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Original Article

Rising Cellular Immune Response after Injection of pVax/*iutA*: A Genetic DNA Cassette as Candidate Vaccine against Urinary Tract Infection

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Abstract

Background: Uropathogenic Escherichia coli (UPEC) are major bacterial agent of Urinary Tract Infection (UTI). This infection is more prevalent among women because approximately half of all women will experience a UTI in their life-time and near a quarter of them will have a recurrent infection within 6–12 months. IutA protein has a major role during UPEC pathogenesis and consequently infection. Therefore, the aim of current study was assessment of IutA protein roles as a potential candidate antigen based for vaccine designing.

Methods: This survey was conducted during 2014-2015 at the University of Tehran, Iran. Chromosomal DNA extracted from *E. coli* 35218 and *iutA* gene amplified by PCR. The amplicon cloned to pVax.1 eukaryotic expression vector and recombinant vector confirmed by sequencing. The *iutA* gene expression in genetic cassette of pVax/*iutA* was evaluated in COS7 cell line by RT-PCR. Then, injected to mouse model, which divided to three groups: injected with pVax vector, PBS and pVax/*iutA* cassette respectively in two stages (d 1 and 14). One week after the second injection, bleeding from immunized mouse was performed and IFN-gamma was measured.

Results: The mice immunized with pVax/*iutA* showed increased interferon-γ responses significantly higher than two non-immunized groups ($P<0.05$).

Conclusion: Cellular immune response has a main protective role against UTI. Raising this kind of immune response is important to preventing of recurrent infection. Moreover, the current DNA cassette will be valuable for more trying to prepare a new vaccine against UTI.

Keywords: Genetic vaccination, Uropathogenic *escherichia coli*, *IutA*

Introduction

Urinary tract infection (UTI) is a most common infection in humans especially in young women (1-3).The major bacterial agent for this infection, UPEC, regularly persists in feces. This pathotype could colonize in the lower urinary tract and cause UTI. Moreover, some strains could make rising infection that can progress to pyelonephritis (3, 4). The UTI is usually self-limiting, but UPEC establishing a persistent infection via penetrate to bladder cells and intracellular amplification (2, 5). Some innate immune mechanisms were made urinary tract to be sterile like urinary flow, sIgA of mucus and bactericidal property of urine epithelial cells. However, pathogenic E. coli bacteria have several virulence factors such as adhesion molecules and toxins (6). These strains

possibly will colonize on the mucosal surfaces of bladder epithelial cells by adhesion molecules such as pili S, adhesion family of Dr, pili p and pili type I (7, 8). On the other hand, iron molecules are really important factor in UPEC pathogenesis. Accordingly, pathogenic strains have an iron acquisition system. IutA, a 84 KD protein is a trans-membrane molecule which has a role for facilitating iron acquisition because this receptor mediate uptake of siderophores. Deletion of the siderophore receptor IreA, heme receptors ChuA or Hma, enterobactin receptor Iha, salmochelin receptor IroN, or aerobactin receptor IutA lead to decrease of UPEC pathogenesis in the murine urinary tract (9-12).

Current treatment of acute urinary tract infection is limited due to the increasing rate of antibiotic resistance strains as well as no protective vaccines (6, 9). While several clinical trials of vaccine candidates have currently been tested against urinary tract infection in human (13-14), no suitable results have been found so far. Therefore, targeting critical molecules such as IutA as a vaccine candidate can lead to an increase in vaccines efficacy (10).

On the other hand, the DNA vaccine has proven to be one of the most promising applications in vaccination against infectious diseases. These kinds of vaccines potentially could raise both of immune responses, humoral and cellular, in animal models. Besides, they considered from early 1990s (15). 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. Additionally, UTI is a high prevalence infectious disease and the major agents of it were shown growing up resistance to antibiotics. Therefore, designing a protective vaccine seems to be necessary.

In the present study, a pVax/*iutA* genetic cassette was constructed and injected to animal model. Consequently, immune response against DNA cassette was evaluated in the murine model.

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. (16), 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.

PCR, Cloning and iutA sequencing

All DNA manipulations were carried out by standard procedures (17). The enzymes and chemicals used for DNA manipulation were purchased from Roche (Germany), Fermentas (Lithuania) and CinnaGen (Iran). Chromosomal DNA was extracted by using the Genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer's recommended protocol.

