Original Article

In silico design of fusion protein of FimH from uropathogenic *Escherichia coli* and MrpH from *Proteus mirabilis* against urinary tract infections

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Abstract

Background: Urinary tract infections (UTIs) caused by uropathogenic *Escherichia coli* (UPEC) and *Proteus mirabilis* are the most important pathogens causing UTIs. The FimH from type 1 pili of UPEC and the MrpH from *P. mirabilis* play critical roles in the UTI process and have presented as ideal vaccine candidates against UTIs. There is no effective vaccine against UTI and the development of an ideal UTI vaccine is required.

Materials and Methods: In this study, we planned to design a novel fusion protein of FimH from UPEC and MrpH from *P. mirabilis*. For this purpose, we modeled fusion protein forms computationally using the Iterative Threading Assembly Refinement (I-TASSER) server and evaluated their interactions with toll-like receptor 4 (TLR4). The best fusion protein was constructed using overlap extension polymerase chain reaction (OE-PCR) and the biological activity of fusion was evaluated by the induction of interleukin-8 (IL-8) in the HT-29 cell line.

Results: Our study indicated that based on the Protein Structure Analysis (ProSA)-web and the docking results, MrpH.FimH showed better results than did FimH.MrpH, and it was selected for construction. The results of bioassay on the HT-29 showed that FimH and MrpH.FimH induced significantly higher IL-8 responses than untreated cells or MrpH alone in the cell line tested.

Conclusions: In the present study, we designed and constructed the novel fusion protein MrpH.FimH from UPEC and *P. mirabilis* based on *in silico* methods. Our bioassay results indicate that the MrpH.FimH fusion protein is active and capable of inducing immune responses.

Key Words: FimH, *in silico*, MrpH, TLR4, urinary tract infection

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INTRODUCTION

Infections of the urinary tract are the second most common type of infectious disease in humans, following respiratory tract infections. A urinary tract infection (UTI) occurs when bacteria contaminate the periurethral area and ascend the urethra to colonize the bladder. Left untreated, uropathogens ascend the ureters and establish the infection in the kidney.[1,2] UTIs account for more than 11 million physician visits, 1.7 million emergency room visits, and almost half a million hospitalizations, so that the social cost of these infections is 3.5 billion dollars annually in the US.[3] Over half of all women will experience at least a symptomatic UTI in their lifetime, and about

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a quarter of the affected women will suffer recurrent UTI within 6-12 months; many have multiple recurrences.[4] Uropathogenic *Escherichia coli* (UPEC) strains are the common cause of community acquired UTI as well as a large portion of nosocomial UTIs.[1] Furthermore, *Proteus mirabilis* (*P. mirabilis*), another common uropathogen, can cause serious complications including kidney stone formation, acute pyelonephritis, and bacteremia, especially in patients with catheterized UTI or patients with abnormalities in the urinary tract.[5,6]

Antimicrobial therapy, the leading treatment for UTI, has become increasingly complex owing to the rise of antimicrobial resistance among uropathogens. In addition, because of the high incidence, recurrent UTIs, and the significant costs associated with UTIs, there is a need for a vaccine to reduce susceptibility to UTIs.[7-9]

Virulence factors produced by both UPEC and *P. mirabilis* pathogens include fimbriae, toxins, flagellae, iron acquisition systems, and proteins that function in immune evasion.[1] Type 1 pili and its adhesin FimH are required for attachment and invasion of UPEC, thus playing a critical role in the UTI process.[10,11] Mannose-resistant, Proteus-like (MR/P) fimbriae, having the MrpH adhesin, are involved in the development of pyelonephritis that mediate the adherence of *P. mirabilis* to uroepithelial cells.[9,12]

Some of the virulence factors of UPEC and *P. mirabilis* tested as vaccine targets against UTI showed limited success. Thus, there is a need to test different antigens and technologies to develop an ideal vaccine against UTI.[8,13]

