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Protective multi-epitope candidate vaccine for urinary tract infection

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ABSTRACT

Urinary tract infections (UTIs) are induced by exogenous organisms including extraintestinal pathogenic such as *Escherichia coli* (ExPEC), *Proteus mirabilis* and *Klebsiella pneumonia*, which are closely related. These organisms can colonize in the urinary tract and cause UTIs. In this study, a cross-reactive multi-epitope vaccine was designed by two constructs to stimulate the immune system (CD8+ and CD4 + T cells) against ExPEC, *Proteus mirabilis* and *Klebsiella pneumonia* strains.

Uropathogenic *Escherichia coli* (UPEC), *Proteus mirabilis* and *Klebsiella pneumoniae* are the main bacterial cause of UTI. They were used for designing experimental candidate vaccine, and their immunogenicity and protectivity were assessed. In this study, conserved antigens from their bacterial genomes were considered, and informatics-based immunological vaccine with cross-protective T and B-cells epitopes was designed and evaluated. The vaccine candidate was used as a broad immune system inducer, and its cross-protective immunity and protectivity were confirmed in *in vivo* experiments.

1. Introduction

Urinary tract infection (UTI) is one of the most common infectious diseases [1]. As one of the common colonizer organisms in the human gastrointestinal tract, *Escherichia coli* is the major cause of UTIs being responsible for 90 % of the cases [2]. Microbial pathogens, such as extraintestinal pathogenic *E. coli* (ExPEC), along with other family members of *Enterobacteriaceae, Proteus mirabilis* and *Klebsiella pneumoniae* have been recognized as the common causes of infectious disease in the urinary tract in the community and healthcare settings [3]. Urinary tract infection (UPEC) pathogens can result in a wide range of infections. ExPEC strains account for over 80 % of UTI cases; the intestinal pathogenic *E. coli* group including many *E. coli* pathotypes such as enterotoxigenic (ETEC), enteropathogenic, enterohemorrhagic (EHEC), enteroinvasive, adherent invasive, and diffusely adherent *E. coli*, may lead to infections in the human intestinal tract [1]. *P. Mirabilis* is responsible for 1–10 % of all recurrent UTIs; this pathogen is difficult to treat as 48 % of its strains are resistant to broad-spectrum of antibiotics [4–6]. Acquisition of antibiotics resistance can also derive from a wide range of *K. pneumoniae* strains [7]. As a consequence of antibiotic resistance, infections such as UTIs have become resistance to treatment giving rise to serious infections which may be life-threatening [7].

The majority of UTIs can be treated by antibiotics, but the drug resistant strains have raised a major challenge in treatment of UTI. This has increased the concerns on the deficient response to antibiotics in complicated UTIs, recurrent UTIs, bacteremia, and sepsis. Therefore, there is a need to develop efficient vaccines to prevent from UTIs. Development of conventional vaccines such as whole cell organisms (inactivated or live attenuated) are not recommended due to unnecessary antigenic load which may lead to the non-specific immune responses [8–10]. A subunit-based vaccine is a peptide-based or epitope-driven vaccine with different antigenic epitopes [8]. Peptide-based vaccines are highly specific and easy to design and formulate compared to whole cell or subunit vaccines [8–10]. With progress of bacterial whole genome sequencing and advances in bioinformatics, reverse vaccinology has been revolutionized [11,12]. Reverse vaccinology targets antigens based on their microbial genome sequence analysis. Vaccination inducing considerable protective immune response could not elicit with an antigen alone [13]. Therefore, the use of multi-subunit or multi-peptide approaches could be advantageous for delivery of high doses of antigens to develop protective immune responses [14]. Using reverse vaccinology in ExPEC and subtractive analysis of nonpathogenic antigen strains analysis, 9 antigens were found to be protective in a mouse challenge model [15]. In this way, development of a broadly protective *E. coli* vaccine could be possible [16]. The gene encoding showed the most of highly

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conserved sequence and protective antigen in most of *E. coli* isolates [15].

