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EVALUATION OF MAMMALIAN CODON USAGE OF FIMH IN DNA VACCINE DESIGN

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Uropathogenic *Escherichia coli* (UPEC) bacteria are the principal cause of urinary tract infections (UTI). Because these bacteria propagate intracellularly, the cellular immune response is an important factor in UTIs. Therefore, we designed a genetic construct to induce a cellular immune response. In order to develop a genetic construct that induces strong cellular immunity against this pathogen, we used the *fim*H synthetic gene according to mammalian codon usage, and the gene expression was compared with wild type codon usage. Initially, we designed two constructs, pVAX/*fim*H mam and pVAX/*fim*H wt, which contain mammalian and wild type codon usage, respectively. The Cos-7 cell line was transfected separately with a complex of pVAX/*fim*H mam-ExGene 500 poly cationic polymer and pVAX/*fim*H wt-ExGene 500 poly cationic polymer. Expression of the *fim*H gene in both constructs in COS7 cells was confirmed by RT-PCR, SDS-PAGE, and Western blotting. Both of the pVAX/*fim*H cassettes expressed inserted *fim*H genes (mam and wt) in Cos-7 cells. Our results suggest that codon optimization successfully expressed the *fim*H gene because the *fim*H gene with mammalian codon usage is compatible with the eukaryotic expression system. Therefore, mammalian codon usage could be appropriate in a pVAX/*fim*H construct as a DNA vaccine.

Keywords: Codon usage, *fim*H, DNA vaccine, uropathogenic *Escherichia coli*

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Introduction

Uropathogenic *Escherichia coli* (UPEC) bacteria are the principal cause of urinary tract infections (UTI), representing one of the most common bacterial infections today [1, 2], accounting for more than 60% of recurrent cystitis cases and at least 35% of recurrent pyelonephritis infections [3]. To establish disease in the urinary tract, UPEC must attach to bladder epithelial cells [4]. Type 1 pili are extracellular organelles expressed by UPEC that are crucial for this interaction [5]. The distal tips of type 1 pili include *Fim*H, an adhesin that binds to mannosylated receptors on the luminal surface of the bladder epithelium (urothelium) [6]. Binding is followed by invasion [7, 8]. Studies in a mouse model of human urinary tract infection (UTI) have shown that, once inside umbrella cells, UPEC rapidly proliferate to form intracellular bacterial communities (IBCs) [9, 10]. Bacteria within the IBCs are secluded; within the IBC pathway the bacteria are protected from clearance by innate host defenses such as polymorphonuclear cell attack and antibiotic treatment. A recurrent urinary tract infection is a symptomatic UTI that follows clinical resolution of an earlier UTI generally, but not necessarily, after treatment. The UPEC pathotype is an intracellular pathogen and causes recurrent UTI [11]. Therefore, the cellular immune response is important in combating such infections.

Antibiotics are the primary means of prophylaxis in patients with recurrent infections [12]. An antibiotic combination that is often used to treat UTIs, trimethoprim-sulfamethoxazole, fails to clear bacteria from bladder tissue. In addition, bacteria are protected, for the most part, from the ex vivo treatment of bladders with gentamycin [13], and concern about the emergence of antibiotic-resistant bacterial strains limits the long-term feasibility of treatment with antibiotics [14]. Overuse also leads to harmful modifications of the normal host microbiota [15]. DNA vaccines have recently been emerging as one of the most promising approaches to vaccination against infectious diseases. However, DNA vaccines utilize host cell molecules for gene transcription and translation into proteins [16]. In mammalian cells, high expression of genes derived from microorganisms cause successful DNA immunization. Mammalian codon usage is distinctive from that of other microorganisms such as *E .coli* [17]. The dramatic differences in codon usage between microorganisms and mammals are a major obstacle for expression of genes derived from non-mammalian organisms in mammalian cells [18]. This vaccine strategy includes the adaptation of codon usage for the genes encoded in a DNA vaccine to a desirable codon bias for augmented expression in mammals. This technique has effective in many systems [19–21], enhancing protein expres-