The toll-like receptor (TLR) family is expressed on the surface of antigen-presenting cells (APCs). However, the recognition of pathogen-associated molecular patterns (PAMPs) by TLRs stimulates the maturation and activation of APCs and the production of pro-inflammatory responses that is a prerequisite for the activation of innate and adaptive immune responses.[14-16] In addition to the role of FimH protein in the pathogenesis of UPEC, several studies have shown the effectiveness of FimH as an adjuvant by interaction with the TLR4 ligand.[17]

In this study, we planned to design a novel fusion protein to act against UTIs by incorporating the FimH from UPEC and MrpH from *P. mirabilis*. In this light, we modeled two fusion protein forms consisting of FimH and MrpH computationally using the I-TASSER server and evaluated the interaction of fusion forms with TLR4. Then, the best fusion protein, presenting the best affinity and pose of interaction to the TLR4 was constructed. Finally, the biological assay of the best model was evaluated by the induction of interleukin-8 (IL-8) in the HT-29 cell line.

MATERIALS AND METHODS

Bacterial isolation

A total of 40 clinical isolates of *Escherichia coli (E. coli)* ($n = 20$) and *P. mirabilis* ($n = 20$) were collected from the urine samples of patients in hospitals in Tehran, Iran. All urine samples were cultured on blood agar and MacConkey agar and incubated at 37°C for 24 h. Bacterial identification was done by routine conventional methods and biochemical tests.

Hemagglutination assay

The bacteria were subcultured three times for 48 h each in Luria broth (LB) at 37°C and then harvested via centrifugation. The pellets were suspended in phosphate buffered saline (PBS) into about 10^9 colony-forming units (CFU)/ml, and then mixed with an equal volume of a 3% v/v suspension of guinea pig erythrocytes or red blood cells (RBCs) in the presence or absence of 50 mM mannose (Sigma Chemical, USA). Rapid clumping of the *E. coli* in the absence of mannose indicated the presence of type 1 fimbriae, and agglutination of *P. mirabilis* in the presence and absence of mannose showed MR/P expression of the isolates.[18] *E. coli* K-12 was used as a negative control.

DNA isolation and gene amplification

All bacterial isolates were cultivated overnight in 5 ml of LB at 37°C. Genomic DNA was extracted using the phenol and chloroform method. Polymerase chain reaction (PCR) amplification of *fimH* and *mrpH* genes was performed by primers designed for the conserved 3' and 5' ends of the genes. The primers [Table 1] were designed based on the *fimH* gene of *E. coli* CFT073 strain (GenBank accession no. NC_004431.1) and the *mrpH* gene of *P. mirabilis* HI4320 strain (GenBank accession no. NC_010554.1). PCR amplifications were carried out in 50 μl volume containing 2 μl of DNA template, 5 μl of 10× reaction buffer, 2 μl of deoxynucleotide triphosphates (dNTPs) (10 mM), 2 μl of $MgCl_2$ (50 mM), 2 μl of each primer (10 pmol) and 1U of *Pfu* DNA polymerase (Fermentas, Germany). PCR conditions were as follows: An initial denaturation for 5 min at 94°C, followed by 10 cycles of denaturation, each consisting of 60 s at 94°C, 60 s at 45°C and 60 s at 72°C, and then 20 cycles, each consisting of 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C, with a final step at 72°C for 5 min.

Cloning of genes into expression vectors

The amplification of *fimH* and *mrpH* genes was performed using primers designed to introduce an

Table 1: Characteristics of primers used in this study

Number	Primer name	Sequence (5´-3´)	Tm (C)	Reference
1	fimH-For	CATGCCATGGCCATGAAACGAGTTATTACC	66.8	This study
2	fimH-Rev	CCCAAGCTTTTGATAAACAAAAGTCAC	63.9	This study
3	mrpH-For	CATGCCATGGCCATGTTTATATTTAAACGATT	63.1	This study
4	mrpH-Rev	CCCAAGCTTAGGCATGGTTAAAATAATTG	62.4	This study
5	fusion-For	ATTTTAACCATGCCTATGAAACGAGTTATT	59.9	This study
6	fusion-Rev	AATAACTCGTTTCATAGGCATGGTTAAAAT	59.9	This study

*Nco*I site at the 5' terminus and a *Hind*III site at the 3' terminus of the genes. The amplified fragments were digested with the *Nco*I and *Hind*III enzymes and cloned into the *Nco*I and *Hind*III sites of the expression vector pET28a (Novagen, USA) with a polyhistidine-tag (6x-His tag) to generate proteins with 6x-His tag at the C-terminus of the proteins. The resultant plasmids were transformed into *E. coli* BL21(DE3) (Novagen, USA). The fidelity of cloning was verified by gel electrophoresis, PCR, restriction endonuclease digestion and sequencing (MWG-Biotech AG, Germany).

