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Original article

Construction of a New Fusion Protein Vector Associated to Fibronectin Binding Protein A and Clumping Factor A Derived from *Staphylococcus aureus* NCTC8325

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Abstract

Objective(s)

Staphylococcus aureus is a leading cause of many nosocomial and community acquired infections. According to many reports, antibiotic therapy can not guarantee the eradication of *S. aureus* infections. Thus designing an adhesin based vaccine could restrain the *S. aureus* infections. This study designed for construction of a new fusion protein vaccine against *S. aureus* infections based on adhesin molecules fibronectin binding protein A (FnBPA) and clumping factor A (ClfA).

Materials and Methods

Bioinformatic experiments were performed using Oligo analyzer and DNAMAN softwares. The fragments corresponding to fnbA binding domain and a C-terminal fragment from *clfA* were amplified from *S. aureus* NCTC8325 genomic DNA. Purified PCR products and the vector, pET15b, were digested with *Nco*I and *Bam*HI. The digested PCR products were hybridized together and then ligated to digested vector. Finally incomplete construct was assembled by Taq DNA polymerase. To quick confirmation of cloning procedure the new construct designated pfnbA-clfA was digested with *Nco*I and *Bam*HI. To further verification, the product was sent for sequencing.

Results

The data based on bioinformatic analysis showed no homology between fusion protein and human proteins. Digestion of new vector with *Nco*I and *Bam*HI confirmed the ligation of fusion protein sequence into pET15b. Sequencing results verified the integrity of target sequences.

Conclusion

This study is the first effort to construct a new fusion protein vector based on *S. aureus* adhesins using a new design. This project is being continued to study the expression and biological activity of the fusion protein in a cell culture model.

Keywords: Cell adhesion molecules, Fusion protein vaccine, *Staphylococcus aureus*

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Introduction

Staphylococcus aureus, an extremely versatile pathogen, remains a frequent cause of community-acquired and nosocomial infections. The broad spectrum of important staphylococcal diseases includes endocarditis, septic arthritis, toxic-shock syndrome, scalded-skin syndrome, and food poisoning (1).

Historically, *S. aureus* infection has been successfully treated with antibiotics. *S. aureus* strains exhibiting multiple antibiotic resistances are isolated in 60% of community and up to 80% of hospital infections (2). For example, *S. aureus* strains with intermediate or full resistance to vancomycin, which is considered the therapy of last resort for methicillin-resistant *S. aureus*, have recently emerged (3-5). The increasing incidence of multiple antibiotic-resistant *S. aureus* strains and the emergence of vancomycin-resistant *S. aureus* have placed renewed interest on alternative means of prevention and control of infection (3). In this regard, members of the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) family of adhesion proteins have come under intensive scrutiny due to their ability to promote adhesion to the extracellular matrix that surrounds and anchors cells in tissue (6, 7). Consequently, this family represents attractive target for therapeutic and vaccination strategies aimed at interfering with colonization (8). Adhesion to host tissue is the first critical step in the initiation of an infection (9). The fibronectin binding protein (FnBP) adhesins of *S. aureus* are members of the MSCRAMM family of microbial proteins, which promote adhesion to tissue extracellular matrix (7, 10). FnBP also promotes attachment to indwelling medical devices, keratinocytes, endothelial cells, and traumatized tissues and internalization by different cell types (7, 11-15). Ligand-binding domain of the FnBP adhesin can be employed to produce adhesion-blocking antibodies (16, 17). *S. aureus* possesses two tandem *fnb* genes, encoding FnBPA and FnBPB (18, 19), each of which possesses three consecutive 37- or 38-amino-acid D motifs, designated D1, D2, and D3. In tandem, these motifs comprise a high-affinity fibronectin binding domain, D1-3 (20).

The ability to bind Fn is located exclusively within the C-terminal 20 amino acids of each D motif (21-23). These amino acids contain the sequence GG (X3, 4) (I/V) DF and change to either of the GG or IDF sequences resulted in loss of Fn binding (7, 10, 22).

Clumping factor A (ClfA) is a fibrinogen-binding adhesin known as a primary factor contributing to the colonization of implanted biomaterials or damaged endothelial surfaces at the site of endovascular infections (24). The biological role of ClfA in such infections has been demonstrated in numerous studies (25-27) and suggests that ClfA is a major virulence factor of *S. aureus*. The *clfA* gene has been shown to be expressed *in vivo* and is present in nearly all clinical *S. aureus* strains examined to date (28, 29). The fibrinogen binding activity of ClfA has been localized to the N-terminal A region of this protein (30).