Table 1: Primers used in present study

iutA forward primer	atgatgataagcaaaaagtatacg
iutA reverse primer	tcagaacagcacagagtagttc
pVax/*iutA* forward primer	ggatccACCACCATGGtgataagc
pVax/*iutA* reverse primer	gaattctcagaacagcacagagtag

* Italic words indicate restriction site of BamHl and EcoRl on forward and reverse primer, respectively

** Capital words are a sign of kozak sequence on pVax/*iutA* forward primer.

*** Underlined words show start and stop codon sequence on forward and reverse primers, respectively

The oligonucleotide primers, designed by using the published sequence for the *iutA* gene (NCBI accession number NC 004431.1) are 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 t 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 the DNA extraction kit (Fermentas, Lithuania). Amplified *iutA* 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 ($CaCl_2$). A recombinant clone (pBluescript/*iutA*) confirmed by Sequencing.

Sub-cloning and construction of pVax/iutA

The primers were used to amplify a target sequence of 2202 bp containing coding region for producing of a 84 KDa *iutA* with signal peptide designed by Gene Runner software (version 2.05; Hastings software Inc.) and described in Table 1. Kozak sequence (18) used on forward primer for optimizing gene expression. PCR product, 2202 bp DNA fragment encoding *iutA*, was cloned to pVax mammalian expression system vector (Invitrogen, USA). The resultant Plasmid (pVax/*iutA*) 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, amphotericin B solution fungizone (250 µg mL^{-1}) and 10% FCS. When cultured cells were grown to 50% confluency, transfected with a complex of pVax/*iutA* (1µg 10^{-5} cell) and ExGen 500 poly cationic polymer (3.3 µLμg^{-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 (CinnaGen 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 manufacture'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 manufacture'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 95C for 10 min to inactivate 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.

Experimental groups of mice and immunization

Six-to-eight wk inbred female BALB/c mice were purchased from Pasteur Institute of Iran (Karaj, Iran). Mice were housed for 1 wk before the immunization, given free access to food and water, and maintained in a light/dark cycle with lights on from 6:00 to 18:00 h. All mouse experiments were performed in agreement with the Animal Care and Use Protocol of University of Tehran. The endotoxin level of extracted pVax and pVax/*iutA* was detected by the limulous amoebocyte lysate test (LAL test) according to the manufacturer's standard protocol (Thermo scientific, USA). Experimental mice (n=18) were divided into 3 groups (n=6). The groups 1 to 3 were intramuscular immunized two times with 2 wk interval with 100 µg of pVax vector, pVax/*iutA* and PBS as a control group.

IFN-γ Cytokine assay

One week after the final immunization, a total number of 3×10^6 splenocytes were seeded into each well of a 24-well plate in complete RPMI-1640 medium and stimulated with 10 µg/ml of IutA protein and incubated at 37 °C in 5% CO_2. Three days later, supernatants were collected and IFN-γ cytokine was assessed through commercial ELISA Kits (Mabtech, Sweden) according to the manufacturer's instructions. The quantity of cytokine was expressed as pg/ml according to the related standard curve.

Statistical analysis

All experiments were performed in triplicate, and the data was expressed as means ±S.D for each experiment. All statistical analyses were carried out by the t-test, one way and two way ANOVA

test and SPSS v18 software (Chicago, IL, USA). In all of the cases, P-values<0.05 were considered to be statistically significant.

Results

Construction of pVax/iutA

The *iutA* gene was amplified by PCR and using *E. coli* 35218 chromosomal DNA as the template. The amplified *iutA* gene was blunt end fragment, purified from gel and inserted into EcoRV digested pBluescript cloning vector yielding pBlue/*iutA* gene and protein sequences of *E. coli* CFT073 strain and 35218 strain aligned by using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2).*iutA* gene has some nucleotides variation among enterobacteriaceae like *E. coli*. Five point mutations in 35218 *iutA* gene (result not shown) and one changed amino acid in 35218 *iutA* protein sequence (Table 2) was found. The sequence of *E. coli* 35218 *iutA* gene was shown more than 97% identity to other *iutA* sequence reports in GenBank.

Table 2: Result of *iutA* proteins alignment

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gi|26249458|ref|NP_755498.1|      MAQRQPEKTAAGGCCFNSLYNKYSGITMMRKKYMPRALGPLLLVVLSPAV 50
IutA                              MAQRQPEKTAAGGCCFNSLYNKYSGITMMRKKYMPRALGPLLLVVLSPAV 50
                                  **************************************************

gi|26249458|ref|NP_755498.1|      AQQNDDNEIIVSASRSNRTVAEMAQTTWVIENAELEQQIQGGKELKDALA 100
IutA                              AQQNDDNEIIVSASRSNRTVAEMAQTTWVIENAELEQQIQGGKELKDALA 100
                                  **************************************************

