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Cloning of PilF & PilQ genes of *Pseudomonas aeruginosa* in *Escherichia coli* and expression by real time PCR

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ABSTRACT

Pseudomonas aeruginosa has emerged as one of the most common causes of hospital-acquired infections among patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants, and intravenous-drug addiction, resulting in considerable annual mortality

rates. Because of biofilm formation and its ability of rapidly acquires of resistance to many antibiotics, *P. aeruginosa* related infections are difficult to treat, and therefore, developing an effective vaccine is the most promising method for combating infection. In the present study, we aimed to transfect PilF and PilQ genes, essential genes to participate in the assembly and regulation of the type-4 pilus system, into *E. coli* strain XL-1 blue. The results of the present study revealed that transfection of PilF and PilQ genes in the PTG-T19 plasmid and *E. coli* XL1-blue strain resulted in the elevation of the mRNA expression level of these genes, suggestive of the success of our transducing method. Moreover, we suggest that TA cloning is a rapid and efficient method for transfecting the aforementioned genes as compared to conventional cloning methods. We also suggested for the first time that due to the presence of protected areas, PilF and PilQ could be considered as a good candidate for developing the recombinant vaccines against infections caused by *Pseudomonas aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, PilF, PilQ, Recombinant vaccine, Antibiotic resistance.

1. INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*), an opportunistic gram-negative pathogen, is one of the most common causes of hospital-acquired infections in the lungs of those who are admitted to the burn, emergency care, and especially those with cystic fibrosis (CF) (Corona-Nakamura et al., 2001 and Gharajelar et al., 2012 and Chang et al., 2007 and Doosti et al., 2013 and Rosenthal, 1968 and Giltner et al., 2006). By producing a variety of virulence and virulence-associated factors, *P. aeruginosa* vast its infection in patients, cause severe secondary infections and notably resists to diverse type of anti-biotics. Given to its multi-drug-resistant characteristics, Pseudomonas infections are difficult to treat and thereby could be considered as a major cause of the death in the hospitalized patients (Giltner et al., 2006). According to these, it is not surprising that tremendous efforts are now on the way to develop and to thrive promising therapeutic approaches for this infection.

Apart from the main biological roles, the cell-associated flagella and pili (Fimbria), important lipopolysaccharide surface components of *P. aeruginosa*, facilitate attachment of the organism to the epithelium of host cells during the infection (Giltner et al., 2006 and Feldman et al., 1998 and Lillehoj et al., 2002). Among different types of pilus on the surface of this pathogen, the type-4 pilus which is the most prevalence pilus identified from different sources, is account for the development of diversity in the genomic structure of bacteria (Dabiri et al., 2014 and Persat et al., 2015). It has been reported that four subspecies of the genes throughout the genome participate in the assembly and regulation of the type-4 pilus system in *P. aeruginosa*, which among them, PilQ and PilF are the most essential ones (Persat et al., 2015). Mounting body of evidence outlined that the assembly of the outer membrane PilQ secretin channel, with the molecular weight of 77kDa, through which the pilus is extruded is the critical step for the biogenesis of pilus. Moreover, PilQ pilotin protein PilF, an outer membrane lipoprotein, plays a major role in the biogenesis of the type-4 pilus through accelerating the entrance and multi-merization of PilQ in the outer membrane (Leighton et al., 2015 and Koo et al., 2013). Based on the tight corporation between PilQ and PilF in the biogenesis of the type-4 pilus, it is reasonable to assume that probably these genes could be considered as an appropriate candidate for the development of an effective recombinant vaccine for the treatment of pseudomonas infections that may be clinically accessible in the near future. In this study, we aimed to clone PilF & PilQ genes of *P. aeruginosa* in *Escherichia coli* and to investigate their expression by real time PCR.

2. MATERIALS AND METHODS

Bacterial strains and culture method

This study was conducted at Pasargad Research Lab (Tehran, Iran) in 60 clinical *P. aeruginosa* strains were collected from 95 patients with burn injuries in Kerman Hospital. Identification of the bacteria was done based on general phenotypic methods including colony pigmentation, Gram staining, oxidase test, catalase test, fermentation of lactose and glucose in TSI medium, mobility on the SIM environment, indole, citrate test, MR/VP and the growth at 42˚C and growth on cetrimide agar (Ansari et al., 2017). For further experiments, the bacterial specimens were inseminated in BHI Broth (Merck Germany), containing 20% glycerol, and kept at -70 ° C. *E. coli* XL-1blue strain was used as cloning and expression host and linear vector of pTG19 synthesized by Biomatik Corporation (Cambridge, Ont., Canada).