In this study, bacteria causing UTI were considered as the most commonly bacterial pathogens: uropathogenic *Escherichia coli* (UPEC), *Proteus mirabilis* and *Klebsiella pneumoniae*. The bacterial virulence factors, such as adhesion, toxins, iron acquisition proteins, metabolic enzymes and structural components were considered for vaccine preparation. In this experiment, nine already-detected antigens in UPEC were searched by in silico studies in *Klebsiella pneumoniae*, and *Proteus mirabilis*. The common antigens were selected by various bioinformatics tools and the most common epitopes were selected. The multi-epitopes were synthesized and then expressed in *E. coli*. The immunogenicity and efficacy of the induced humoral and cellular responses (by the constructed fusion protein with adjuvant) was evaluated in mice models. The expressed proteins were then assessed for their *in vivo* protectivity in animal models. The aim was to design a candidate vaccine for stimulating the cellular immune system (CTL and T helper) against ExPEC, *K. pneumonia,* and *P. mirabilis* to concur a broad spectrum of UTI-inducing bacteria and recurring UTI infectious disease.

2. Materials and methods

2.1. Identification and selection of vaccine targets

Epitope prediction and selection was performed on nine candidate proteins [15]. The verified immunogenic epitopes were designed by *in silico* tools using available data from reference sequences [15]. In this experiment, conserved domains for each candidate were obtained from NCBI databases [17]. Epitope prediction was carried out using the IEDB (immune epitope database and analysis resource) server. IEDB is a free resource providing experimental data on characterization of antibody and T cell epitopes in humans, and non-human primates, as well as other animal species. Epitopes involved in infectious disease, allergy, autoimmunity and transplant were included. IEDB tools also assisted in prediction and analysis of B cell and T cell epitopes [18]. MHC class I and II prediction were conducted and the length of amino acid epitopes was selected [18]. Efficient epitopes were determined for each nine candidates. Then the selected epitopes were examined by Blast and total alignment in the whole genome of *K. pneumonia* and *P. mirabilis* strains. Common epitopes in *E. coli, K. pneumonia* and *P. mirabilis* strains were determined.

2.2. T helper and CTL construct

The significant pattern and frequency of each epitope were assessed by evaluating the immunization ability of the designed vaccines. In T helper (Th) and Cytotoxic T Lymphocytes (CTL) construct, the fusion of epitopes was assembled by EpiTool Kit 2.0 server for the novel epitope-based candidate vaccine [19]. In the CTL construct, cholera toxin B subunit was selected as the adjuvant; while domains of flagellin antigen and (universal T helper pan DR epitopes) PADRE sequence served as the carrier adjuvants in T helper construct [20]. Adjuvants enhanced phagocytosis, up-regulation of MHC-II, survival of T cells and memory responses. Effect of linkers on stability, proper folding, structural flexibility and protein functional dynamics was also assessed. Alpha helix-forming linkers with the sequence of EAAAK amino acids linker was chosen as the adjuvant carrier which was connected to both constructs. EAAAK linker can increase the stability and folding of the constructs. Sequences of GPGPG linker were selected in the T helper construct. Physicochemical characteristics of both CTL and T helper constructs were checked by ExPASY server (http://web.expasy.org/protparam/).

The constructs were designed with particular fusion of epitopes with proper adjuvant linkers, and carriers. Physicochemical characteristics, post-translational modifications (PTMs) solubility, allergenicity and antigenicity as well as reverse translation, codon optimization and second and tertiary structure prediction were also assessed for the two designed candidate vaccines (http://www.cbs.dtu.dk/services/, http://www.biocuckoo.org/, and http://web.expasy.org/) [21,22]. Reverse translation and codon optimization were evaluated by mEMBOSS 6.0.1 (http://www.ebi.ac.uk/Tools/st/emboss_backtranseq/) program; while second and tertiary structure prediction was carried out using I-TASSER server (Software Swiss-PdbViewer version 4.1.0).

The GenBank accession numbers were obtained for the newly designed T helper and CTL constructs (MG242033 and MG242034).

