*Original Article*

# Cloning and expression of PMI1945 involved in iron acquisition as a promising vaccine candidate against *Proteus mirabilis*

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## ABSTRACT

**Introduction:** *Proteus mirabilis* is one of the common pathogens of urinary tract infections. Iron scavenger receptors from *P. mirabilis* are considered as important virulence factors of this strain and have the properties of an ideal vaccine candidate. In this study, the frequency of *P. mirabilis* iron receptor 1945 (PMI1945) was evaluated in the isolates and then its expression was conducted in pET28a-BL21. **Methods:** Amplification of *PMI1945* was performed by PCR using *P. mirabilis* isolates genomic DNA. Cloning of *PMI1945* gene was done in pET28a-BL21 system. After transformation, the expression of the cloned gene was induced by IPTG. The expression of this protein was then evaluated by SDS-PAGE and Western blot techniques. **Results:** The frequency of *PMI1945* gene in the isolates was 76%. The cloning of *PMI1945* gene into pET28a vector was confirmed by electrophoresis, PCR, enzyme digestion and sequencing. The sequencing of the cloned gene showed 100% identity with other sequences of *PMI1945* gene in GenBank. SDS-PAGE and Western blot results showed that 77 kDa PMI1945 protein was successfully expressed in BL21 (DE3) host. **Conclusion:** Cloning and expression of PMI1945 was done as the first step for evaluation of a novel vaccine candidate against UTIs caused by P. mirabilis.

**KEYWORDS:** *Proteus mirabilis*, Iron scavenger receptors, PMI1945, Cloning, Expression.

## INTRODUCTION

Urinary tract infection (UTI) is one of the most common infections in the world [1] that may lead to bladder (cystitis) and kidney (pyelonephritis) infections [2]. UTIs account for approximately 7 million clinic visits annually in the United States [3] and it is estimated that around 40% of women may experience at least one UTI in their lifetimes [4]. *Proteus mirabilis* is considered as one of the main causes of UTI, especially in people with defect in their urinary tract [5]. *P. mirabilis* strains use a variety of virulence factors for pathogenesis in the urinary tract such as fimbriae, urease, iron adsorption systems and toxins. Among them, iron uptake mechanisms can be considered as one of the most effective ways to affect the urinary tract [6]. Common antibiotics are often used for the treatment of UTIs which have resulted in antibiotic resistance against the bacterium. Thus, designing an efficient vaccine against UTIs can play an important role in decreasing the antibiotic resistance and reducing the morbidity rate among the patients [7, 8]. In spite of efforts to design a functional prophylactic, there is still no effective vaccine against UTI; therefore, more researches in this field to find new antigens and adjuvants are required [9].

Despite the lack of iron reservoirs in the urinary tract of human, UTI pathogens need to encode several iron receptor genes in order to adsorb iron from the environment [10]. The importance of these iron adsorption factors have been shown in the pathogenesis of *P. mirabilis* strains in various mutation studies, [10, 11]. PMI1945 is a putative TonB-dependent ferri-siderophore receptor that is involved in transportation of ferri-siderophores into the cytoplasm of *P. mirabilis* strains [12]. Because of the important role of PMI1945 and its presence on the surface of *P. mirabilis* strains as an outer membrane protein (OMP), several studies proposed this factor as a suitable candidate against UTIs caused by *P. mirabilis* [13, 12, 14].

Therefore, in the present study, the frequency of iron receptor gene *PMI1945* in hospital isolates of *P. mirabilis* was estimated and a selected sample was prepared for cloning and expression

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in pET28a-BL21 system which would finally be considered for evaluation as a potential vaccine in animal models.

## MATERIALS and METHODS

**Collection of the samples**

Totally, 50 *P. mirabilis* isolates were collected from people who had referred to Loghman Hospital in Tehran, Iran. The patients were informed about this project and were required to consent in writing. Confirmation of the isolates in our laboratory was performed by routine diagnostic tests for *P. mirabilis* including cultivation in differentiation medium as well as differential biochemical tests including fermentation of sugars, H2S production, motility, indole production, urea and citrate consumption, methyl red and voges-proskauer (MR-VP test) (Merck co, Darmstadt, Germany).

**Purification and PCR amplification**

The genomic DNA of the bacterial isolates were extracted and purified using phenol and chloroform method. PCR amplification of the *PMI1945* gene was performed by forward (5′- CGCGGATCCATGAAATTTAAAATAAGTGC- 3′) and reverse (5′- CCGCTCGAGAAAACTATATTTGATATTTGC- 3′) primers designed to introduce *BamH*I and *Xho*I sites at the conserved 5′ and 3′ ends of the gene. PCR mixture 2.5 mM $MgCl_2$, 50 pmol of each primer and 0.5 U *Pfu* polymerase (Fermentas, Lithuania). The PCR program for the amplification was initial denaturation for 5 min at 94˚C, and then 30 cycles of 1 min at 94˚C (denaturation), 1 min at 57˚C (annealing temperature) and a final extension at 72˚C for 5 min, using an Eppendorf thermocycler. After amplification, 5 µl of the samples were subjected to electrophoresis on 1% agarose gel to confirm the presence of the amplicon.

