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ORIGINAL ARTICLE

Cloning, expression and purification of outer membrane secretin $PilQ_{406-770}$ of *Neisseria meningitidis* serogroup B

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ABSTRACT

Background: *Neisseria meningitidis* is a major cause of bacterial septicemia and meningitis. Currently, there are no vaccines to prevent disease caused by strains of *N. meningitidis* serogroup B, since cross-reactivity of the serogroup B capsule with human tissue has hampered efforts to develop a reliable vaccine. PilQ is an antigenically conserved outer membrane protein which is essential for meningococcal pilus expression at the cell surface.

Materials and methods: In the current study, we selected a 1095bp fragment of C-terminal of secretin *pilQ* and evaluated the immunogenicity of this recombinant fragment. This fragment was amplified by PCR from genomic DNA isolated from *N. meningitidis* serogroup B and cloned into the pET-28a expression vector. PilQ406-770 was over-expressed with IPTG and then affinity-purified by Ni2+-Sepharose resin. The recombinant PilQ406-770 was reacted with rabbit anti-*N. meningitidis* polyclonal antibody in western-blot analysis. Mice were immunized subcutaneously with purified $rPilQ_{406-770}$ mixed with an equal volume of Freund's adjuvant and evaluated specific serum antibody responses.

Results: Our results show $pilQ_{406-770}$ cloned in pET28a vector, while the cloning of $pilQ_{406-770}$ was confirmed by colony-PCR and enzymatic digestion. SDS-PAGE analysis showed that our constructed prokaryotic expression system pET28a-pilQ406-770-BL21efficiently produces target recombinant protein with molecular weight of 43 kDa in the form of dissoluble inclusion body.

Conclusion: Our results confirmed that a prokaryotic expression system for PilQ406-770 protein was successfully constructed.

Keywords: Cloning, E*xpression, Neisseria meningitidis, $PilQ_{406-770}$, Recombinant protein*.
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INTRODUCTION

Neisseria meningitidis is a major cause of bacterial meningitis and septicemia and remains an important public health problem (1). Currently, there are no vaccines to prevent disease caused by strains of *N. meningitidis* serogroup B, because cross-reactivity of the serogroup B capsule with human tissue has hampered efforts to develop a reliable vaccine (2,3). Therefore, non-capsular antigens such as outer membrane proteins are being evaluated as candidates for vaccine (4). Among the many virulence factors of *N. meningitidis,* type IV pili are most important in the early stages of infection of human hosts (5). Initial interaction of

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N. meningitidis with host cells is thought to be mediated by type IV pili (6).

Type IV pili are involved in a variety of bacterial processes; in addition to their role in host cell attachment, pili are also involved in bacterial auto-agglutination, twitching motility, biofilm formation, bacteriophage infection and natural competence for DNA uptake (7). The biogenesis of type IV pili requires the complex interactions of 15 proteins. The outer membrane secretin PilQ plays an essential role in these processes, mediating pilus translocation across the outer membrane (8). Neisserial PilQ-null mutants are not piliated and are non-competent for natural transformation. Structural analysis suggest the 82 kDa PilQ monomers are assembled into a symmetrical dodecamer, which has a large central cavity closed at the poles by 'plug' and 'cap' structures (9). PilQ is a member of the GspD secretin superfamily and is unique among them because of its abundance in the outer membrane and the presence in its N-terminal domain of four to seven copies of an octapeptide, PAKQQAAA, termed small basic repeats (SBRs) (10). The C terminal of protein which is conserved between members of secretin superfamily and is responsible for secretin oligomer formation has been predicted to contain 13 β-strands, which could be embedded in the outer membrane (11).

Immunization with PilQ complex has been shown to elicit bactericidal antibodies and protect mice against experimental infection (12). Although PilQ is quite abundant on meningococcal cells, recent studies have shown that OMVs produced from a strain in which the *pilQ* gene was up-regulated induced higher anti-PilQ antibody titers in mice (13). PilQ is considered a reliable candidate antigen as a part of multi-component recombinant protein vaccines because it has a relatively conserved sequence, it is present on most meningococci and it is abundant on cell surface (9).

Since epitope-based vaccines represent a new strategy for eliciting a specific immune response against the selected epitopes, in the present study we choose a conserved C-terminal fragment of *pilQ* gene ($pilQ_{406-770}$) and a prokaryotic high-level expression system for protein production was successfully established.