sion in vitro and antigen specific responses in vaccinated animals. A recent study that designed pcDNA/*fim*H as a DNA vaccine against UTI reported that the construct was expressed successfully in Cos-7 cell line [22]. Our previous study showed that the injection of pcDNA/fimH DNA cassette in mice could raise INF-gamma titer (not published). Thus, in view of the adhesion role of FimH protein during UPEC attachment to urothelial cells, and the role of anti-adhesin antibody for attachment inhibition, a DNA candidate vaccine could activate cellular immune response, a critical step in the limitation of recurrent UTI. In the current study, we investigated whether codon optimization of the *fim*H gene could enhance its expression and thus the immunogenicity of the DNA vaccine. Therefore, we have compared two expression constructs (pVAX/*fim*H) for the *fim*H gene that differ solely in codon usage.

Materials and Methods

Cloning of fim*H and sequencing*

To construct the pVAX/*fim*H (*fim*H wt), genomic DNA of *E. coli* 35218 was used as the template in PCR. The oligonucleotide primers, which were designed by using the available sequence of *fim*H gene (NCBI accession number NC 007946), contained the upstream primer 5'-CT *GGATCC* ACC ACC ATG GTT GTA ATG A-3' and the downstream primer 5- TGT *GAATTC* TTT ATT GAT AAA C-3' (restriction sites are indicated in italics, and underlined words show start and stop codon sequences on forward and reverse primers, respectively). A Kozak consensus sequence [23] was used on the forward primer to optimize gene expression. PCR was carried out using pfu DNA polymerase as follows: 94°C for 4 min; followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; followed by a final 5 min at 72°C. The PCR product was isolated from agarose gel using a DNA extraction kit (Fermentas-Germany). The amplified *fim*H gene was purified from the gel and inserted into an EcoRV digested pBluescript cloning vector yielding pBlue/*fim*H and confirmed by sequencing.

Construction of pVAX/fimH

The sequences for both native *fim*H (*fim*H *wt*) and synthetic *fim*H designed according to mammalian codon usage (*fim*H mam) are shown in Figure 1. They

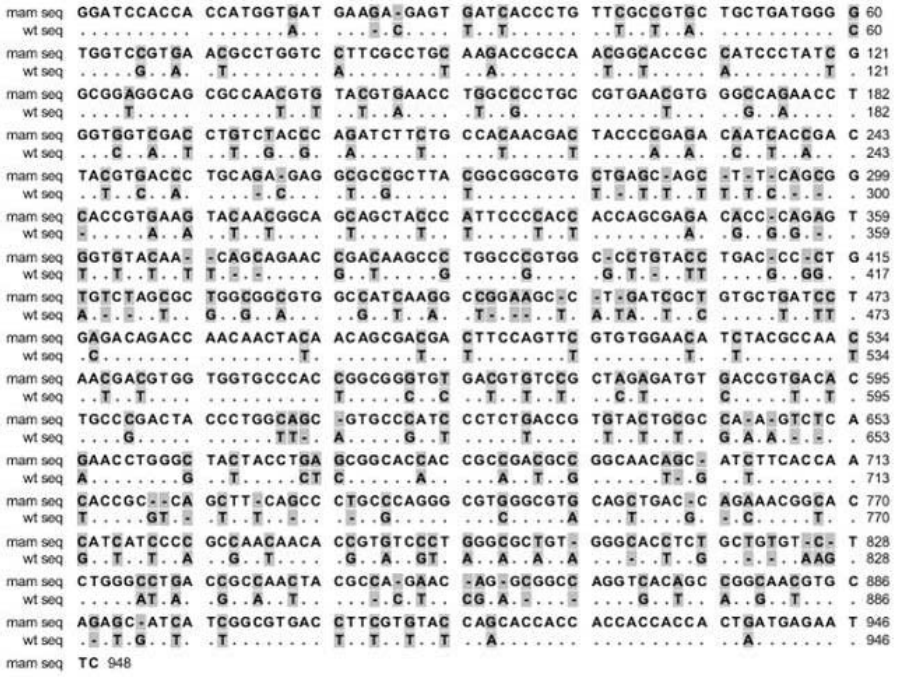

Figure 1. Nucleotide sequences. Alignment of synthetic (mam) and wild type (wt) *fim*H gene. Nucleotides in *fim*H gene that differ from the mammalian codon (mam seq) are shown below the wild type *fim*H gene (wt seq)