ClfA-fibrinogen interaction is crucial for the bacterial virulence and thus disease outcome. The impact of the fibrinogen binding of ClfA on the ability of *S. aureus* to evoke septic arthritis and sepsis was investigated (31). The ability of ClfA to bind fibrinogen was associated with enhanced virulence in terms of the ability to cause septic death (31). Furthermore, the biological impact of targeting ClfA was demonstrated by Josefsson and colleagues, who found that active immunization with recombinant ClfA protein and passive immunization with human polyclonal anti-ClfA antibodies protected mice against *S. aureus* septic arthritis and sepsis-induced death (32).

As elucidated above, FnBPA and ClfA are important adhesins characterized as vital virulence factor. So construction of a fusion protein based on these two molecules could be an authentic project. This study is designed to construct a new fusion protein vaccine against *S. aureus* infections based on adhesin molecules.

Materials and Methods

Bioinformatics

Bioinformatic experiments were performed to control the integrity of final product. Blastp was performed to test homology between final

product and human proteins. The primers were designed using Oligo Analyzer and DNAMAN softwares. Restriction analysis for *fnbA* binding domain and *clfA* binding domain were performed using DNAMAN software.

Bacterial strains and plasmid vector

S. aureus NCTC8325 was selected as a target for *fnbA* and *clfA* genes. *Escherichia coli* DH5a (CinnaGen, Iran) was selected as cloning host. pET15b (Novagen, USA) was used as cloning/expression vector. *E. coli* strains were grown in LB broth (Hi-Media, India) or on LB agar (Hi-Media, India) at 37 °C with appropriate antibiotics. *Staphylococci* were grown in brain heart infusion broth (Hi-Media, India) with constant shaking, or on brain heart infusion agar at 37 °C overnight

PCR

Genomic DNA was extracted from *S. aureus* strain NCTC8325 using lysostaphin (Sigma-Aldrich) (1 mg/ml) followed by phenol-chlorophorm standard method.

A *fnbA* gene fragment was amplified from chromosomal DNA of *S. aureus* strain NCTC8325 using PCR with the forward primer (Fw-fnbA-2) 5'-ATCCATGGATGGGCCAAAATAGCGGTA ACCAG-3' which contained AT flag for efficient restriction of *Nco*I, restriction cleavage site *Nco*I, and a start codon ATG respectively (underlined) and Reverse primer (Rv-fnbA-2) 5-TAAATCACCGCTTACTTTTGGAAGTGTAT C-3', which contained an overlap flag which is homologous with the first 3 amino acids from clfA binding domain corresponding to amino acids GDL. Fw-fnbA-2 and Rv-fnbA-2 spanned D1-D3 region (2149-2493, 345 bp) in *fnbA* gene and generated a PCR product of 365 bp (345 bp+20 bp corresponding flags).

The reaction mixture contained 150 ng target DNA, 10 pm of forward and reverse primers, 5 mM $MgCl_2$, 200 µM of each dNTP, 10X buffer, and 1 U Taq DNA polymerase (CinnaGen, Iran). PCR reactions for *fnbA* were initiated at 95 °C for 5 min and carried out with a 1-min denaturation step at 94 °C, a 1-min annealing step at 58 °C, and elongation for 15 sec at 72 °C. This cycle was repeated 40 times.

A *clfA* gene fragment was amplified from chromosomal DNA of *S. aureus* strain NCTC8325 using PCR with the forward primer 5'- AAAGTAAGCGGTGATTTAGCTTTACGT TCA-3' which contained an overlap flag which is homologous with the final 3 amino acids from *fnbA* binding domain corresponding to amino acids KVS and reverse primer 5'-ATGGATCCTTACTCTGGAATTGGTTCAA TTTC-3', which contained AT flag for efficient restriction of *Bam*HI, the restriction cleavage site *Bam*HI, and a stop codon respectively (underlined). Fw-clfA and Rv-clfA spanned terminal region of clfA binding domain (1501-1677, 177 bp) in *clfA* gene and generated a PCR product of 197 bp (177 bp+ 20 bp corresponding flags).

The reaction mixture contained 100 ng target DNA, 10 pm of forward and reverse primers, 3 mM $MgCl_2$, 200 µM of each dNTP, 10X buffer, and 1 U Taq DNA polymerase (CinnaGen, Iran) and was amplified for 40 cycles, initiated at 94 °C for 5 min and each cycle consisting of a 1-min denaturation period at 94 °C, a 1-min annealing at 65 °C, and a 15- sec extension period at 72 °C.

PCR products were electrophoresed on 1% agarose (Fermentas) and photographed under gel documentation system (UVP).