2.3. Synthesis of constructs

The designed multi-epitope synthetic genes were synthesized with *HindIII* restriction enzyme sites (Gene Fanavaran). Amplification of the genes was performed by designed forward and reverse primers with Polymerase Chain Reaction (PCR). PCR reactions were performed using Eppendorf thermo-cycler; PCRs were carried out in 50 μl volume containing 3 μl of DNA template, 2 μl of forward primer, 2 μl of reverse primer, 25 μl master mixture and 18 μl DDW (double-distilled water). The PCR condition for amplification of the synthetic gene included initial denaturation for 5 min at 95 °C, followed by 20 cycles involving 1 min at 95 °C, 1 min at 60 °C and a final extension at 72 °C for 1 min. The presence of the amplified products was evaluated by electrophoresis on 1% agarose gel. Plasmid vector, pET28a (Novagen) was digested with the restriction enzymes (Fermentas) and ligated at 22 °C overnight by T4 DNA ligase (Fermentas). The result was assessed by 1% agarose gel electrophoresis. Expression of recombinant proteins containing 6xHis tags in C-terminal was applied as mentioned in previously described protocols [21].

2.4. Bacterial strains

Antigens were selected based on different criteria from *ExPEC* (IHE3034, 536, and CFT073) and *P. mirabilis* HI4320 strain. TOP10 strain was used for the plasmid *Escherichia coli* transformation according to the manufacturer's instructions [21]. Bacterial clones containing the recombinant plasmid were cultured. The plasmids were isolated and the presence of the desired fragment was examined by digestion assay according to the manufacturer's protocol [21]. The Mac Conkey and agarose plates containing kanamycin (100 μg/μl) were used for selection of transformed colonies. The white transformed colonies were selected following an overnight culturing. They were then checked by plasmid extraction and PCR. The cloned fragments were sequenced as the selected recombinant plasmids (MWG DNA sequencing service, Biotech AG). Competent *E. coli* Top10 cells were transformed with the ligation mixture. Recombinant colonies were evaluated in 1% agarose by electrophoresis after digestion with the restriction enzymes (Fermentas).

2.5. PCR amplification

DNA of genomic constructs was transfected to the competent bacteria (Top10). It was then purified from bacteria cultured overnight (at 37 °C in humidified 5% CO2) in LB (Difco). DNA concentration was calculated by optical density determination at 260 nm. Primers were designed in conserved DNA region and the genes were amplified using external primers. For the amplification, 100 ng of DNA was used as template. The amplification enzyme was Vent® DNA polymerase (Invitrogen). PCR conditions were as

Table 1
Sequence of MHC class I and MHC class II inducer epitopes were selected based on the lowest score by IEBD, BioEdit, Kyte and Doolitle programs.

MHC class I antigen 1–9		
Antigen no.	Oligonucleotide sequence	Epitope Score
1.	FIPPFQHFG	9.3
2.	NICAYQFRL	4.5
3.	YMYSRGLGV	0.4
4.	WMAGVNTFI	0.8
5.	SNYFWLRSDITVNEI	1.28
6.	SLGNGVAGV	1
7.	NLFDKTYYT	0.4
8.	ILSDGTNTV	0.5
9.	YLSGYGHHI	0.5

MHC II Antigens 1–9		
Antigen no.	Oligonucleotide sequence	Epitope Score
1.	PDCGLRSTISVISVL	0.98
2.	DQRYISIRNTDTIWL	0.70
3.	CNQLGYMYSRGLGVE	2.05
4.	RNFITGMATAKANQE	1.25
5.	KQLKTILSVDSNYFW	1.04
6.	EARWFSLTTRNVNDG	1.18
7.	QTFMPQSSIASYIGD	0.25
8.	IHLQDCILSDGTNTV	0.54
9.	NDLIMYKAEGNVLSI	0.60

follows: 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 20 s and elongation at 72 °C for 3 min. PCR products were then purified according to the manufacturer's instructions [21].