Reagents and growth media

All enzymes for DNA manipulations were obtained from NEB (USA). The anti-His (C-Term)-HRP monoclonal antibody was obtained from Invitrogen (USA). Ni2+-NTA agarose was purchased from Qiagen (USA). The strains were cultured in LB broth or on agar (Merck, Germany) at 37 °C with or without 30 µg kanamycin/ml (Bioscience, Canada) (Cunliffe et al., 1995).

DNA extraction and PCR

Total DNA from *Pseudomonas aeruginosa* specimens was extracted using a DNA extraction kit (Genetic Reserve Center, Iran) according to the manufacturer's recommendation. The quantity of DNA samples was assessed spectrophotometrically using Nanodrop ND-1000 (Thermo Scientific, USA) at a wavelength of 260 nm. Moreover, to verify the purification of DNA, the absorption at 280/250 nm was measured. To design the primer, the nucleotide sequences of the PilQ and PilF genes were first obtained from the NCBI site. Oligonucleotide primers were then designed by the Oligo7 program and then were synthesis by Macrogene. The sequences of the primers used for PCR upstream region of pilF was amplified from genomic DNA using the primers Put_for1 (EcoRI) (ATACGAATTCTAGGGAGGAGCGGATCTAC) and Put_rev1 (AAGCATAAGCGGCCGCAAATTTAAACCATGGTCATCTCGTCACACCC) and the 545-bp downstream region was amplified using the primers PilQ_for1 (PstI) (AATACTGCAGAGGAGGAAAGCGATGCCCAAAG) and PilQ_rev (HindIII) (CCGTAAGCTTCTTGTGGAAGAACTCTATGGG) that C-terminal hexahistidine tag-fused PilQ fragment into the PstI and HindIII sites of the pTG19 vector (Invitrogen, San Diego, CA) (Hackbarth and Hodges, 2010).Thermal cycling conditions included an initial activation step for 3 minutes at 95°C followed by a denaturation step at 54 ° C for 30 seconds, a combined annealing/extension step for 1 minute at 72°C and final expansion at 72 ° C for 10 minutes. Afterwards, the PCR product was analyzed using 1% electrophoresis agarose gel. The genes are purified using a gel purification kit (Sina Clone, Iran) according to the manufacturer's protocol.

Analysis of gene expression by quantitative real-time PCR

The cloning of PilQ and PilF genes, TA Cloning kit (Sina Clone, Iran) was used and tests were performed according to the kit's instructions. For digestion of DNA from recombinant clones, a Sina Clone (Miniprep) plasmid extraction kit (EX6111) was used to assure the quality of cleaved plasmid DNA. After separating the DNA of plasmid, the standard promoter, such as M13 becomes sequenced using a sequencing primer (Bioneer, Germany).

The real time PCR method was used to investigate the mRNA expression level of two *Pseudomonas aeruginosa* virulence genes, PilQ and PilF in *E. coli* strain XL-1blue. For this purpose, PCR assay was performed in an ultimate volume of 20 µl of reaction mixture containing 10 µl of SYBR Green master mix, 2 µl of cDNA product, 0.5 µl of each forward and reverse primer (10 pmol) and 7 µl of nuclease-free water. Thermal cycling conditions were an initial activation step of 30 s at 95°C followed by 40 cycles including a denaturation step of 15 s at 95°C and a combined annealing/extension step of60 s at 60°C. Melting curves were analyzed to validate single PCR product of each primer, and the values for the relative quantification were calculated based on $2^{-\Delta\Delta ct}$ relative expression formula.

Statistical analysis

Data are expressed as the mean ± SD of three independent experiments. All tests were performed in triplicate. The significance of differences between experimental variables was determined by the use of two-tailed Student's t-test and by one-way variance analysis. Statistically different values were defined as significant at *P* value less than 0.05.

3. RESULTS

Identification of Pseudomonas aeruginosa and DNA extraction

Through Gram staining, colony pigmentation and differential biochemical tests, PAO1 strains of *Pseudomonas aeruginosa* were identified. Then, the DNA was extracted from the specimens and the quality and quantity of DNA extracted from the strain PAO1 of *Pseudomonas aeruginosa* was evaluated. The concentration of DNA extracted was determined to be 200 µg/ml. The absorption was also measured at 260/280nm, 1.71 wavelengths. As presented in Figure 1, the results of electrophoretic PCR production of PilF and PilQ genes from 60 strains of *Pseudomonas aeruginosa* showed 1203bp and 579 bp for PilF and PilQ genes, respectively. Our results also delineated that among 60 *Pseudomonas aeruginosa* strains, only 3% of bacteria did not expressed PilF and PilQ genes, introducing these genes a good candidate for development of an effective recombinant vaccine against infections caused by *Pseudomonas aeruginosa* (Figure 2).