gi|26249458|ref|NP_755498.1|      QLIPGLDVSSQSRTNYGMNMRGRPLVVLIDGVRLNSSRSDSRQLDSVDPF 150
IutA                              QLIPGLDVSSQSRTNYGMNMRGRPLVVLIDGVRLNSSRSDSRQLDSVDPF 150
                                  **************************************************

gi|26249458|ref|NP_755498.1|      NIDHIEVISGATALYGGGSTGGLINIVTKKGQPETMMEFEAGTKSGFNSS 200
IutA                              NIDHIEVISGATALYGGGSTGGLINIVTKKGQPETMMEFEAGTKSGFNSS 200
                                  **************************************************

gi|26249458|ref|NP_755498.1|      KDHDERIAGAVSGGNDHISGRLSVAYQKFGGWFDGNGDATLLDNTQTGLQ 250
IutA                              KDHDERIAGAVSGGNDHISGRLSIAYQKFGGWFDGNGDATLLDNTQTGLQ 250
                                  **********************:***************************

gi|26249458|ref|NP_755498.1|      HSNRLDIMGTGTLNIDESRQLQLITQYYKSQGDDNYGLNLGKGFSAISGS 300
IutA                              HSNRLDIMGTGTLNIDESRQLQLITQYYKSQGDDNYGLNLGKGFSAISGS 300
                                  **************************************************

gi|26249458|ref|NP_755498.1|      STPYVSKGLNSDRIPGTERHLISLQYSDSDFLRQELVGQVYYRDESLRFY 350
IutA                              STPYVSKGLNSDRIPGTERHLISLQYSDSDFLRQELVGQVYYRDESLRFY 350
                                  **************************************************

gi|26249458|ref|NP_755498.1|      PFPTVNANKQATAFSSSQQDTDQYGMKLTLNSQLMDGWQITWGLDAEHER 400
IutA                              PFPTVNANKQATAFSSSQQDTDQYGMKLTLNSQLMDGWQITWGLDAEHER 400
                                  **************************************************
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One amino acid substitution was occurred at Valine 198 to Isoleucine

RT-PCR Assay

Confirmed pVax/*iutA* DNA cassette was transfected to cultured COS7 cell line using 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 fluorescence light inverted microscope (Fig. 1). Total RNA extracted and RT-PCR was done (Fig. 2).

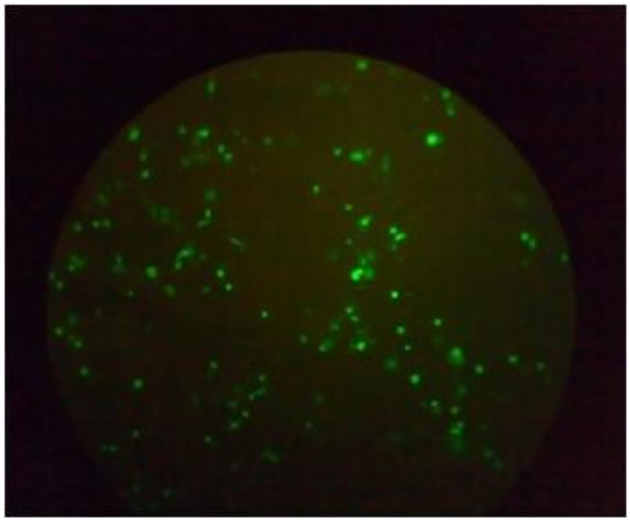

Fig. 1: GFP expression in transfected COS7 as transfection positive control

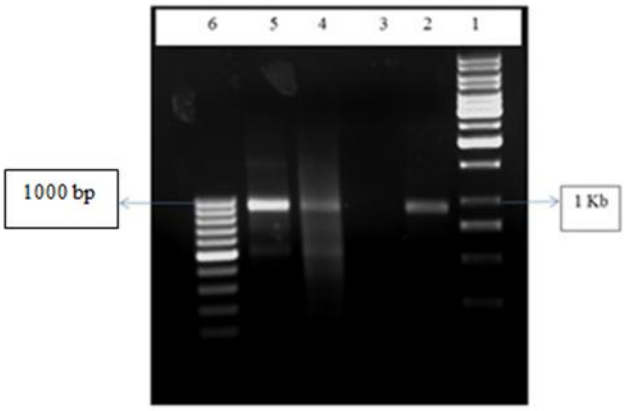

Fig. 2: The result of RT-PCR
Lane 1 (Ladder l kb), Lane 2 (positive PCR for *intA*), Lane 3 (RT-PCR of DNase treated RNA), Lane 4 and 5 (RT-PCR for 24 and 48h transfected cells), Lane 6 (Ladder 100 bp)
Expression of *intA* during 48h was more than 24h (lane 4 and 5), because the intensity of related band of lane 5 on agarose gel was more than the lane 4.

