Construction of pcDNA/fimH Cassette as a DNA Vaccine Candidate Against Urinary Tract Infection and Evaluation of fimH Transcripts in COS7 Cell Line

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Abstract: Uropathogenic *Escherichia coli* is one of the major agents of urinary tract infection. Since it has intracellular propagation, cellular immune response is so important in this case. Accordingly, a genetic construct for inducing of cellular immune system was designed. At first, chromosomal DNA extracted from *E. coli* 35218 and *fimH* gene amplified with this template by PCR. PCR product inserted to pcDNA.1 eukaryotic expression vector and confirmed the recombinant vector by sequencing. The COS7 cell line transfected with a complex of pcDNA/fimH and ExGen 500 poly cationic polymer. Expression of *fimH* gene in COS7 was confirmed by RT-PCR. Consequently, pcDNA/fimH cassette could express inserted *fimH* gene in eukaryotic cells and is a valuable DNA candidate cassette for urinary tract infection vaccination. This is the first prompt to designing a DNA vaccine against urinary tract infection that caused by Uropathogenic *Escherichia coli*.

Keywords: Genetic vaccination, uropathogenic *Escherichia coli*, pili type I, *fimH*

INTRODUCTION

Urinary Tract Infections (UTI) present a disease process that is mediated (or assisted or otherwise induced) by the attachment of bacteria to cells. *Escherichia coli* is the most common pathogen of the urinary tract, accounting for more than 85% of cases of asymptomatic bacteriuria, acute cystitis and acute pyelonephritis, as well as greater than 60% of recurrent cystitis and at least 35% of recurrent pyelonephritis infections (Mysorekar and Hultgren, 2006). Furthermore, approximately 25-30% of women experience a recurrent *E. coli* urinary tract infection within the first 12 months following an initial infection but after a second or third infection the rate of recurrence increases to 60-75% (Hooton, 2001).

Given the high incidence, continued persistence and significant expense associated with *E. coli* urinary tract infections, there is a need for a prophylactic vaccine to reduce susceptibility to this disease.

On one hand, in a murine model, it was shown that UPEC is able to replicate intracellularly, forming loose collections of bacteria termed bacterial factories. Bacteria within the superficial umbrella
cells can escape into the bladder lumen in a process termed fluxing. The bladder epithelium responds to bacterial invasion via a Toll-like receptor 4 (TLR4) pathway that triggers the influx of polymorphonuclear leukocytes (PMNs). In addition, the superficial umbrella cells undergo apoptosis-like cell death and exfoliate, releasing bacteria into urine. Despite these strong host defenses, a subpopulation of UPEC is able to persist for months in murine model in a quiescent reservoir state which may serve as a seed for recurrent infections. So, induction of cellular response in immunized murine model is important and lead to reduce recurrent infection (Justice et al., 2004).

On the other hand, the DNA vaccine has proven to be one of the most promising applications in vaccination against infectious diseases. Due to its unique ability to readily induce humoral as well as cellular immune responses, it attracted great interest when the concept was first confirmed in the early 1990s (Cui, 2005). With more highlighting on, one of the advantages of the DNA vaccine is that it can induce cellular immune responses, including a CTL response. A DNA vaccine against UTI will have potential role to reduce recurrent UTI. Hence, designing a genetic cassette as a DNA vaccine against UTI was started. This is the first report in this field.

**MATERIALS AND METHODS**

**Bacterial Strain and Culture Conditions**

The strain used in this study was *E. coli* 35218 (purchased from Pasteur Institute of Iran). This strain was grown using standard techniques. According to Martinez et al. (2000), for all *in vitro* studies, overnight (typically 16 h), aerated cultures were diluted 1:250 into fresh Luria-Bertani (LB) broth and grown statically at 37°C for 24 to 48 h to induce type 1 piliation.

**Evaluation of *E. coli* 35218 Piliation by Hemagglutination Assay**

Hemagglutination assay was done as described by Snyder et al. (2005). A 3% (v/v) solution of guinea pig erythrocytes was used to determine type 1 fimbrial mannose-sensitive hemagglutination. Approximately 10^9 cfu of bacteria, from nutrient broth resuspended in Phosphate-Buffered Saline (PBS), was serially diluted twofold in round-bottom 96-well microtiter plates. An equal volume of erythrocyte solution was mixed with the bacterial suspension.