Nucleotide and amino acid sequence analyses

The nucleotide and deduced amino acid sequences of the *fimH* and *mrpH* genes were aligned with the available sequences of the previously reported UPEC and *P. mirabilis* (available on GenBank and ExPASy tools), and analyzed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2) and BLAST (www.ncbi.nih.gov) tools.

Primary and secondary structure analyses

Different parameters of the primary structure including molecular weight, theoretical isoelectric point (pI), amino acid composition, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) were computed using the ExPASy ProtParam online tool.[19] The protein secondary structure prediction was performed using the GOR (Garnier-Osguthorpe-Robson) and PSIPRED (Psi-blast based secondary structure prediction) methods.[20,21]

Tertiary structure prediction and validation

Nucleotide and protein sequences of UPEC FimH and *P. mirabilis* MrpH were obtained from the NCBI database (Genbank accession nos. NP_757248.1 and YP_002150050.1). The sequence of FimH was placed at the N- and C-termini of the MrpH protein to design the two fusion forms FimH.MrpH and MrpH.FimH, respectively. For modeling of the fusion proteins, an online version of I-TASSER, a hierarchical modeling approach based on multiple threading alignment was used, which generates 3D models along with their confidence score (C-score).[22] The quality and reliability of the built models was evaluated by the Protein Structure Analysis (ProSA) server (to determine Z-scores) and the PROCHECK and RAMPAGE programs, structure verification programs for Ramachandran plot analysis.[23,24]

Interaction analysis of fusion proteins with TLR4

The tertiary structure of human TLR4 was obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB: 3FXI). Docking studies were carried out using the Hex docking server to find out how modeled fusion proteins interact with TLR4.[25] Total interaction free energies were calculated based on shape and electrostatic information as a correlation type, and the final search was set to 25 (n = 25). Other parameters were set to default values. Based on the total dock energy values, the best fusion form was chosen.

Construction of the MrpH.FimH fusion gene

For construction of fusion MrpH.FimH, the *mrpH* and *fimH* genes with the highest sequence similarity to *mrpH* and *fimH* in GenBank were selected. Construction of the MrpH.FimH fusion gene was performed using overlap PCR according to the procedure previously described.[26] All primer sets are listed in Table 1.

Then, the amplified fusion gene was cloned into a pET28a vector, and the selected recombinant plasmids were subjected to sequencing by universal and internal primers.

GenBank submission

The sequences of *fimH*, *mrpH*, and fusion genes obtained via PCR from our local isolates have been submitted to GenBank.

Bioactivity assay

The HT-29 cell line was purchased from the Cell Bank, Pasteur Institute of Iran. The HT-29 cell line is a human colorectal epithelial cell line that expresses TLR4. The cell line was used to test the bioactivity of purified FimH and the fusion protein MrpH.FimH. The TLR4 activity of the proteins was evaluated based on the induction of IL-8. Briefly, HT-29 cells were cultured in 24-well plates (Greiner, Germany) at a density of 5×10^4 cells/well in 1 ml fresh Dulbecco's

modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biosera, North America) and antibiotics (Biosera, North America). After overnight incubation, the cells were incubated for 5 h with 10 μg/ml of sterilized of FimH and fusion MrpH.FimH. Then, supernatants were collected and the expression of IL-8 was evaluated using the enzyme-linked immunosorbent assay (ELISA) (R and D systems, USA).

Statistical analysis

The one-way analysis of variance (ANOVA), the student's *t*-test, and Tukey's honest significant difference (HSD) test were used to compare the differences between the mean values of the groups using the SPSS software (SPSS version 16). The value $P < 0.05$ for all results was considered significant.