**Preparation of BL21 (DE3) competent cells**

Totally, 5 ml of overnight culture of BL21 (DE3) was added to 50 ml of Luria Bertani (LB) broth medium and cultured to reach the O.D 600 nm and incubated on ice. Then, the culture was centrifuged in a sterile tube. After re-suspension in 100 mM ice-cold $CaCl_2$ and vortex, the cells were centrifuged at 5000 rpm for 10 min. The supernatant was removed and the pellet was suspended in ice-cold $CaCl_2$ containing glycerol 20%. Then, the mixture was divided in sterile micro-tubes and stored at -80°C for further use.

**Ligation of the *PMI1945* gene into the pET28a vector**

The PCR products were gel-purified (Gel purification kit, Fermentas) and digested with *BamH*I and *Xho*I restriction enzymes (Fermentas, Lithuania), according to the protocol of the manufactures. The digested products were cloned into the *BamH*I and *Xho*I sites of the digested pET28a expression vector (Novagen, USA) to generate proteins with a His-tag at the C-terminal of the protein. The ligation mixture was included 50 ng of double digested vector, 37.5 ng of double digested insert, and 1 U of T4 ligase (Ferments, Lithuania) in a total volume of 20 µl.

**Transformation and expression of PMI1945 protein**

In order to express the PMI1945, the ligated plasmids were transformed into competent BL21 (DE3) cells (Novagen, USA). Briefly, ligated plasmids were added to the competent cells and after a heat shock at 42°C for 90 s and chilling on ice, this mixture was incubated at 37°C for 1 h. Then, different volumes (50, 100 and 200 µl) of the mixture were streaked on LB agar plates containing 100 µg/µl kanamycin and the plates were incubated overnight at 37°C. A random selection of the colonies were subjected to the plasmid extraction. The extracted plasmids were analyzed by gel electrophoresis, PCR, restriction analysis and sequencing. For the induction of protein, BL21 (DE3) cells containing confirmed recombinant plasmid were grown in LB medium containing kanamycin (5 µg/ml). On the following day, 500 ml of LB medium was inoculated with 5 ml of the overnight culture of BL21 (DE3) cells and the expression was induced by adding IPTG (Fermentas, Lithuania) in different concentrations (0.1 to 2 mM). After incubation for 3 to 24 h, the cells were harvested by centrifugation at 4°C and stored at -20°C till further use [15].

**SDS-PAGE and Western blot analyses**

The expressed protein was subjected to SDS-PAGE. The bacterial pellets were re-suspended in loading buffer containing loading dye, SDS 10X and 2ME, heated at 95°C and 30 µl of each sample was subjected to 12% SDS-PAGE gel. After electrophoresis of the samples, the protein bands were transferred to a nitrocellulose membrane (Schleicher and Schuell, Germany) using a liquid transfer system (Bio-Rad, USA). The membrane was blocked with skimmed milk (Merck, Germany) and incubated at 4ºC. After several washes, the membrane was incubated with the horseradish peroxidase (HRP) conjugated His–tag antibody (Sigma, USA). The membrane was washed and then the reaction was developed by the chromogenic substrate solution containing DAB (Invitrogen, USA) and H2O2 (Sigma, USA).

## RESULTS

**Amplification of *PMI1945* gene**

*P. mirabilis* isolated from the outpatients were confirmed by the routine diagnostic tests and their genomic DNA was extracted and used for PCR assay. The PCR results showed that *PMI1945* gene existed in 38 of the 50 isolated samples (76%). Electrophoresis of the amplicons showed that the length of PCR fragment of *PMI1945* gene was approximately 2 kbp (Fig. 1).

**Cloning of *PMI1945* gene**

One of the PCR product of *PMI1945* gene was selected, purified and digested with *BamH*I and *Xho*I restriction enzymes and then ligated into the pET28a vector. After transformation of the plasmid containing *PMI1945* gene into the pET28a vector, the first step in confirmation of the cloning was electrophoresis of the extracted plasmids from a selection of the clones on 1% agarose gel as shown in Fig. 2.

Then, a selection of the clones was amplified by PCR using the designed primers that the result is shown in Fig. 3.

The plasmid extracted from of a confirmed colony by electrophoresis and PCR was subjected to digestion with *BamH*I and *Xho*I restriction enzymes and the result is shown in Fig. 4.