MATERIALS and METHODS

Bacterial strains and vector: The *N. meningitidis* serogroup B strain CSBPI, G-245 was prepared from Pasteur Institute of Iran. *E. coli* strains DH5α and BL21 were obtained from Invitrogen and Novagen (USA), respectively. Plasmid pET-28a as expression vector was provided from Novagen. Bacteria were cultured in LB broth or on agar (Merck, Germany) with or without 30μg kanamycin/ml (Sigma, USA).

Preparation of DNA template and PCR: Genomic DNA of *N. meningitides* serogroup B strain CSBPI, G245 was extracted by routine phenol-chloroform method and then was solved in TE buffer; concentration and purity of extracted DNA were determined by spectrophotometry. The specific primers were designed according to pilQ sequences of *N. meningitidis* from NCBI. The sequence of forward primer with an endonuclease site of *Sac I* and reverse primer with an endonuclease site of *Hind III* were 5'-TTCGAGCTCATGCGCCAGCAAGGGAATATC GTCAAC-3' and 5'-CAGCAAGCTTTCAAT AGCGCAGGCTGTTGCCGGC-3', respectively.

For amplification, the reaction mixture contained: 0.5μM of each primer, 10μl 5X prime STAR buffer, 0.2mM concentration of each dNTP, 2.5U of prime STAR DNA polymerase (Takara, Japan) and 200ng genomic DNA in a final volume of 50μl. PCR amplification was performed with an initial denaturation at 98°C for 4 min, followed by 35 cycles of 98°C for 10 sec, 63°C for 15 sec and 72°C for 90 sec, and 10 min at 72°C for final extension. PCR products were analyzed by electrophoresis on 1 % (w/v) agarose gel (Fermentas), and then the 1095bp DNA fragments

were recovered from the gel using PCR purification kit (Bioneer, Korea).

Cloning, Expression and Purification of PilQ$_{406-770}$: After enzymatic digestion of purified pilQ$_{406-770}$ fragment and pET-28a (Novagen, United States) expression vector with responsible endonucleases, ligation reaction was performed between insert and vector using T4 DNA ligase (Fermentas). The products of the ligation reaction were used to transform *E. coli* strain DH5α. The integrity of the recovered plasmid was confirmed by restriction endonuclease digestion and sequencing of the pilQ$_{406-770}$ insert by a commercial facility using universal forward and reverse T7-promoter and T7-terminator primers (TAG Copenhage A/S Symbion, Denmark).

The cells of competent *E. coli* strain BL21 were transformed with recombinant pET28a-pilQ$_{406-770}$ in the presence of kanamycin (30µg/ml). Bacterial cells were grown in the presence of kanamycin at 37ºC with shaking (250 rpm) to an A650 of 0.7. Expression was induced by the addition of IPTG to a final concentration of 1mM, and the cells were incubated for a further 2.5h before being harvested.

Cells were harvested by centrifugation, resuspended in 50ml lysis buffer (1% Triton X100, 20mM Tris–HCl, 10mM EDTA, pH 7.5) and frozen at -20° C. Frozen bacterial pellets were lysed by sonication in the presence of PMSF (1mM) as a protease inhibitor until the solution cleared. After centrifugation, supernatant and precipitate were examined by SDS-PAGE to verify the location of expressed recombinant protein.

rPilQ$_{406-770}$ was purified by Ni-NTA affinity chromatography under combination of denature and native conditions by binding, washing and eluting steps according to manufacture protocol (Qiagen) with some modifications.

The eluted proteins were immediately dialyzed against PBS, pH 7.4 to remove imidazole. Protein concentrations were determined by Bradford analysis and the purity was determined by SDS-PAGE and Coomassie blue staining.

Anti PilQ$_{406-770}$ polyclonal antibodies production: To produce *N. meningitidis* anti PilQ$_{406-770}$ polyclonal antibody, adult females New Zealand white rabbits (Pasteur research institute, Tehran, Iran) were immunized subcutaneously with approximately 1mg of *N. meningitidis* PilQ$_{406-770}$ protein in Freund's incomplete adjuvant (Sigma). Booster doses were also given on days 14 and 28 in Freund's incomplete adjuvant. 10 days after the last immunization animals were exsanguinated and the serum was separated and stored at -20°C until required for use.