**PCR, Cloning and *fimH* Sequencing**

All DNA manipulations were carried out by standard procedures (Sambrook et al., 2001). The enzymes and chemicals used for DNA manipulation were purchased from Roche (Germany), Fermentas (Lithuania) and CimaGen (Iran). Chromosomal DNA was extracted by using the Genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer’s recommended protocol. The oligonucleotide primers, which were designed by using the published sequence for the *fimH* gene (NCBI accession number NC 007946) showed in Table 1. PCR was carried out by *pfu* DNA polymerase as follows: 94°C for 4 min; followed by 35 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; followed by a final 5 min at 72°C. PCR product was isolated from agarose gel by using

<table>
<thead>
<tr>
<th>Table 1: Primers used in present study</th>
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<tr>
<td><em>fimH</em> forward primer</td>
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<tr>
<td><em>fimH</em> reverse primer</td>
</tr>
<tr>
<td><em>fimH</em>CDNA forward primer</td>
</tr>
<tr>
<td><em>fimH</em>CDNA reverse primer</td>
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*Italic words indicate restriction site of BamH1 and EcoRI on forward and reverse primer, respectively. **Capital words be a sign of knock sequence on *fimH*CDNA forward primer. ***Underlined words show start and stop codon sequence on forward and reverse primers, respectively.*

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the DNA extraction kit (Fermentas). Amplified fimH gene cloned to digested pBluescript II SK by EcoRV restriction enzyme. After ligation and transformation, Plasmids were introduced into E. coli Top10F’ strain by chemical method (CaCl2). A recombinant clone (pBluescript/fimH) confirmed by Sequencing.

Subcloning and Construction of pCDNA3/fimH

The primers were used to amplify a target sequence of 903bp containing coding region for producing of a 29KDa fimH with signal peptide designed by Gene Runner software (version 3.05; Hastings software Inc.) and described in table one. Kozak sequence (Kozak, 2002) used on forward primer for optimizing gene expression. PCR product, 903 bp E. coli DNA fragment encoding fimH, was cloned to pCDNA.1 mammalian expression system vector (Invitrogen, USA). The resultant plasmid (pCDNA/fimH) confirmed by sequencing.

Transfection of COS7 Cell Line and RT-PCR

COS7 cell line (purchased from National Cell Bank, Pasteur Institute of Iran) cultured in DMEM medium with 50 U of penicillin, 50 µg of streptomycin, amphotericine B solution fungizone (250 µg mL⁻¹) and 10% FCS. When cultured cells were grown to 50% confluency, transfected with a complex of pCDNA/fimH (1 µg 10⁻⁵ cell) and ExGen 500 poly cationic polymer (3.3 µL µg⁻¹ DNA) (Fermentas Co.). Moreover, pCDNA3.1/NT-GFP-TOPO (Invitrogen Co.) was used as positive control of right transfection and gene expression.

After 24 and 48 h, for the extraction of total RNA, 1 mL RNX solution (Cinagen Co.) added to every six wells of cell culture plate. Cells were lysed and RNA was extracted by using the RNeasy kit (QIAGEN) and following the manufacturer’s recommended protocol. Moreover, total RNA was extracted from untransfected COS7 cell line. Following elution, nucleic acid concentrations were determined by spectrophotometer (NanoDrop) and residual DNA contamination was removed by incubating the samples with RNase-free DNase (Fermentas) according to the manufacturer’s instruction. After DNase inactivation, the RNA was recovered, quantified and used as a template for cDNA synthesis. cDNA was then synthesized from total RNA of transfected and untransfected cells by using RevertAid ™ First Strand cDNA Synthesis kit in the one-step protocol according to the manufacturer’s instructions (Fermentas). As a negative control, a reaction tube heated at 95°C for 10 min to inactivate the RT was included to test for DNA contamination of the RNA sample. Also, cDNA of untransfected cells RNA source, used as template in PCR. In addition, the gene in question was amplified from genomic DNA by PCR, using the same primers as a positive control of the PCR and as a size marker.