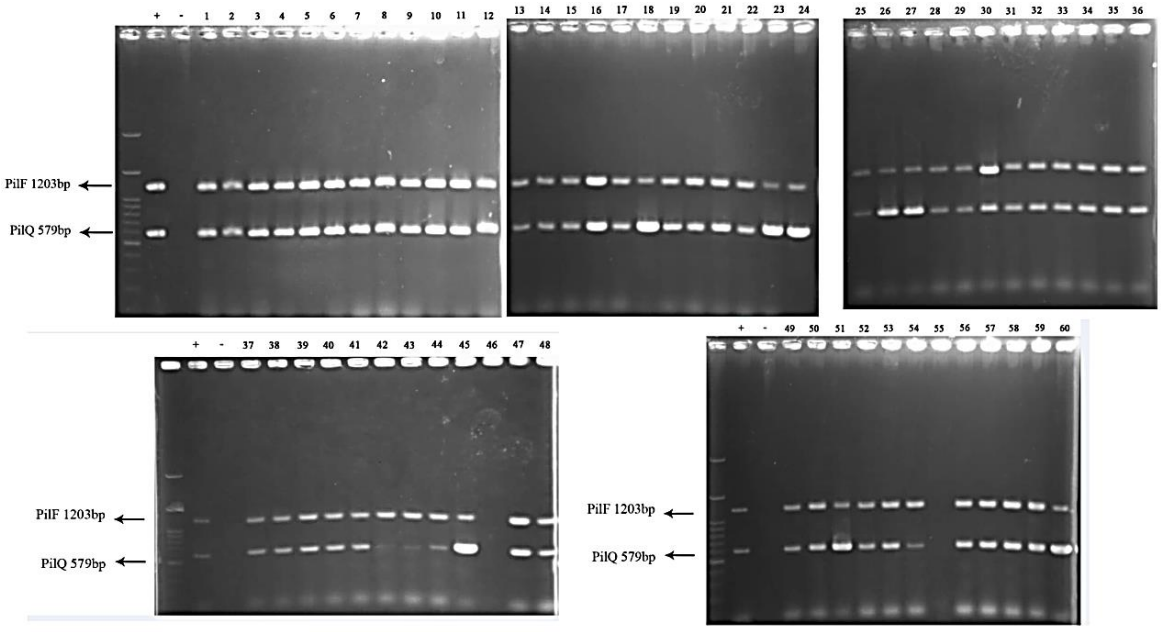

Figure 1 The results of electrophoretic PCR production of PilF and PilQ genes from 60 strains of *Pseudomonas aeruginosa* showed 1203bp and 579 bp for PilF and PilQ genes.

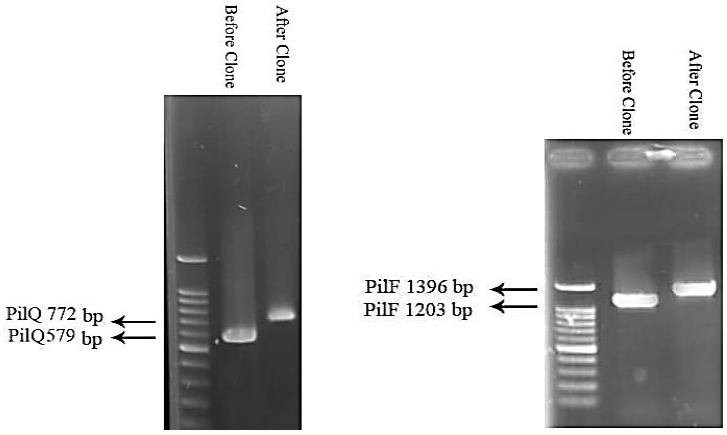

Figure 2 Agarose gel electrophoresis analysis of recombinant pTG19 with restriction enzyme digestion; Quadruple digested recombinant vector with BamHI and XhoI buffers, respectively Amplification of PilQ and PilF genes from 60 *Pseudomonas aeruginosa* strains. As depicted, both PilF and PilQ is expressed in almost 97% of the strains.