RESULTS

Sample collection and PCR amplification

A total of 40 clinical isolates of *E. coli* (n = 20) and *P. mirabilis* (n = 20) species were collected from UTI patients. After amplification, the *fimH* and *mrpH* genes were present in all of the *E. coli* and *P. mirabilis* isolates tested, respectively. The length of the PCR fragments was approximately 900 bp and 827 bp for *fimH* and *mrpH* genes, respectively [Figure 1a]. The purified PCR products were ligated to the pET28a vector. In addition, the confirmation of cloning of the genes by enzyme digestion is shown in Figure 1b. Our results showed that all of the *E. coli* agglutinated RBCs in the absence of mannose and all of the *P. mirabilis* isolates agglutinated RBCs in the presence of mannose. Thus, it is demonstrated that the *E. coli* and *P. mirabilis* isolates expressed type 1 (FimH) and MR/P (MrpH) pili, respectively.

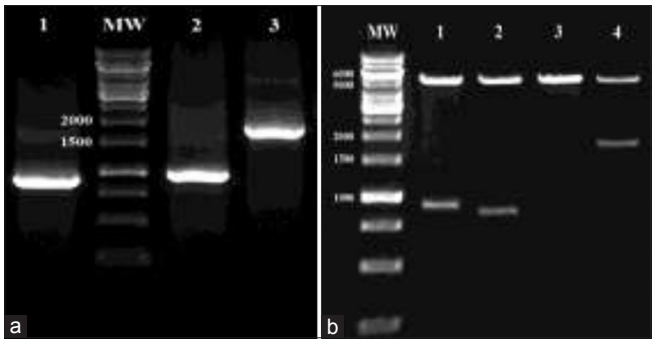

Figure 1: The construction of the fusion gene *mrpH.fimH*. After the amplification of the *mrpH* (a) line 1 and *fimH*, line 2 genes, the fusion gene *mrpH.fimH* was constructed using overlap PCR, line 3. The confirmation of cloning of *fimH (*b) line 1, *mrpH* line 2, and fusion *mrpH.fimH* line 4 into the pET28a vector is shown by enzyme digestion with *Nco*I-*Hind*III. MW: Molecular weight marker (1 kb ladder DNA)

Sequence analyses

Nucleotide and amino acid homology alignment of the sequenced *fimH* and *mrpH* genes with those published in ExPASy and GenBank showed that these sequences were highly conserved among UPEC and *P. mirabilis* [Supplementary data, Figure S1]. Furthermore, the sequences of *fimH* and *mrpH* genes showed significant homology among themselves.

Primary and secondary structure analyses

The analysis of physiochemical parameters of the FimH.MrpH or MrpH.FimH fusion proteins showed molecular weight of 60618.8 Daltons and theoretical isoelectric point (pI) of 8.85 (pI > 7, revealing the basic nature of the protein). The instability index (II) is computed to be 35.05, and that classified the proteins as stable. The biocomputed half-life was greater than 10 h. The aliphatic index of the fusion proteins was 88.64. The N-terminus of the sequence is considered to be M (Met). The negative GRAVY of 0.097 indicates that the proteins were hydrophilic. Large amounts of glycine (G), valine (V), threonine (T), and alanine (A) were found in the fusion proteins.

A "secondary structure" refers to the arrangements of the primary amino acid sequence into motifs such as α-helices, β-sheets, and coils. The results showed total residues numbering 575 for the fusion proteins, which were made up of 164 strands, 76 helices, and 335 random coils [Figure 2].