RESULTS

Cloning of fimH and Sequencing

The fimH gene was amplified by PCR and using E. coli 35218 chromosomal DNA as the template (Fig. 1). The amplified fimH gene was blunt end fragment, purified from gel and inserted into EcoRV digested pBluescript cloning vector yielding pBluefimH. fimH gene and protein sequences of E. coli UT089 strain and 35218 strain aligned by using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2). fimH gene has some nucleotides variation among enterobacteriaceae like E. coli. Seven point mutations in 35218 fimH gene (result not shown) and one changed amino acid in 35218 fimH protein sequence (Table 2) was found. The sequence of E. coli 35218 fimH gene was shown more than 97% identity to other fimH sequence reports in GenBank.
Fig. 1: Result of PCR on genomic DNA of 35218 E. coli strain. The PCR was done with gradient annealing temperature at 52-57°C (lane 1-6, respectively). Amplicons are 107 4bp. Lane 7 is 1 kb DNA ladder and arrow shows 1 kb band.

Table 2: Result of fimH proteins alignment

<table>
<thead>
<tr>
<th>FimH35218</th>
<th>UTI89</th>
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<tr>
<td>1 5 MRKVITLPAVLLMLGWSVNHWFACKTANGTRIPIGGSANVYNNLAPAVN</td>
<td>1 5 MRKVITLPAVLLMLGWSVNHWFACKTANGTRIPIGGSANVYNNLAPAVN</td>
</tr>
<tr>
<td>51 VGSNLAVDLSSTQIFCHYDPEIVTDDYTLQRGAYGGVLSSFSGTVKYN</td>
<td>51 VGSNLAVDLSSTQIFCHYDPEIVTDDYTLQRGAYGGVLSSFSGTVKYN</td>
</tr>
<tr>
<td>101 SYPFETTSETPRVYNYNSTDKPWNVALYLVIPVSSAGVVAIKAGSLIAVL</td>
<td>101 SYPFETTSETPRVYNYNSTDKPWNVALYLVIPVSSAGVVAIKAGSLIAVL</td>
</tr>
<tr>
<td>151 ILQCNYNDODGQFWJNYNNCVVPTTGGCVDSSRDVTYLPFGSV</td>
<td>151 ILQCNYNDODGQFWJNYNNCVVPTTGGCVDSSRDVTYLPFGSV</td>
</tr>
<tr>
<td>201 PIPLTVYCAKSLQNYLGSITDAGNSFTTASFSPAQGVQLTRNG</td>
<td>201 PIPLTVYCAKSLQNYLGSITDAGNSFTTASFSPAQGVQLTRNG</td>
</tr>
<tr>
<td>251 TIPANNTVSALGTSASVLGLTANYARTGSGQTANGNSQSIIGTVQ</td>
<td>251 TIPANNTVSALGTSASVLGLTANYARTGSGQTANGNSQSIIGTVQ</td>
</tr>
<tr>
<td>301 QITANSGLAVGVSASVLGLTANYARTGSGQTANGNSQSIIGTVQ</td>
<td>301 QITANSGLAVGVSASVLGLTANYARTGSGQTANGNSQSIIGTVQ</td>
</tr>
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*Serin 33 in fimH 35218 is Alamine in fimH UTI89. Underlined words show difference amino acid A in UTI89 fimH and S in fimH 35218

Construction of pCDNA3/fimH

After confirmation of pBluescript/fimH via sequencing, it was used as template for amplifying exact fimH gene with signal sequence. The 903 bp PCR product (Fig. 2) inserted to pCDNA 1 expression vector. The resultant plasmid (pCDNA/fimH) confirmed through sequencing. The result of sequencing of inserted fimH gene to pCDNA showed right frame of it with kozak sequence.