PilF and PilQ cloning

The transfection of PilF and PilQ genes was performed using the TA cloning kit according to the manufacturer's instructions. The recombinant vector successfully transferred in *E. coli* strain XL-1 blue. At this stage, the cells were cultured into a plate containing antibiotics, including ampicillin, X-gal and IPTG. Synthesis of recombinant plasmid in *Escherichia coli* XL-1 blue strain was successfully performed. As presented in Figure 3, the cultured bacteria are grown in blue and white colonies. It is worth to mention that the blue colonies represent the absence of the plasmid containing the desired gene fragment, and the white colonies represent the presence of plasmid with the desired fragments. The DNA extract from the recombinant clones was purified using plasmid extraction kit (Sina Clone, Iran) and sequenced using sequencing primers M13.

Evaluating the mRNA expression level of PilF and PilQ using RQ-PCR

To evaluate whether the transfection of PilF and PilQ genes into *Escherichia coli* XL-1 blue strain was associated with the expression aforementioned genes, the mRNA level of both PilF and PilQ was analyzed by applying RQ-PCR using vivantis kits. NTC without cDNA samples confirmed the absence of DNA contamination in all specimens. The amount of CT in all samples were similar,

suggesting that there was no difference in the amount of nucleic acids at the beginning. After transfecting cells with the plasmid, we found that there is different in the mRNA expression level of PilF and PilQ in transfected and un-transfected groups. This finding suggested that the expression level of both PilF and PilQ increased in transfected group in comparison with un-transfected one, suggestive of the success of plasmid transfection (Figure 4).

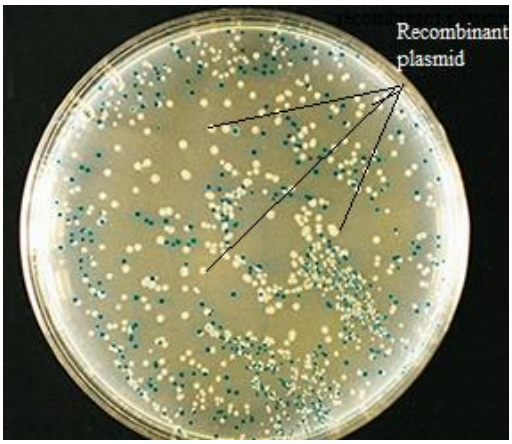

Figure 3 Transfection of recombinant vector in *E.Coli* XL-1 blue strain. The white colonies are representative of the presence of the recombinant plasmid and the blue ones are representative of the absence of recombinant plasmid.

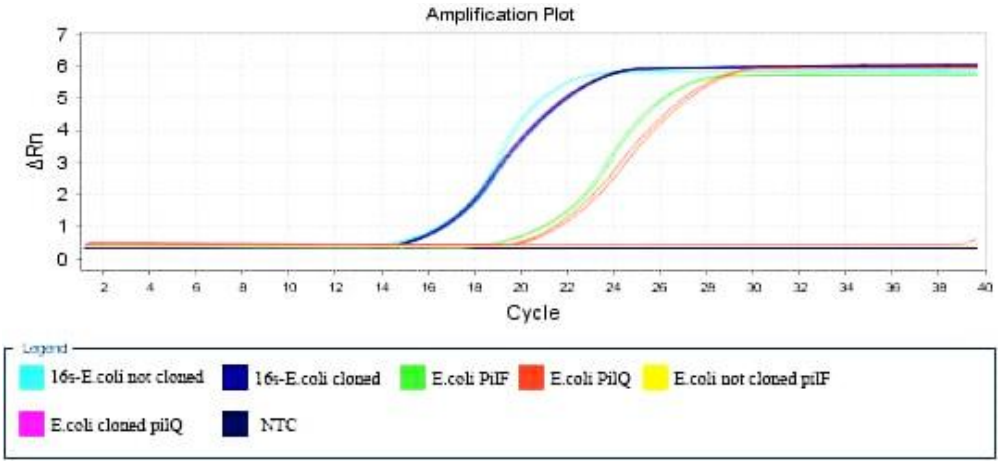

Figure 4 Amplification curve for investigating the expression level of PilF and PilQ genes in *Pseudomonas aeruginosa* strains using Real Time PCR. The amount of CT of the PilF and PilQ genes is different in transfected and un-transfected groups. However, in transfected groups, there is no significant difference in the amount CT of PilF and PilQ genes.