Fusion protein modeling and validation

The tertiary structures of the FimH.MrpH and MrpH.FimH fusion proteins were modeled using the I-TASSER server by placing FimH at the N- or the C-terminus of MrpH. In addition, 3D structures of control (FimH and MrpH) were modeled. The I-TASSER server generated five full-length 3D models of each query sequence, along with the C-score. Among the offered models, the best modes of each fusion protein with the highest C-score were selected for validation analyses. The modeled structures of FimH.MrpH and MrpH.FimH are shown in Figure 3a and b, respectively. Evaluation of the selected models was performed using ProSA-web and PROCHECK. The Z-scores obtained from ProSA-web were within the range of scores typically found for native proteins, indicating the better quality of the MrpH.FimH model compared with FimH. MrpH [Figure 3c and d]. Evaluation of the quality of the structures was performed by Ramachandran plot analysis, and our results showed that the fusion proteins had <3% residues in disallowed regions [Supplementary data, Figure S2].

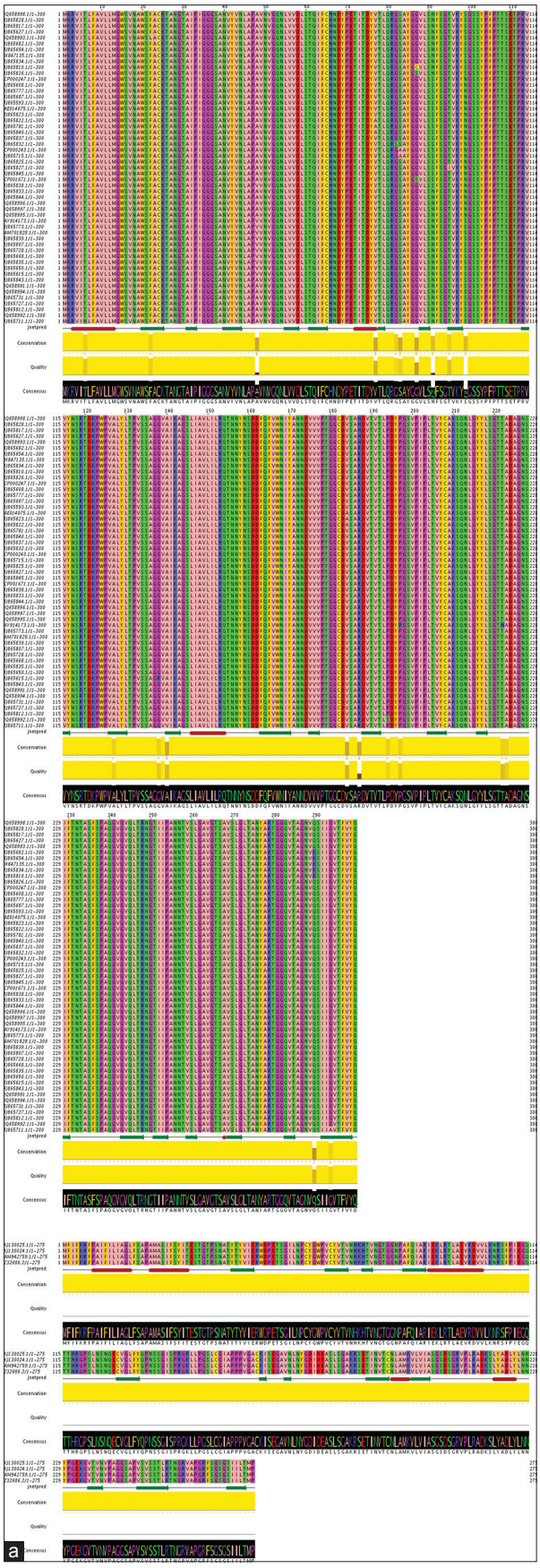

Figure S1: Multiple alignments of amino acid sequences of (a) FimH of UPEC

Docking analysis

After fusion protein modeling, interaction between the best models of the fusion proteins with TLR4 was performed by using the Hex docking server. The interaction free energies and docking conformations of the fusion models with human TLR4 are shown in Table 2. According to Table 2, the structure MrpH.FimH presents the best interaction tendency to TLR4 (-1115 kJ/mol) based on the total free energy.

Construction of the fusion gene MrpH.FimH

After sequence comparisons, the fusion gene MrpH.FimH was constructed from an UPEC and *P. mirabilis*, with the highest homology to the *fimH* and *mrpH* sequences in GenBank. The fusion gene, consisting of the *mrpH* gene, was linked to the N-terminus of the *fimH* gene using the overlap PCR method [Figure 1a]. Sequencing of the cloned MrpH.FimH gene by internal and universal primers confirmed the precise construction of the fusion gene.