2.6. Cloning and protein expression

PCR amplification was conducted from the genomic DNA templates cloned in pET28a vector (Novagen); they were then transformed in BL21 (DE3) competent cells for protein expression. Two multi-gene synthetic candidates were cloned and expressed as His-tagged fusion proteins and the proteins purified as previously described [21]. Synthesis of multi-epitope constructs was induced by adding different concentrations of isopropyl-beta-thio galactopyranoside (IPTG). Then, the protein expression was assessed by 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by western blotting using monoclonal HRP-conjugated His-specific-antibody (Cyto Matin Gene). Briefly, recombinant proteins separation was performed on a SDS-PAGE gel and blotting was conducted into a nitrocellulose membrane. The membrane was blocked and the conjugated His-specific antibody was added (1:1000 dilution) for development. The expressed proteins were purified by His-tag affinity chromatography on Ni-NTA column (Qiagen) with a denaturation system applying triton X-114 to remove the lipopolysaccharide (LPS) from the recombinant proteins. The LPS level of the purified proteins was measured using the chromogenic Limulus Amebocyte Lysate test (LAL), according to the manufacturer's protocol (Lonza). Then, the purified proteins were dialyzed against different concentrations of urea and the final concentrations were measured using Bradford assay with concentrated Bradford solution (BioRad).

2.7. Mice immunization and challenge studies

Female BALB/c mice of 6–8 weeks old (10 females per group, at least three groups per antigen) were purchased from Pasteur Institute of Iran. The animal tests were performed according to the European community council directive of 24 November 1986 (86/609/EEC). The mice were divided into 5 groups which underwent the injections (20 μg of recombinant protein in 150 μL of saline solution) three times with 2-week intervals (on days 1, 14, and 28). Negative control mice were immunized with saline solution. Immunized animals were challenged at day 49 with a lethal dose of homologous strains. Heparinised-blood samples were collected from the survived mice 20 h after challenge to determine their bacteremia levels; the mortality was monitored 4 days after challenge. The vaccinated mice were consequently used for cytokine and challenge experiments.

Antigen-specific serum and urine responses including total IgG, IgA and IgG isotypes (IgG1 and IgG2a) were determined by standard enzyme-linked immunosorbent assay (ELISA). In a typical procedure, the ELISA 96-well plates (Greiner, Germany) were coated with purified constructs (Th, CTL, and mixed constructs) (10 μg/mL in PBS) and incubated overnight. The plates were blocked with 3% bovine serum albumin (BSA) and incubated with two-fold serial dilutions of immune serum (1:50–1:6400) and mucosal samples (undiluted, 1:5 and 1:10) in 1% BSA. Afterwards, HPR-conjugated goat anti-mouse IgG, IgG1, IgG2a and IgA (Zymed) were used as secondary antibodies. The plates were incubated with the TMB substrate to visualize the antibody reactivity at 450 nm using an ELISA reader.

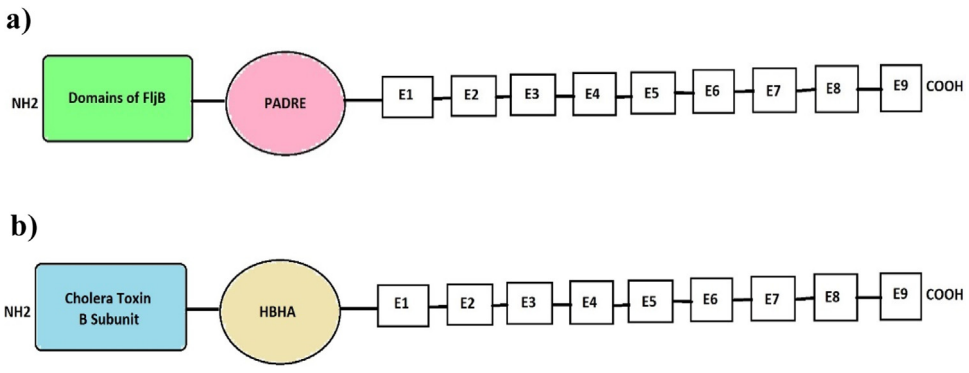

Fig. 1. a) T helper (Th) construct with flagellin (FljB) and PADRE sequence as carrier.
FljB: phase 2 flagellin, strain: *Salmonella enterica* subsp. Enteric aserovar *Typhimurium* str. LT2, TLR5 agonist, gi|16766083|ref|NP_461698.1|
PADRE: Universal T helper pan DR Epitope
Linkers: EAAAK, GPGPG, AAA
b) CTL construct with cholera toxin B subunit carrier.
Cholera toxin B subunit, strain: *Vibrio cholerae* O1 biovar El Tor str. N16961, gi|15641467|ref|NP_231099.1|
HBHA: heparin binding hemagglutinin, strain: *Mycobacterium tuberculosis* H37Rv, gi|15607616|ref|NP_214989.1|, TLR4 agonist