IFN-γ cytokine assay

Assessment of the IFN-γ cytokine in the experimental groups showed that immunization with pVax/*intA* increased the IFN-γ cytokine as compared to the control group which injected with PBS (P=0.0008) (Fig. 3).

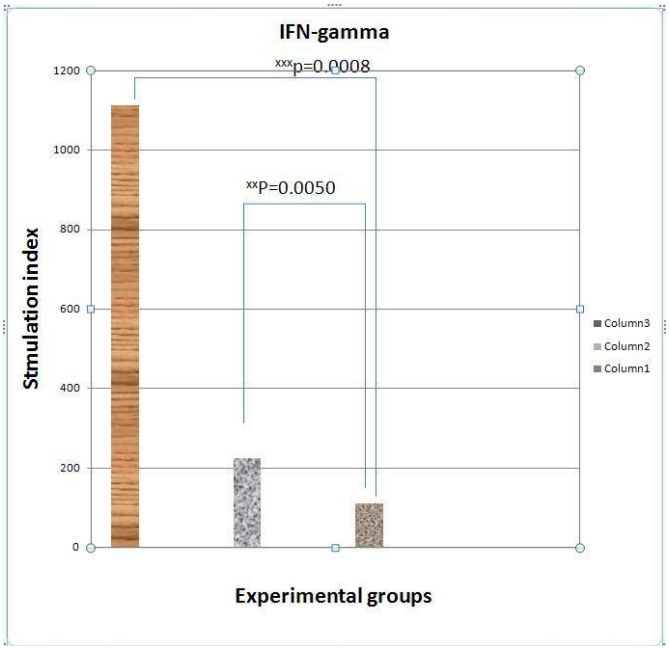

Fig. 3: IFN-γ cytokine assay of immunized mouse groups
Colume 1 showed the stimulation index of IFN-γ cytokine titration of mouse which injected with pVax/*intA* genetic cassette. Colume 2 is related to groups whicg received pVax vector and Colume 3 represent of reaction of groupe which injected with PBS.

Discussion

UPEC pathotype have several virulence factors for establishing UTI in bladder tissue. The first step of pathogenesis is colonization of this pathotype on urinary epithelium (19). In Gram negative bacteria, uptake of ferric siderophores and other iron complexes is facilitated by specific outer membrane receptors. In this regard, a number of studies have pointed to a role of iron acquisition in pathogenicity and using involved proteins as candidate vaccine against UTI. One of the most important outer membrane receptors is IutA, an 84 KDa protein which forming as a trans-membrane receptor and involve in iron acquisition (12).
Russo et al. tested the hypotheses that the siderophore receptor IroN is antigenic and that an IroN-specific antibody response confers protec-

tion in vivo (20). After injection of denatured IroN via subcutaneous route, just immunoglobulin G (IgG)-specific response in serum ($P<0.0001$) was increased without any rising for systemic or mucosal IroN-specific IgA response. UPEC outer membrane iron receptors have a potential role for protecting mouse model against UTI when they are intranasal used as immunogenic protein vaccines (14). Toward this aim, the authors practiced a current plan based on functional vaccinology approach and focused on all factors which engaged in iron acquisition as a major step of UPEC pathogenesis. After whole genome sequencing of *E. coli* CFT073, it was cleared 5379 predicted proteins may express within this genome. These candidate targets were ChuA, Hma, Iha, IreA, IroN, and IutA. Finally, they found that intranasal immunization with Hma, IreA or IutA, induced specific protective antibodies, IL-17 and IFN-γ and provides significant protection against experimental infection with UPEC (21-24).

In this study, a pVax/*iutA* genetics cassette was constructed and assessed for induction of cellular immune response in mouse model. The pVax/*iutA* was injected to tree groups of mouse. Immunization of mice with pVax/*iutA*, increased lymphocyte proliferation compared to the control groups. Lymphocyte proliferation as a cellular immunity marker, showed the ability of pVax/*iutA* in the induction of cellular immunity.

Cellular immune responses play important roles in the clearance of infection in UTI (11-14) and *IutA*, as a vaccine candidate, can successfully induce cellular immune responses. Cytokine analysis showed that genetics cassette of pVax/*iutA*, as a candidate vaccine, could strongly induce IFN-γ secretion and polarize the Th1 pattern.

Conclusion

The results of cytokine assay confirmed the ability of pVax/*iutA* to induction of Th1 cytokine profile, and the possible potency of this vaccine to raising the protective immune response against UTI. This is the first approach for designing a DNA candidate vaccine based on iron acquisition compound versus UTI.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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