GenBank submission

The determined sequences of the *fimH*, the two *mrpH*, and the fusion genes were submitted to GenBank and have been assigned under GenBank accession numbers JX847135.1, KJ130024.1, KJ130025.1, and KJ182940.1, respectively.

Biological activity of FimH and fusion MrpH.FimH

To evaluate the biological activity of the fusion protein MrpH.FimH, the level of pro-inflammatory cytokine IL-8 in the HT-29 cell line was measured. As shown in Figure 4, FimH alone and in fusion form (MrpH.FimH) induced a significantly higher IL-8 response than did untreated or MrpH protein alone ($P < 0.01$). However, there was no significant difference between the IL-8 levels induced by FimH and those induced by MrpH.FimH in the cell line ($P > 0.05$).

DISCUSSION

UPEC and *P. mirabilis* are among the most common causes of UTI, especially in case of complicated UTIs. The UTIs caused by the uropathogens represent a significant healthcare burden, which could be

Table 2: Hex docking results for fusion forms FimH.MrpH and MrpH.FimH, based on Energy (E-total) and docking conformation (Pose) in two different views

Receptor (TLR4)	Ligand	Energy (kJ/mol)	Pose
Whole molecule	FimH.MrpH	−600.2	
Whole molecule	MrpH.FimH	−1115.0	

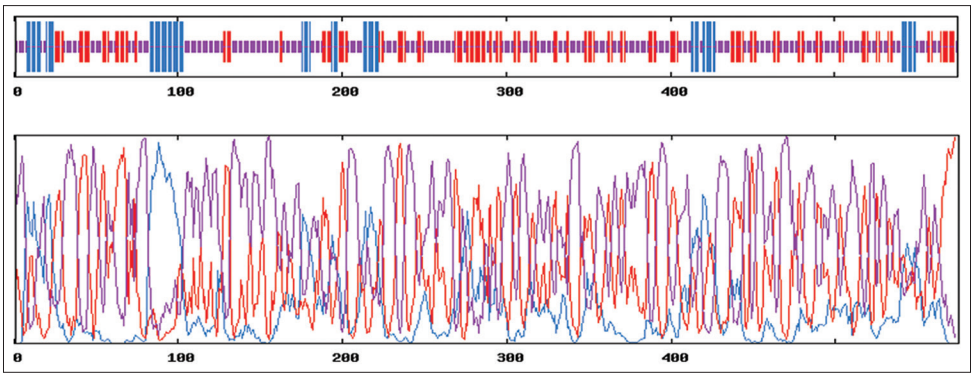

Figure 2: Graphic results for secondary structure prediction of chimeric protein. Extended strand: purple, coil: red, helix: blue

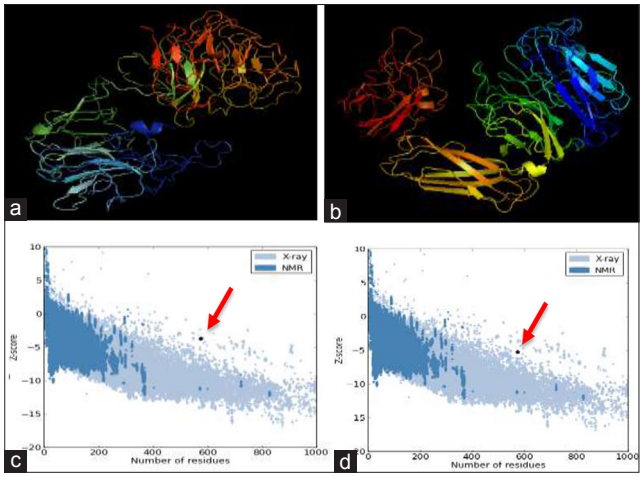

Figure 3: Fusion protein modeling. Modeled structures of the fusion proteins FimH.MrpH (a) and MrpH.FimH (b) by I-TASSER. ProSA-web Z-score of fusion proteins FimH.MrpH (c) and MrpH.FimH (d) plot. The Z-scores indicate overall model quality. ProSA-web Z-scores of all protein chains in PDB determined by x-ray crystallography (light blue) or nuclear magnetic resonance (NMR) spectroscopy (dark blue) with respect to their length. The Z-scores of FimH.MrpH (Z = -3.75) and MrpH.FimH (Z = -5.28) are highlighted as large dots. The values are in the range of native conformations