RT-PCR Assay

Confirmed pCDNA/fimH DNA cassette transfected to cultured COS7 cell line with use a poly cationic polymer. Moreover, a mammalian expression vector which harbor GFP coding gene used as positive control of transfection and gene expression. After 48 h, GFP expression was determined by
Fig. 2: Result of PCR on pBluescript/finH as template. This figure shows a gradient annealing temperature PCR at 49-57°C (lane 1-9, respectively). The arrow shows 1 kb band

Fig. 3: The result of RT-PCR * Lane 1 (Ladder 1kb), Lane 2 (positive PCR for finH), Lane 3 (RT-PCR for DNAse treated RNA), Lane 4 and 5 (RT-PCR for 24 and 48h transfected cells), Lane 6 (100 bp Ladder)

fluorescence light inverted microscope. Total RNA extracted and RT-PCR was done (Fig. 3). Expression of finH during 48 h was more than 24 h (lane 4 and 5), because the intensity of related band of lane 5 on agarose gel was higher than the lane 4.

DISCUSSION

While many factors contribute to the acquisition and progression of E. coli urinary tract infections, it is generally accepted that colonization of the urinary epithelium is a required step in the infection process (Johnson, 1991). In a typical course of E. coli urinary tract infection, bacteria originate from the bowel, ascend into the bladder, and adhere to the bladder mucosa where they
multiply and establish an infection (cystitis) before ascending into the ureter and kidney. Thus, disruption or prevention of pilus-mediated attachment of *E. coli* to urinary tract epithelial cells may prevent or retard the development of urinary tract infections (Langemarn and Ballou, 2001). In this regard, a number of studies have pointed to a role for pili in mediating attachment to host bladder mucosal cells.

Briefly, Hopkins *et al.* (1999) used a vaccine containing killed *Escherichia coli* and other uropathogenic bacteria and Langernann *et al.* (1997, 2000) found that immunization with *FimH* reduced in vivo colonization of the bladder mucosa by more than 99% in a murine cystitis model and in monkeys were inoculated with *FimCH* adhesin-chaperone complex. Yong-Hwa *et al.* (2003) expressed a fusion protein based on fusing *FimH* adhesion to the cholera toxin subunit A2B (CTXA2B).

In all studies just IgG titer, as an indicator of humoral immune response, was measured. But, Reigstad *et al.* (2007) reported that UPEC can form intracellular bacterial communities (IBCs) in residual umbrella cells. Therefore, cellular immune response will be important to prevent recurrent UTI. In this research, a candidate DNA vaccine for inducing cellular immune response in mice model was designed.

For uropathogenic *E. coli*, *FimH* is a critical determinant of tropism for the urinary tract epithelium. Thus, *fimH* gene selected for constructing in designed pC1 DNA/fimH cassette gene. Indeed, it was shown that different structural variants of *FimH* vary in the strength of their binding to uroepithelial cells (Sokurenko *et al.*, 2004). It showed *E. coli* 35218 *FimH* has a single amino acid difference with *E. coli* UTI89 *FimH* protein. It was a substitution of Alanine (UTI89) to Serine (35218) at residue 33, but it does not any effect on level of binding to tri-mannosidic structures (M1α1–3, α1-6-D-mannotriose).

Kozak Consensus sequence for the context of translation initiation codon is useful to optimize the gene expression in eukaryotic cells (Kozak, 2002). The most general and accepted kozak sequence is ACCACATGG. Hence, this sequence was used in forward primer for amplifying *fimH* gene in subcloning procedure.

As the first codon after ATG in *fimH* gene is AAA, if ATG of kozak sequence had been used as start codon, the following G of kozak sequence would have been substituted to A and so, the second codon of *fimH* gene would be GAA instead of AAA Which would lead to change lysine (codon aae), a basic amino acid, with glutamic acid (codon gag). Therefore, it can be concluded that the ATG of the kozak sequence could not be used as start codon and it was preferred to use the original ATG of *fimH* gene as start codon in forward primer.

Finally, according to the RT-PCR assay results, prepared pC1 DNA/FimH construct could successfully express the *fimH* cloned gene at mRNA level in cos7 cell line and now, it is ready to be used for animal immunization trial studies.

ACKNOWLEDGMENTS

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REFERENCES