Western blot analysis: The separated proteins by SDS-PAGE transferred to 0.45µm pore size PVDF membrane (Hi-bond Amersham Biosciences, USA) by using a semidry blotter unit (Labconco, Kansas City, Mo.). The membrane was blocked by 1% (w/v) skim milk according to standard procedures. The native immune serum was diluted to 1:1,000 in phosphate-buffered saline (PBS)-0.1% (v/v) Tween 20 and incubated 3h at 4°C with shaking. Block membranes were washed with PBS-Tween 20 and then incubated with affinity purified goat anti-rabbit immunoglobulin G (heavy and light chain) horseradish peroxidase (HRP) conjugate antibody (Bio-Rad), at a 1:2,500 dilution in PBS-Tween 20.

Membranes were then washed three times with PBS-Tween 20 and development using DAB solution (Sigma, USA).

Immunization: Groups (six mice each) of five-week-old female mice (BALB/c) were immunized subcutaneously with 10µg of purified PilQ$_{406-770}$ mixed with an equal volume of Freund's adjuvant (complete FA for the first dose and incomplete FA for subsequent doses) at weeks 0, 2 and 4. Control groups consisted of either unimmunized (naive) mice or animals receiving Freund's adjuvant alone. Sera were collected at weeks 0, 2, 4, and 5 to determine the antibody responses. Aliquots of serum were stored at -70°C for assay.

Determination of serum antibody levels to purified $PilQ_{406-770}$: Antibody titers (total IgG) against $PilQ_{406-770}$ were determined by ELISA as previously described (14). The wells of a microtiter plate were coated with 1µg/100µl of $PilQ_{406-770}$ in PBS and incubated overnight at 37°C. The coated plates were first blocked with 5% (wt/vol) non-fat milk in PBS and then incubated with antisera (1:500 diluted in PBS/BSA). After incubation (2h at room temperature), the plates were washed and rabbit anti-mouse immunoglobulin G (IgG)–peroxidase conjugate (1:7,000 diluted in PBST/BSA) was added. After 2h at room temperature, TMB substrate was added, and the absorbance at 405nm was measured after 30 min.

RESULTS

DNA extraction and PCR amplification for $pilQ_{406-770}$: Genomic DNA of *N. meningitidis* serogroup B strain CSBPI, G245 extracted by routine phenol-chloroform method. The 1095-bp DNA fragment amplified from genomic DNA is shown in figure 1.

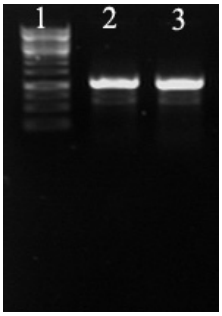

Figure 1. Electrophoresis of $PilQ_{406-770}$ gene amplified from *N. meningitidis* on agarose gel (1% w/v). *Lane 1*: 1kb DNA size marker; *Lane 2,3*: single expected band of $PilQ_{406-770}$ (approximately 1095bp).

Identification of the recombinant pET28a-$pilQ_{406-770}$ by PCR and enzymatic digestion: The 1095-bp PCR product was digested with *Sac I* and *Hind III* restriction enzyme (fig 2) and ligated into the corresponding sites of digested pET-28a. Having transformed competent *E.coli* DH5α with ligation product, plasmid extraction from single colonies appeared in LB-agar plate after 18-24 h incubation in 37°C with the use of plasmid extraction kit (Bioneer, Korea). Resulted plasmid was digested with *Sac I* and *Hind III*. Agarose gel analysis showed that the extracted plasmids contained the objective gene.

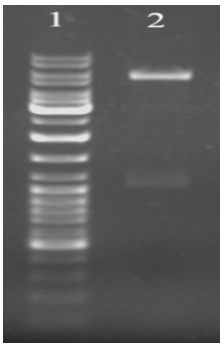

Figure 2. Agarose gel electrophoresis analysis of recombinant pET28a-$PilQ_{406-770}$. *Lane 1*: 1 kb DNA size marker; *Lane 2*: Double digestion of recombinant pET28a-$PilQ_{406-770}$ with Sac I and Hind III.

Additionally, PCR with specific primers and extracted plasmid as template DNA have done and presence of target gene in recombinant vector confirmed by electrophoretic detection of amplified 1095-bp DNA fragment from extracted plasmids.