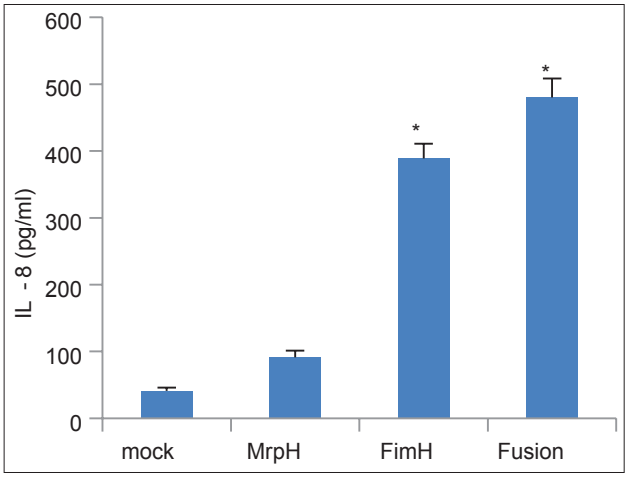

Figure 4: Evaluation of the biological activity of FimH, MrpH, and MrpH. FimH fusion proteins. The HT-29 cell line was treated with 10 μg/ml of FimH, MrpH, and MrpH.FimH proteins, and the supernatants were analyzed with ELISA for the production of pro-inflammatory cytokine IL-8. Single asterisks indicate a statistical significance of IL-8 over MrpH and untreated (mock) groups (P < 0.01). Bars represent mean ± S.D. from three independent experiment

removed by the development of an ideal vaccine. The limited success of monovalent UTI vaccines designed against known virulence factors highlights the need for other UTI vaccines.[8,13,27,28] The development of an ideal vaccine that simultaneously prevents UPEC and *P. mirabilis* would provide an advantage over the monovalent vaccines that are available. In this way, new strategies such as fusion technology are required for the design of vaccines to act against UTIs.[15]

An ideal vaccine target should be widely distributed among clinical isolates, have a surface-exposed site, and possess epitopes that are conserved among the pathogens.[29] Thus, we compared the *fimH* and *mrpH* sequences of UPEC and *P. mirabilis* isolated from UTI patients with the sequences published in ExPASy and GenBank, revealing that the sequences are 98-100% identical. Despite the presence of the characteristics in FimH of UPEC and in MrpH of *P. mirabilis*, we constructed a fusion protein consisting of the virulence factors as a novel vaccine candidate against UTI.

Nowadays, advances in bioinformatics and biotechnology have opened new doors for the characterization of novel genes and vaccine design. In this context, the computational methods for the prediction of fusion protein formation and protein-protein interactions accelerate the analysis and design of fusion proteins as vaccine candidates.[30,31] In this study, we used different *in silico* methods to predict the structure of the fusion proteins and protein-protein interaction.

The physico-chemical parameters of the fusion proteins FimH.MrpH and MrpH.FimH were first analyzed. Then, the secondary structure of the proteins was computed using different servers like PSIPRED and