Finally, this confirmed clone was sent for nucleotide sequencing (Genfanavaran, Iran). The sequence of the cloned gene with universal and specific primers showed that this gene sequence had 100% identity with *PMI1945* gene sequence deposited in the GenBank (Accession number: NC_010554.1), and no mutation or other firms of alterations were observed (data not shown).

**Confirmation of PMI1945 protein expression**

The expression of encoded protein by *PMI1945* coned in pET28a was optimized by parameters such as different concentrations of IPTG and incubation times. The expression of PMI1945 was analyzed using 15% SDS-PAGE with Coomassie blue staining and then by Western blotting using anti-His tag

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antibody (Sigma, USA). Fig. 5 shows the optimum expression condition, obtained with 1 mM IPTG and 6 h incubation time.
Furthermore, the final confirmation of the expressed protein using Western blot technique is shown in Fig. 6. As shown in this figure, the analysis of Western blot of the protein showed a single band at the size of approximately 77 kDa for PMI1945 that matches the theoretical size of of this protein in pET28a (DE3) system.

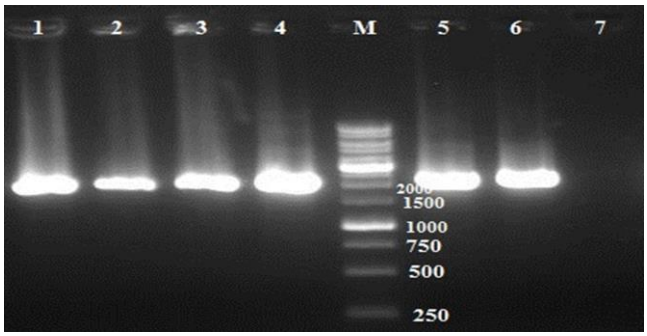



**Fig. 1.** A sample of *PMI1945* gene amplification results by PCR. Lanes 1 to 5 illustrate positive samples, lane 6 shows standard strain *P. mirabilis* HI4320 as a positive control and lane 7 indicates *E. coli* K12 strain as a negative control. The marker (M) is 1 kb ladder mix type (Fermentas, Lithuania).

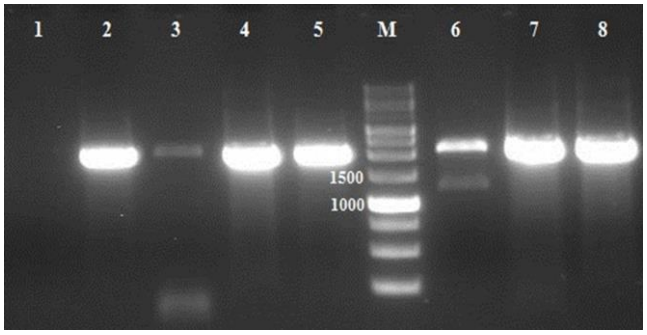



**Fig. 3.** Confirmation of the *PMI1945* gene cloning in pET28a vector. Lanes 2 to 8 are related to clones containing the relevant genes, and lane 1 has no gene. The marker (M) is 1kb ladder mix type as specified above.

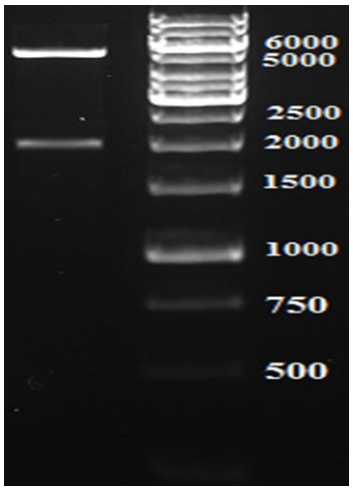



**Fig. 4.** Confirmation of the *PMI1945* gene cloning in pET28a vector by enzyme digestion. The marker is 1kb ladder mix type as specified above.

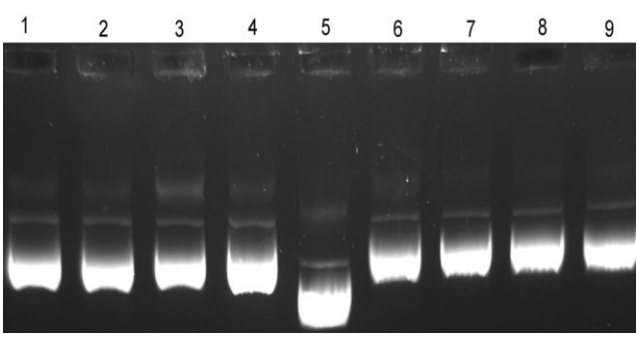



**Fig. 2.** The results of electrophoresis of clones on agarose gel. Lanes 1 to 4 and 6 to 9 illustrate positive clones containing the inserted gene, and lane 5 shows pET28a vector without an inserted gene.