were subcloned from pBlue/*fim*H wt and synthetic pMA/*fim*H mam synthesized by MilleGen company (France), into pVax1 (Invitrogen, USA) by using the EcoRI and BamHI sites to create pVax/*fim*H *wt* and pVax/*fim*H mam (Figure 2). The recombinant plasmids, designated as pVax/*fim*H wt and pVAX/*fim*H mam, were transformed to *Escherichia coli* TOP10 F' strain (Invitrogen, USA) by chemical methods (CaCl2), extracted and purified using a GeneJET plasmid Miniprep Kit (Fermentas, Germany). The sequences of the pVax/*fim*H constructs were confirmed by DNA sequencing analysis (Macrogen Research, Seoul, Korea). The qualities of resulting plasmids were assessed by the ratio of light absorption (260 nm/280 nm) and by 1% agarose gel electrophoresis. The purified plasmids were dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA at pH8.0). Plasmids were stored at –2ºC before use.

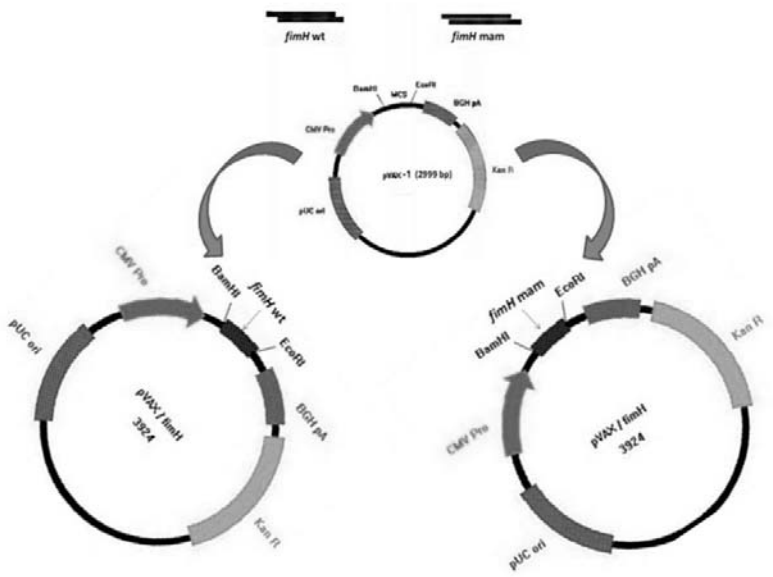

Figure 2. Diagram of the pVax1 vectors with the *fim*H genes (synthetic and native) insert of 948 bp. pUC ori: origin of replication; CMV pro: CMV immediate-early promoter; *fim*H mam and *fim*H wt: fimbriae H gene with eukaryotic and prokaryotic codon usage, respectively. Insert: BGH pA: bovine growth hormone gene (for polyadenylation signals), Kan R: Kanamycin resistance gene. Double digestion with bamHl and EcoRl produces 948 bp and 2976 bp bands