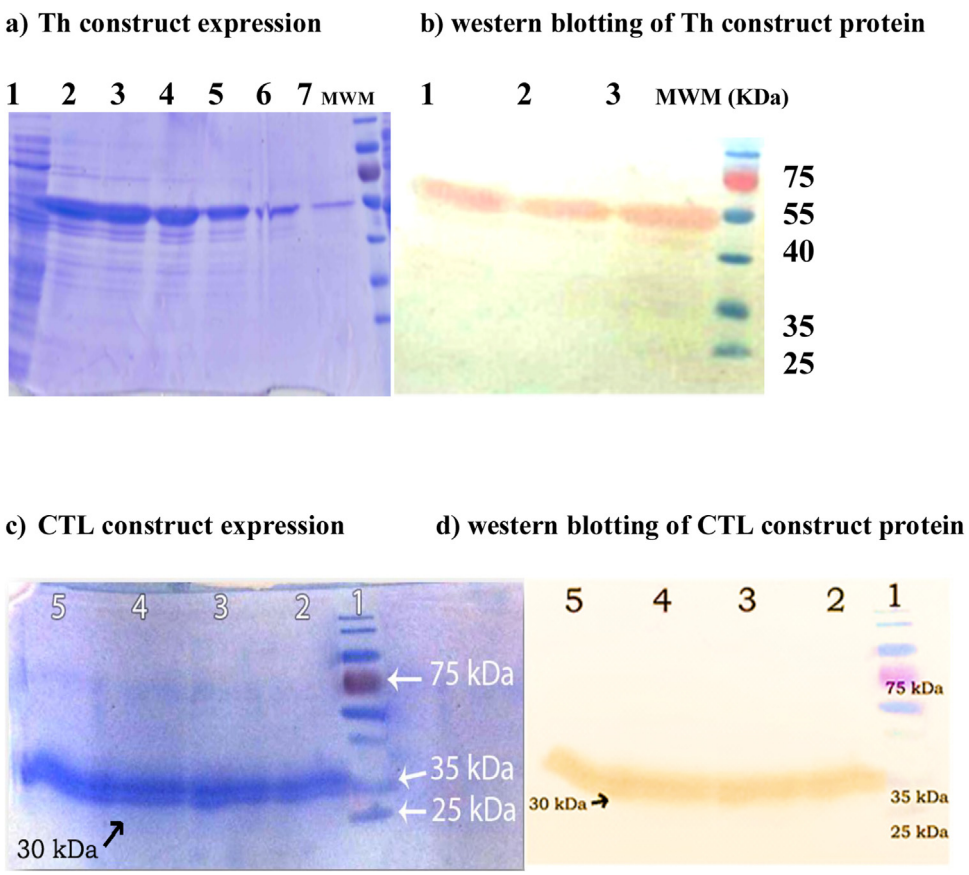

Fig. 2. Expression evaluation of the recombinant *E. coli* cell lysate and purified proteins by SDS-PAGE and western blotting analysis. The recombinant proteins from Th and CTL constructs were purified using the nickel resins and evaluated by Western blot using the His-specific monoclonal antibody.
a) Th construct expression (55 KD); lane 1: Negative control, lane 2–7: protein expression
b) Western blotting of Th construct protein; lane 1–3 protein expression confirmation
c) CTL construct expression (30 KD); lane 1: MW, lane 2–5 protein expression
d) Western blotting of CTL construct protein; lane 1: MW, lane 2–5 protein expression confirmation

2.8. Cytokine assay

Six mice from each group were sacrificed two weeks after the last vaccination to evaluate the production of interferon-γ (IFN-γ), interleukin-4 (IL-4) and IL-17 cytokines using monoclonal antibodies against the cytokines in the supernatant of cultured splenocytes. In Summary, the spleen cells (3 × 105 cell/well) were cultured in 24-well microtiter plates (Greiner, Germany) under sterile conditions. Then 10 μg/mL filtered proteins were incubated for 72 h to collect the supernatants. Finally, the cytokine levels in the supernatants were measured using Mouse DuoSet ELISA kit according to the manufacturer's instructions (R&D Systems).