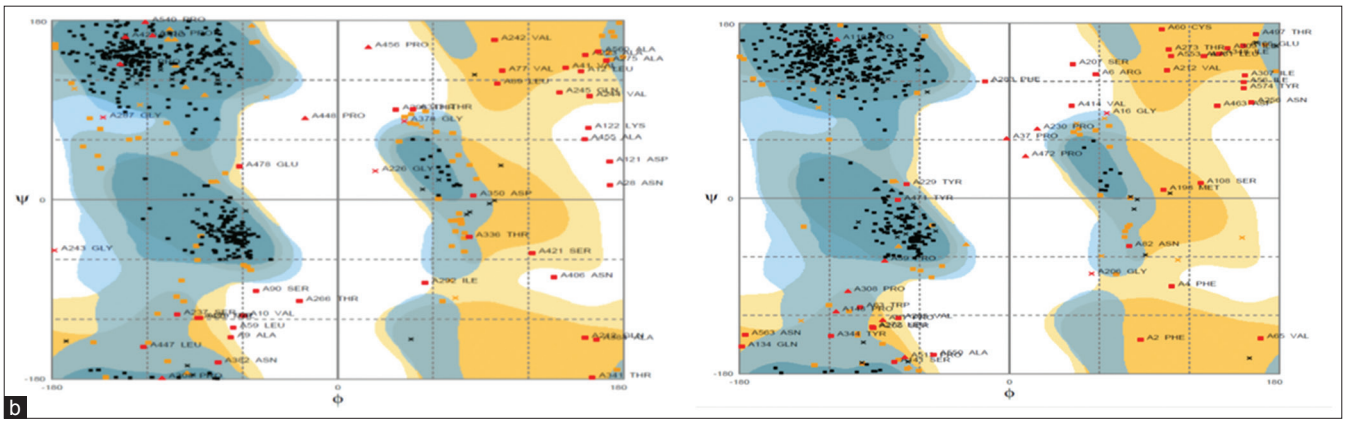

Figure S2: (b) MrpH of Proteus mirabilis among different strains obtained from GenBank and ExPASy tools, showing conservation of the proteins among the strains

GOR-IV to check the presence of alpha helix and beta plated sheets in the fusion structures [Figure 2]. Our results showed that when the secondary structure of the fusion proteins was compared with the 2D structure of the single proteins FimH or MrpH, no significant changes were observed.

Furthermore, the study of protein secondary structure plays an important role in the prediction of protein —3D structure with the *ab initio* method or protein fold recognition by providing additional constraints.[31]

The predicted 3D structure will provide more insight in understanding the structure and function of the proteins. Moreover, this structure can be used for drug development or understanding the interaction between proteins.[31]

As a part of the present study, *ab initio* methods were used for predicting the 3D structure of the fusion proteins. As shown in Figure 3a and b, our results showed that *ab -initio* I-TASSER software could predict the folds as well as provide a good resolution model for the fusion proteins.

Assessment of the accuracy and reliability of experimental and theoretical models of protein structures is necessary. For the evaluation of the predicted models, ProSA-web (Z-score) was used. The Z-score from ProSA-web indicates overall model quality and measures the deviation of the total energy of the structure with respect to an energy distribution derived from random conformations.[23] Z-scores outside a range characteristic for native proteins indicate erroneous structures. As shown in Figure 3c and d, protein structure analysis indicated that fusion MrpH.FimH formed a 3D structure similar to the native protein with a Z-score in the range of native conformations. Our fusion structures showed a desirable protein stability based on Ramachandran plot predictions. In Ramachandran plot analysis, a negligible 2.8% of the residues were found to be in the outlier region, which could probably be due to the presence of chimeric junctions.

The studies demonstrated that FimH adhesin binds specifically to TLR4 present on immune cells and on the surface of uroepithelial cells.[32,33] To find the best fusion form that had highest affinity to TLR4, we studied the interaction of the fusion proteins with TLR4 using the Hex docking server. According to our docking results, MrpH.FimH showed the best interaction tendency to the receptor [Table 2]. After the expression of fusion protein MrpH.FimH, we showed that the purified fusion protein is active and capable of inducing immune response and the secretion of the pro-inflammatory cytokine IL-8 [Figure 4]. These results suggest that fusion MrpH.FimH retains the necessary characteristics to induce immune responses against UTIs.

CONCLUSION

In conclusion, our data indicate that between these two fusion proteins predicted by *in silico* methods, MrpH.FimH can be considered as a vaccine candidate against the UTIs caused by UPEC and *P. mirabilis*. *In vitro* and *in vivo* studies concerning the immunogenicity and protection of the fusion protein against UTIs are in progress.

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