Transfection of recombinant cassettes into the Cos-7 cell line

Cos-7 cells, purchased from the National Cell Bank Pasteur Institute of Iran, were cultured in RPMI supplemented with 10% foetal bovine serum (FBS), penicillin 100U/ml, and streptomycin 100µg /ml and were incubated upon 50–80% confluency. Transfectamin® (ExGen 500) reagent (Invitrogen, USA) was used for transfection of the Cos-7 cells according to the manufacturer's instructions. The day before transfection, 0.8–2.4 $\times 10^5$ cells per well of a 6-well plate were seeded and incubated at 37ºC in a 5% CO_2 incubator until the cells were 50% confluent. For each well, a mixture of 1µg recombinant plasmid DNA in 100 ml NaCl 150 mM and 3.3 µg ExGen 500 polycationic polymer (Fermentas,Germany) was incubated at room temperature for 10 min to allow DNA–ExGen 500 complexes to form. Then, 900 µl of medium were added to each well of the 6-well plate. The complexes were mixed gently and added to the medium. Moreover,

pcDNA3.1/NT-GFP-TOPO (Invitrogen, USA) was used as a positive control for correct transfection, and the gene expression that resulted is shown in Figure 3. Cell extracts were assayed for transient gene expression 48 h after the start of transfection.

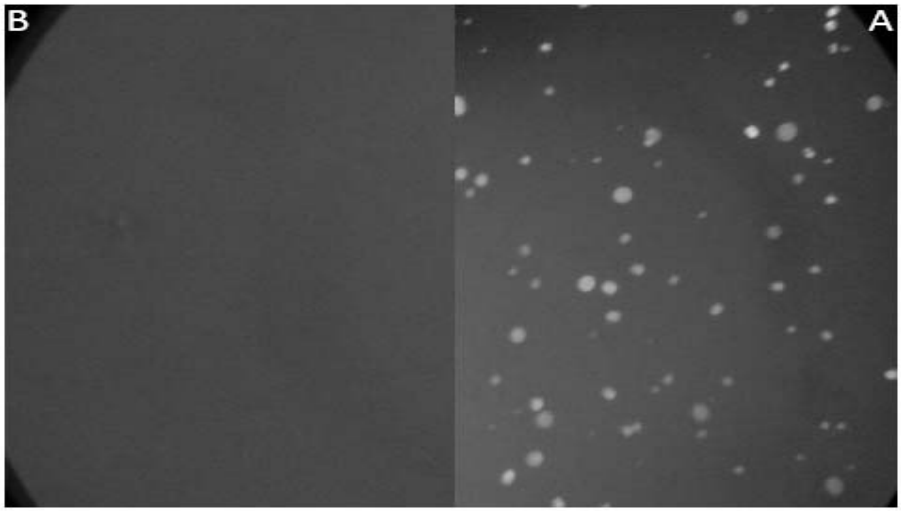

Figure 3. Result of GFP expression in transfected Cos-7 cells 48 h after using the pcDNA3.1/NT-GFP-TOPO (A) construct and negative control (B)

Extraction of total RNA and RT-PCR analysis

Cos-7 cells transfected with pVAX/*fim*H (mam and wt) were incubated for 48 h, and then the cells were collected separately. Total RNA was extracted by using TriPure Isolation reagent (Roche, Germany) in accordance with the manufacturer's protocol. Moreover, total RNA was extracted from untransfected COS-7 cells as a negative control. Following elution, nucleic acid concentration was determined by spectrophotometer (NanoDrop-1000, Wilmington, DE). The RNA was quantified and used as a template for cDNA synthesis. The cDNA was then synthesized from the total RNA of transfected and untransfected cells by using AccuPower® CycleScript RT PreMix (dN6) kit in the one-step protocol according to the manufacturer's instructions (Bioneer, Korea). RT-PCR was used to detect the *fim*H mRNA transcript.

SDS-PAGE and Western blot analysis

Expression of the *fim*H gene in the recombinant cassettes, transfected to Cos-7 cells, was analyzed after 72 h by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [17]. For Western blot analysis, 20 µl of cell lysate were electrophoresed and transferred onto a polyvinylidene difluoride membrane (Hi-bond Amersham Biosciences, USA) (125 V) within 1.5 h using the Mini Trans-Blot Electrophoresis Transfer Cell System from Bio-Rad (Hercules, CA, USA) in Tris/glycine buffer, pH 8.4, containing 20% (v/v) methanol. Then, the membranes were blocked with a solution containing 5% skim milk and 0.1% Tween 20. The blocked membranes were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and incubated with a rabbit anti-*fim*H polyclonal antibody at room temperature for 1 h according to the supplier's instructions (Roche, Germany). Afterwards, the membranes were washed four times with PBS containing 0.1% Tween 20 and incubated with peroxidase-conjugated goat-rabbit antibodies for 1 h. The membranes were then washed four times with PBS containing 0.1% Tween-20 and finally developed with DAB (Sigma, St. Louis, MO, USA).