Construction of a chimeric fnbA-clfA gene

Purification of PCR products were performed on 1% LMP agarose (Fermentas) and recovered using phenol and chloroform extraction method. Plasmid extraction was performed using alkali lysis standard method (33). The purified PCR products corresponding to *fnbA* and *clfA* were digested with *Nco*I and *Bam*HI respectively. pET15b was double digested with *Nco*I (Fermentas) and *Bam*HI (Fermentas) too. The digested products were mixed together and boiled for 5 min. During the cooling to hybridization temperature, 48 °C, the other reagents (ligation buffer, dATP, and T4 DNA ligase) were added to mixture as well as double digested pET15b. The mixture was incubated at 16° C for 16 hr. Finally PCR mixture containing 200 µm dNTP, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 10X buffer without any

primers and template DNA were added to ligated products. The hybridized-ligated complex finally was repaired using Taq DNA polymerase at 72 °C for 15 min. Briefly, in this kind of design four steps including digestion, hybridization, ligation, and repair are required to structure of the fusion protein construct designated pfnbA-clfA. Schematic presentation of cloning process is outlined in Figure 1.

The newly constructed plasmid vector, pfnbA-clfA, was transformed to fresh overnight culture of *E. coli* DH5α using $CaCl_2$ standard methods (33).

Transformants were incubated at 37 °C for 1 hr and then subcultured on LB agar containing 100 µg/ml ampicillin and incubated at 37 °C for 18-24 hr.

The transformant colonies were marked on LB agar plate and a bit of flagged colonies inoculated in LB broth containing 100 µg/ml ampicillin and incubated at 37 °C for 18-24 hr. Plasmid extraction was performed using alkali lysis standard method. Purified plasmids were digested with *Bam*HI and *Nco*I separately and electrophorsed on 1% agarose for quick verification of cloning procedure. pET15b was digested with *Bam*HI and *Nco*I separately as negative control. Selected vector was sent to sequencing for final confirmation of cloning process.

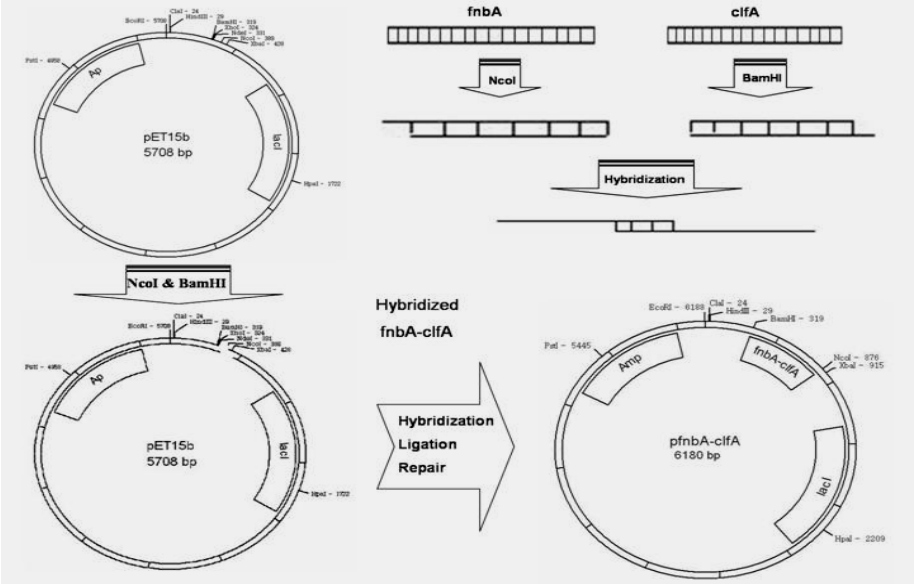

Figure 1. Schematic presentation of cloning process.

Results

Bioinformatics

According to blastp results there was no homology between final product and human proteins. Restriction analysis on *fnbA* binding domain and *clfA* binding domain sequences using DNAMAN software showed that there were no restriction sites corresponding to *Nco*I and *Bam*HI within the final fusion protein sequence.

PCR

The size of PCR product fragment for *fnbA* and *clfA* were 365 bp and 197 bp respectively. The amplified fragments related to *fnbA* and *clfA* binding domains are shown in Figures 2 and 3.

Cloning

The results obtained from digestion of plasmid vector revealed the integrity of cloning method. The results related to digested pET15b and pfnbA-clfA are shown in Figure 4. Sequencing results verified the integrity of targeted sequence on *fnbpA* and *clfA* genes.