4. DISCUSSION

Exposing to the bacterial infections either through the contaminated environmental recourses or infected people, every year, compelling number of people in the world become infected by *Pseudomonas aeruginosa* and this pathogen still remains a threat to human health (Zeraik and Nitschke, 2012). Despite dramatic improvements in the anti-microbial treatment strategies over the past decades, *Pseudomonas aeruginosa* infections still remains one of the leading causes of person-years of life lost worldwide (18-61%) (Langton and Smyth, 2014). Given to the natural tendency of the pathogen for the drug resistance, recently World health organization (WHO) has classified *Pseudomonas aeruginosa* as a chief priority with critical urgency for developing novel, as well as, effective treatments (Rice, 2006). In the era of vaccinology approaches, substantial efforts are being made to identify the prospective candidates for evolving a potent and cost-effective vaccine with strong activity against *Pseudomonas aeruginosa* infection in order to improve cure rate, especially in developed countries. However, due to the contribution of several virulence factors in the

pathogenesis of *Pseudomonas aeruginosa*, exploiting an appropriate candidate turned into a huge obstacle for vaccine development (Organization, 2017). Recently, mounting body of evidence has declared a critical role for pilus not only in the pathogenesis of *Pseudomonas aeruginosa*, but also in the acquisition of the drug-resistance phenotype; thereby, introducing it as a promising target for vaccine development (Koo et al., 2013 and Rashid et al., 2017). Among different types of pilus, type-4 pilus, encoded by PilQ and PilF, plays a fundamental role in both the pathogenesis and the attachment of bacteria (Hogan and Kolter, 2002 and Hahn, 1997). In the present study, we cloned these aforementioned genes into the prokaryotic cells and investigate the effect of their expression, proposing them as a suitable step for the preparation of a recombinant vaccine.

Herein, for the first time in Iran, we used PilQ and PilF genes for cloning in a PTG-T192 vector and the expression of these genes were investigated in *Escherichia coli* strain named XL-1blue. In 2013, the functional map of PilF and PilQ were designed in Canada to determine the characteristics role of these two structural component genes (Koo et al., 2013). Moreover, the results of the mutation of the PilQ gene indicate that although the specific domain is not necessary for multi-merization, it could facilitate the formation of the type-4 pilus (Bitter et al., 1998).

In this study, a fast and efficient cloning method of PCR products with 3dA endpoints from the linear PTG-19 vector with a 3-dT endpoint has been used, which results in a high percentage of recombinant clones (Faezi et al., 2017 and Yoon and Leitner, 2014 and Barnini et al., 2004). In comparison with other vectors, the existence of the M13 primer site for TA cloning, screening and sequencing make our vector more effective and beneficial. In additional, the presence of LacZ gene allows this vector to select the blue/white colonies and the BamH1 limiting enzyme to release the insert fragment. Since cloning TA has been removed in the enzyme digestion process, it has the benefits of cloning the PCR product and is able to reproduce the PCR product with high performance by using the thermophilic DNA polymerase transferase, such as the enzyme taq polymerase (Koo et al., 2013). This method requires the T4 DNA ligase to attach to the complementary DNA fragment and the linear plasmid to form a simple ring molecule capable of replicating in the *E. coli*. The TA cloning technique can be also used for cancer treatment through propagation and cloning of the gene in an expression vector for the production of a recombinant protein (Barnini et al., 2004).

Overall, our results showed that the target expression construct was well and significantly expressed in the target strains, and this is important for new vaccines. Therefore, the target genes can be cloned by this method and considered as a candidate for P. aeruginosa vaccine after immunization study.

5. CONCLUSION

The results of the present study revealed that cloning and expression of PilF and PilQ genes in the PTG-T19 plasmid and *E. coli* XL1-blue strain have been successfully performed. In addition, TA cloning is a rapid and efficient method for transfecting the aforementioned genes as compared to conventional cloning methods. We also suggested for the first time that due to the presence of protected areas, PilF and PilQ could be considered as suitable genes for developing the recombinant vaccines against infections caused by *Pseudomonas aeruginosa*. Because *P. aeruginosa* has posed therapeutic challenges worldwide as some strains have become resistant to nearly all front-line antibiotics, our results provided the basis for an alternative strategy for the prevention of *P. aeruginosa* infection by immunization.

List of abbreviations

PCR: Polymerase Chain Reaction
P. aeruginosa: *Pseudomonas aeruginosa*
E. coli: *Escherichia coli*
TA *cloning:* Known as rapid *cloning* or T *cloning*
CF: Cystic fibrosis
DNA: deoxyribonucleic acid
BHI Broth: Brain-Heart Infusion Broth

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Conflicts of Interest:
The authors declare no conflict of interest.

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