Results

Confirmation of correct PCR product

The *E. coli* 35218 chromosomal DNA samples were subjected to PCR, and the results revealed that all of the samples produced the expected *fim*H amplicon of 948 bp. The *E .coli* 35218 *fim*H gene was shown to have more than 97% identity to other *fim*H sequences reported in GenBank [22]. The PCR product was cloned into the pBluescript plasmid and confirmed with automated sequencing.

Cloning of fimH gene (mammalian and wild type) into pVax

The *fim*H segment from the pBlue/*fim*H was then subcloned into the BamHl and EcoRl sites of linearized pVax. Upon double digestion analysis with BamHl and EcoRl, the purified pVax/fimH mam plasmid showed a distinct *fim*H band at 948 bp and the linearized plasmid at 2.9 kb. The pVax/*fim*H wt plasmid

showed similar bands after digestion. The production of the 948 bp band proved that the insert was in the correct orientation (Figure 2). Highly purified pVax/*fim*H mam and pVax/*fim*H wt with A260/A280 ranging from 1.7 to 1.9 and extracted using the GeneJET plasmid Miniprep Kit (Fermentas, Germany) were used for the transfection experiments. Sequence analysis results proved that the cloned *fim*H gene contained no mutations or extra ATG sequences upstream of the initiation codon, and it was in the correct orientation for transcription.

Evaluation of fimH gene expression in recombinant cassettes

To determine whether the *fim*H gene in the DNA vaccine constructs (pVAX/*fim*H) was expressed in vitro, Cos-7 cells were transiently transfected with the plasmid constructs, and expression at the transcriptional and protein levels was detected by RT-PCR or SDS-PAGE, and Western blotting, respectively. After extracting the total RNA from the transfected cells, RT-PCR was performed with the AccuPower® CycleScript RT PreMix(dN6) kit in the one-step protocol, and then PCR was carried out with specific primers. The DNA band of 948 bp for the *fim*H gene was amplified and detected on an agarose gel. Subsequently, band intensity detection of both related DNA bands of fimH wt and mam assessed by TotalLab software gel analysis. The result showed more expression of mam *fim*H (158 ng/band). than fimH wt (80 ng/band), respectively. Western blotting analysis indicated that *fim*H (wt and mam) proteins were detected in transfected Cos-7 cells

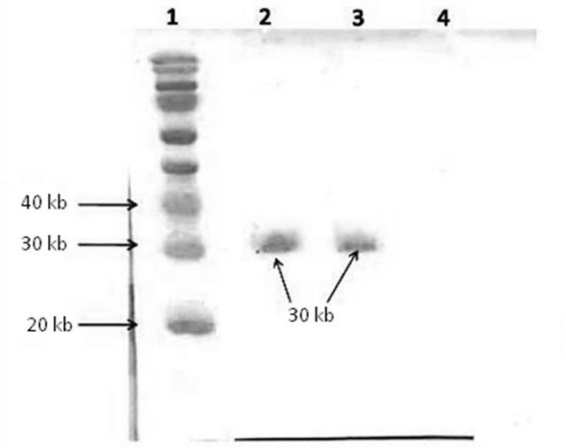

Figure 4. Western blotting analysis of the target protein FimH from Cos-7 cells. Lane 1 molecular weight markers. Lane 2 FimH (mam). Lane 3 FimH (wt). Lane 4 negative control (pVAX1-transfected Cos-7 cells)

(Figure 4). As expected, FimH (wt and mam) proteins were not detected in the control pVAX1-transfected Cos-7 cells (Figure 4). Consequently, these results showed that expression of *fim*H gene with mammlian codon usage was similar to expression of the wild type *fim*H gene.