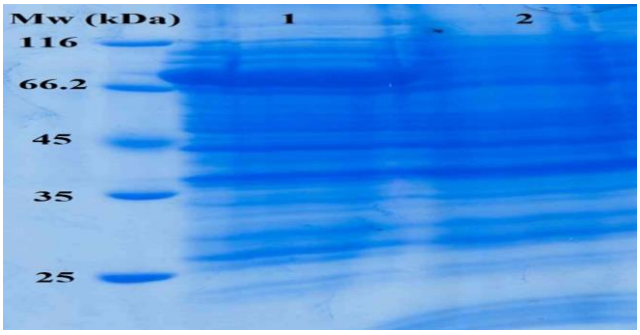



**Fig. 5.** The SDS-PAGE results of the expressed PMI1945 protein. Lane 1 shows the overexpressed protein in presence of 1 mM IPTG (6 h), and lane 2 represents uninduced clone (6h; negative control). MW is the protein marker (Fermentas, Lithuania).

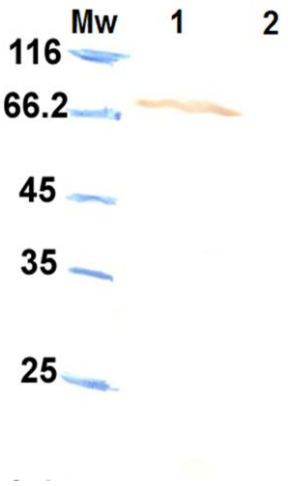



**Fig. 6.** The result of the Western blot assay. Lane 1 represents expressed protein in the presence of 1 mM IPTG 1 (6 h) and lane 2 represents an uninduced clone (6h; negative control). Mw is protein Marker in kDa.

## DISCUSSION

UTI is one of the most common infections diagnosed in patients in Iran and all over the world [16]. *P. mirabilis* have been among the most common pathogens found in the urinary tract [17, 5]. Furthermore, *P. mirabilis* plays a major role in complicated catheter-associated urinary tract infections (CAUTIs) [18]. The emergence of antibiotic resistance and ineffectiveness of available vaccines have complicated the situation [19]. Despite the high efforts to produce a vaccine against UTI, there is still no promising vaccine for preventing or treating UTIs [20, 4]. These issues point to more efforts that are required to find new approaches for the treatment or prevention of UTI.
In the past, candidates such as MrpH, MrpA, Uca, and Pta proteins from *P. mirabilis* were evaluated by other researches



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against UTIs [21-23, 9, 8, 24]. These targets were successfully designed, constructed and expressed in prokaryotic expression system, and some of them had the capability to be introduced as candidates for vaccine against UTIs caused by P. mirabilis. Few studies have been conducted on iron adsorption factors in *P. mirabilis* strains collected from UTIs [13]. In one of these studies, D'Alessandro et al. [14] used a proteomic method to evaluate in vivo expression of outer membrane proteins (OMPs) of *P. mirabilis* in iron-limited conditions and they concluded that PMI1945 was detected in high levels in *P. mirabilis* strains under iron-restricted conditions. Furthermore, Western blot analysis has also indicated that this protein is highly immunogenic during the infection [14]. In another study, Himpsel et al. [12] have also demonstrated that PMI1945 was up-regulated during iron limitation.

There is no general published study on the prevalence of iron adsorption factors in *P. mirabilis* collected from patients with UTI; while in this study the prevalence of iron adsorption receptor *PMI1945* (*IreA*) was evaluated in a population of *P. mirabilis* isolated from outpatients and our results indicated that the prevalence of *PMI1945* gene was high in *P. mirabilis* isolated from Iran. The presence of this gene with high prevalence among these isolates indicated that this factor may play a crucial role in the pathogenesis of *P. mirabilis* strains which may have been preserved during the evolution of this bacterium.

In this study, pET28a vector was used, which includes a strong T7 promoter for the expression of the genes and His tag for purifying the expressed proteins. This vector was also used because there is no need to the specific primers for the sequencing of cloned fragments. The inducer of this system is IPTG, and this vector, unlike the vectors such as pBADgIIIA does not have a sequence signal, resulting in the production of a protein in the cytoplasmic area. This can reduce the energy consumption to move the protein from the cytoplasmic space to the periplasmic space, which can be considered as a good factor for the expression of proteins in the pET28a system.

In conclusion, this study showed the high frequency of iron adsorption receptor; *PMI1945* in *P. mirabilis* isolated from outpatients and it was expressed as a recombinant candidate in prokaryotic expression system with high yield. To prove the effectiveness of this novel candidate as a vaccine against *P. mirabilis* strains, further studies such as evaluations of the purified protein in animal models are required which are currently underway.

## ACKNOWLEDGEMENT

We appreciate all who helped us to perform this study. This work was financially supported by Pasteur Institute of Iran.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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