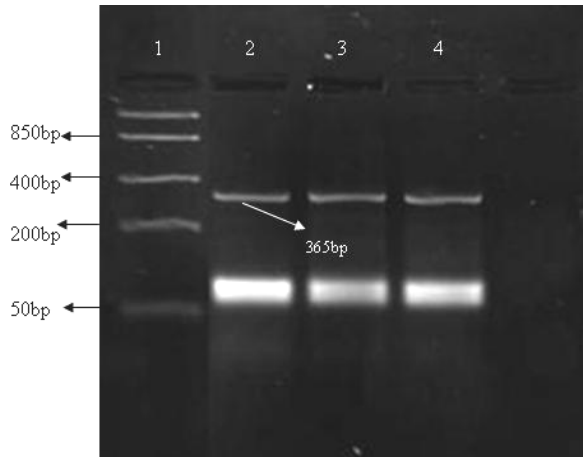

Figure 2. PCR for *fnbA* binding domain.
Lane1: DNA ladder FastRuler, low range (Fermentas)
Lane 2-4: *fnbA* PCR products

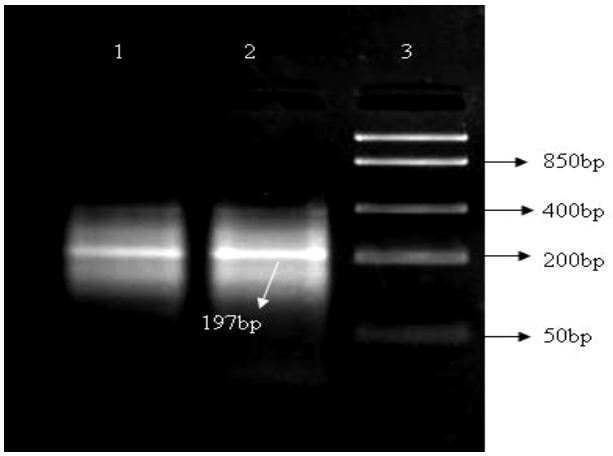

Figure 3. PCR for *clfA* binding domain.
Lane1 and 2: *clfA* PCR products
Lane3: DNA ladder FastRuler, low range (Fermentas)

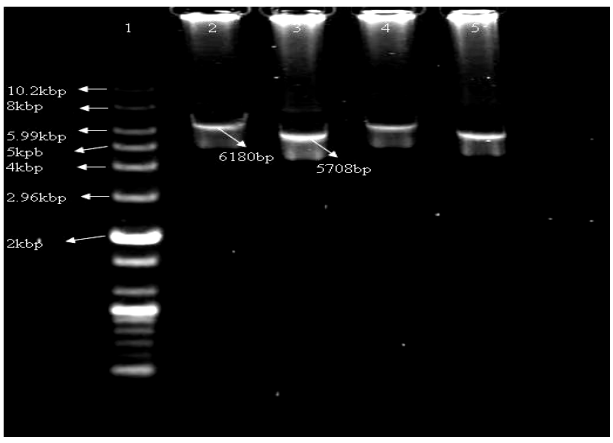

Figure 4. Digestion of pET15b and pfnbA-clfA for quick verification.
Lane1: DNA ladder 100bp plus (Bioneer)
Lane 2: *Bam*HI digested pfnbA-clfA
Lane3: *Bam*HI digested pET15b
Lane4: *Nco*I digested pfnbA-clfA
Lane5: *Nco*I digested pET15b

Discussion

The dramatic increase in methicillin-resistant bacteria, coupled with the recent emergence of vancomycin-resistant isolates, has accelerated and broadened the interest in developing novel therapeutics against *S. aureus* (34). Adhesion to host tissue is the earliest vital step in the commencement of an infection (9).

S. aureus can express an array of surface proteins including FnBPs, collagen binding protein, fibrinogen binding proteins (FgBP), vitronectin binding protein and elastin binding protein (35). Adhesions intervene bacterial binding to plasma proteins, components of the extracellular milieu, and cell surface molecules which promote colonization of various anatomical locations and donate to virulence (6, 37).

During the past decade and recent years researchers have focused their studies on adhesion proteins of *S. aureus* mainly fibronectin- and fibrinogen-binding proteins and developing immunogens base on these proteins (7, 10, 17-32, 34).

The efficacy of protection against *S. aureus* infections has been studied with the assist of FnBPA or ClfA separately but the results could not meet the full requirements according to active immune protection (17, 19, 20, 28, 32, 34). Arrecubieta and his colleagues found that the combination of protein antigens ClfA, FnBPA, and FnBPB appeared to provide enhanced protection against prosthetic-device infection (35).

It seems that combination of adhesin antigens may increase stimulation of the immune response system. Our construct was designed to gather the characteristic of FnBPA and ClfA together. As the complete protein is not needed, binding domain of *fnbA* gene (D1-D3) and a C-terminal 59Aa from *clfA* binding domain were selected to construct a new fusion protein vector (7, 10, 17-32, 34).

We proposed that simultaneous production of antibodies against binding domains of FnBPA and ClfA may boost the immunological response against forthcoming *S. aureus* infections.

Conclusion

This study is the first effort to construct a new

fusion protein vector based on *S. aureus* FnBPA and ClfA molecules with a new design. This work is being continued to study the expression and biological activity of fusion protein in a cell culture model.

Acknowledgment

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