Discussion

There are many factors that contribute to the acquisition and progression of *E. coli* urinary tract infections. To initiate an infection, UPEC must first be able to colonize in the urinary epithelium of the host. For colonization to begin, UPEC attachment to receptors expressed by umbrella cells lining the luminal surface is necessary. The apical side of the umbrella cells consists of a detergent-insoluble membrane containing a family of integral membrane proteins termed uroplakins [24]. Uroplakins act as receptors for FimH, the tip adhesion molecule of UPEC type 1. FimH was an attractive candidate for use in vaccination because of its inherent adhesive properties, high numbers on the cell surface, and ease of purification when FimH protein is used along with DNA vaccine. In addition, Kelly and colleagues [25] revealed by microscopy that the UPEC remained diffusely spread throughout the superficial umbrella cells and did not immunostain with anti-FimH antibodies, displaying the absence of type 1 pili.

Most of the vaccines developed against UTI by using several adherence antigens, Dr fimbria [26], Type 1 fimbria [27], FimC-H or FimHt [5], FimHt [28], FimC-H [29], Fim peptides [30], P fimbria [27, 31], PapD-G [32], and Pap peptides [33] could only induce a humoral immune response. However, previous studies [34–36] have shown that some gene sets in a plasmid as a DNA vaccine could induce not only humoral immunity but also a cellular immune response. Therefore, because DNA vaccines can also stimulate a cellular immune response, our experiment could be important in preventing recurrent UTI.

As has been already mentioned, UPEC can form intracellular bacterial communities (IBCs) in residual umbrella cells. Therefore, a cellular immune response will be important in preventing recurrent UTI. A DNA vaccine could be a good choice for combating this problem.

There are several methods for improving DNA vaccine efficacy against infection: prime-boost immunizations, multivalent vaccines, genetic adjuvants, or codon optimization [37]. We used mammalian codon usage and native codon usage for comparison; uropathogenic *E. coli* genes are rich in A:T codons, whereas mammalian codons are more G:C rich [37]. The *fim*H G+C content in the mamma-

lian gene was 62.3%, and that in the wild type gene was 50.4%. Usage of synonymous codons in protein coding genes is not necessarily haphazard, and codon composition could be biased towards codons that would match the tRNA pool of the host organism [38]. Translational selection causes the unequal usage of synonymous codons in protein coding genes in a broad variety of organisms, which is one of the most subtle effects of molecular evolution [19]. In addition, many studies, like those of Uchijima et al. [39], have found that the optimized Listeriolysin O (LLO 91–99) DNA sequence showed substantially higher translation efficiency than the wild-type sequence in mammalian cells. In another study, Ko et al. [40] demonstrated that the optimization of the *M. tuberculosis* Ag85B coding sequences to those of highly expressed mammalian genes resulted in substantial improvement in protein expression by mammalian cells. Likewise, a DNA vaccine with optimized codon usage was able to induce Th1-like and cytotoxic immune responses in BALB/c mice (reference is missing). In the present study, we investigated how codon optimization affected the translational efficiency of expression *fim*H gene in Cos-7 cultured cells and showed the expression of FimH protein from pVax 1 carrying the *fim*H mam gene in COS-7 cells. Our results indicate that the effects of codon usage on gene expression were the same in the construct with the wild type *fim*H gene (prokaryotic) or with the fimH (mam) gene. Hence, it may be helpful to use mammalian codon usage in the DNA vaccine, because codon usage affects the CTL induction and cytokine expression [39], and mammalian codon usage is compatible with an eukaryotic expression system. This expression study also shows that the pVax/*fim*H mam construct that we developed was able to function in vitro even with a low amount of protein expressed. The functionality of the pVax/*fim*H mam in vivo is currently under study. It is hoped that the *fim*H mam will establish adequate levels of cellular immunity when expressed in animals and remove the intracellular bacterial communities (IBCs) in residual umbrella cells.

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