Expression and purification of target recombinant protein: The BL21 competent cells were transformed with confirmed recombinant vectors and induced with IPTG (final concentration=1mmol) to express target recombinant protein. All proteins were electrophoresed on 10% SDS-PAGE gel and stained with Coomassie blue and recombinant protein with approximate molecular weight of 43 kDa (fig. 3A). The large scale culture and induction were achieved and after sonication the expressed protein was mainly presented in inclusion bodies. The resulted protein was purified by Ni^{2+}affinity chromatography under denature condition (fig. 3B).

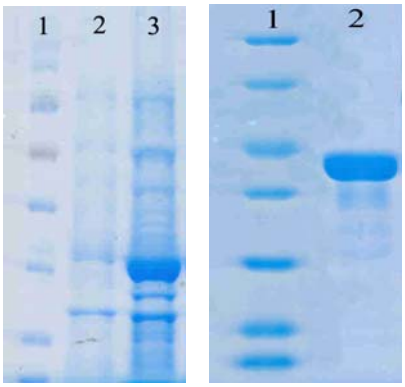

Figure 3. **(A)** SDS-PAGE (12% w/v) analysis of expression product of pET28a-PilQ406-770 in BL21. *Lane 1*: Protein marker; *Lane 2:* None induction sample; *Lane 3*: Induction of pET28a-PilQ406-770 by treatment with 1mM IPTG (43 kDa). **(B)** pET28a-PilQ406-770 recombinant proteins purified by Ni-NTA column

Western blot analysis: Western blot analysis was performed to detect antigenicity of expressed protein. Applying both *N. meningitidis* anti PilQ antibody and goat anti-rabbit immunoglobulin G horseradish peroxidase (HRP) conjugate antibody, brown strip corresponding to the site of the recombinant protein appeared on PVDF membrane after adding DAB solution which confirmed the antigenicity of our protein.

Serum antibody responses following immunization with $PilQ_{406\text{-}770}$*:* BALB/c mice immunized subcutaneously with 10µg of purified recombinant $PilQ_{406\text{-}770}$ with Freund's adjuvant exhibited good $PilQ_{406\text{-}770}$ specific serum IgG titers (fig 1).

DISCUSSION

The outer membrane proteins in gram negative bacteria have particular significance as a potential target for protective immunity (15). Meningcoccal PilQ is an antigenically conserved, abundant outer membrane protein which forms a large multimer composed of 10 to 12 and is a key component of the type IV pilus secretion machinery. The PilQ complex is critical for the surface expression of Tfp and mutants that express defective forms of the protein lack pili and do not have pilus-associated phenotypes. Structural analysis has demonstrated that meningococcal PilQ is surface exposed and naturally expressed at very high levels (16). A total of 200 to 300 conserved C-terminal residues of PilQ exhibits identity with members of GspD superfamily required for translocation of macromolecules across the outer membrane (17). Halliwell and colleagues demonstrated that immunization with PilQ complex elicits bactericidal antibodies and protects mice against experimental infection (12). Outer membrane vesicles produced from a strain in which the *pilQ* gene was up-regulated induced higher anti-PilQ antibody titers in mice (13).

Wilde and colleagues demonstrated that antibodies raised to the PilQ multimer had bactericidal activities (18). Therefore, PilQ is considered a reliable candidate antigen as a part of multi-component recombinant protein vaccines.

The cloning of genes encoding the outer membrane proteins has facilitated the production of pure proteins free from other *Neisseria* antigens for investigation as potential vaccine candidates (19).

Among the many system available for protein production, the gram negative bacterium *E. coli* remains one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, its well characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains (20).

In the present study we were able to clone and express conserved C-terminal fragment of pilQ in *Escherichia coli*; the 1095bp fragment of pilQ ($pilQ_{406\text{-}770}$) was amplified by PCR. PCR product was cloned in pET-28a and then transformed into *E.coli* strain BL21. Cloning of $pilQ_{406\text{-}770}$ was confirmed by colony-PCR and enzymatic digestion. The results of SDS-PAGE showed that our constructed prokaryotic expression system *p*ET28a-$pilQ_{406\text{-}770}$–BL21 efficiently produces target recombinant protein in the form of dissoluble

inclusion body and carefully purified with affinity chromatography with Ni-NTA agarose. The output of $rPilQ_{406-770}$ was approximately 50% of the total bacterial proteins. This highly expression might be beneficial to industrial production.

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