overlap PCR and showed an approximately 900 bp segment [Table/Fig-4]. Sequencing of the fusion t*mrpH.tfimH* showed the fusion gene was constructed correctly.

Cloning and expression: After amplification, fusion gene was cloned in pET28a vector system and transformed into *E. coli* BL21 (DE3). Cloning PCR, digestion by *Nco*I and *Hind*III restriction enzymes and sequencing confirmed cloning and transformation. Expression of t*mrpH.tfimH* was optimised by parameters such as different concentration of IPTG, incubation temperature and time. Optimum expression was obtained with IPTG 0.5 mM and incubation time of 4 hours at 37°C. The level of expression was analysed by SDS-PAGE, then confirmed by Western blot [Table/Fig-5,6]. The analysis of SDS-PAGE and Western blot of the proteins showed specific band at the size of approximately 30 kDa for fusion protein tMrpH.tFimH.



[Table/Fig-4]: PCR and Overlap PCR. Lane 1: Negative control (master mix without DNA template), lane 2: Molecular Weight marker (1kb), lane 3: truncated *mrpH* gene: 405 bp, lane 4: truncated *fimH* gene: 495 bp, lane 5: fusion gene t*mrpH.tfimH*:900 bp.

[Table/Fig-5]: SDS-PAGE analysis of total cell lysate of *E. coli* BL21 DE3 containing pET28a and PET28a-recombinant proteins: lane 1: Unstained Molecular Weight marker (size range 14.4-116 kDa), lane 2: MrpH.FimH (approximately 30 kDa) induced by 0.2 mM IPTG, lane 3: negative control, lane 4: MrpH.FimH (approximately 30 kDa) induced by 0.5 mM IPTG. (Images from left to right)



[Table/Fig-6]: Western bloting analysis: lane1: Prestained protein Molecular Weight marker (size range 10-180 kDa), lane 2: negative control (*E. coli* strain BL21), lane 3: fusion protein MrpH.FimH(approximately 30 kDa). **[Table/Fig-7]:** Protein purification: lane 1 and 2: FimH.MrpH protein ~ 30 kDa (denatured purification, lower protein concentration), lane 3: Unstained Molecular Weight marker (size range 14.4-116 kDa), lane 4 and 5: fusion protein MrpH.FimH ~30kDa (modified hybrid purification, higher protein concentration). (Images from left to right)

Protein purification and LPS free test: After purification, SDS-PAGE analysis showed a high level of the concentration of purified protein. We obtained more than 4.56 mg/L from 1 L culture by modified method while 3.5 mg/L protein was obtained by conventional method [Table/Fig-7]. The level of LPS in the protein preparations was ≤0.01 EU/mL.

DISCUSSION

Nowadays development of an ideal vaccine that simultaneously prevents UPEC and *P. mirabilis* would provide an advantage over the monovalent vaccines that are available. Therefore, it is the need of the hour to test and try different strategies such as fusion technology for designing vaccines against UTIs [19].

FimH of UPEC is a two-domains protein in which the N-terminal domain contains the receptor-binding site and the C-terminal domain is required for organelle integration [6]. Therefore, we hypothesised that a fusion protein containing of N-terminal domain of FimH and N-terminal domain of MrpH can be used as vaccine targets to prevent the majority of infections due to UPEC and *P. mirabilis*.

In this study, purification conditions of protein were modified to obtain higher yield products, as a result of this modification, the yield of protein was 1.3 folds higher than conventional method. pET system was selected because it is a very powerful system developed specially for the cloning, expression and purification of recombinant proteins in *E. coli* and also has been utilized to overexpress exogenous proteins for decades [20].

Inclusion body form has disadvantages due to aggregation, but using modified method yields solubilized protein. In this condition, the recombinant protein lyses and binds under denaturing condition, washes and elutes under native conditions. Recently, high-throughput protein refolding methods such as, dilution, dialysis and solid-phase separation for renaturation of inclusion bodies have been developed [21-23]. In the present study dialysis method have been selected because in comparing to other methods, dialysis method showed ideal results. In the dialysis membrane, a decreased gradient of urea was used for the gradual removal of urea and renaturation of recombinant protein. The modified purification protocol yielded higher MrpH.FimH protein in pET28a as compared with conventional method. In the present study, not only it had been evaluated the efficiency of pET28a vector for the high level expression of the MrpH.FimH but also simultaneously developed a highly reproducible and efficient procedure for purification and scalable production of the recombinant protein with high yield and high purity.

LIMITATION

Animal study is required to assay efficacy and safety of fusion protein so that it will complete findings of this study.

CONCLUSION

The recombinant fusion protein tMrpH.tFimH can be used as a novel vaccine candidate against UTIs caused by *P. mirabilis* and UPEC isolates. Moreover, the modified purification process employed in this study can be useful in the efficient purification of other recombinant proteins highly expressed in *E. coli* as inclusion bodies.

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