The protection efficacy of the induced immune responses was examined in the vaccinated groups. In this UTI model, 6 mice in each vaccinated group were anesthetized and their bladders were emptied with gentle pressure and transurethrally challenged with 1 × 108 cfu/mL of bacteria using sterile polyethylene catheter. One week after inoculation, the mice were sacrificed and their homogenized bladders and kidneys were cultured in different dilutions to determine their bacterial loads.

2.9. Statistical analysis

Two-tailed Fisher's exact test was used to compare the number of survived animals in vaccinated groups with those of negative control groups. P values below 0.05 were considered as significant. Analysis of the immune responses was performed by One Way Analysis of Variance (ANOVA), Student t-test, and Tukey HSD tests. Challenge results were analyzed in different groups using Prism (GraphPad) program Version 6, which compared the median results obtained in different groups with the Kruskal-Wallis test (Dunn's multiple comparison tests).

3. Results

3.1. Design and synthesis of multi-epitope candidate vaccine

The virulence *E. coli* antigens were selected based on protective antigens studied in the mouse model [15]. Protective antigens including hypothetical proteins, c0975 (gi|26246865), c1275 (gi|26247149), and c5321 (gi|26251128) from *Escherichia coli CFT073*, bacterial Ig-like domain (group 1) ECOK1_0290 (gi|386598028), general secretion pathway protein K ECOK1_3374 (gi|386600983), putative lipoprotein ECOK1_3385 (gi|386600994), TonB-dependent siderophore receptor ECOK1_3457 (gi|386601066), fimbrial protein ECOK1_3473 (gi|386601082) from *Escherichia coli IHE3034* and hemolysin A (gi|110643969) from *Escherichia coli 536* were considered. Amino acids sequences of the nine antigens were compared between *P. mirabilis* and *K. pneumonia* strains; the most MHC I and MHCII inducers were selected based on the lowest score from IEBD, BioEdit, Kyte and Doolitle programs (Table 1). T helper (Th) and CTL constructs were designed as T helper with flagellin and PADRE sequence linker as a carrier adjuvant and CTL construct with cholera toxin B subunit carrier adjuvant (Fig. 1) [22–26].

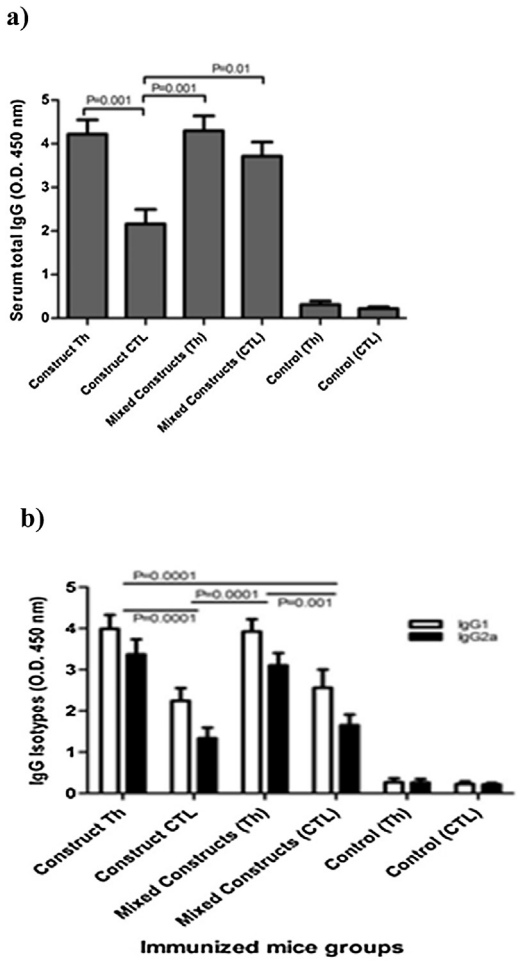

Fig. 3. Evaluation of IgG responses in mice.
Mice were immunized with Th and CTL purified proteins. The control group was injected with PBS. P values were calculated between groups received expressed proteins and a P value below 0.05 were considered as significant.
a) Total IgG antibody response in different groups.
b) Serum IgG1 and IgG2a antibody responses in different groups.
The results are the average of three independent experiments. Bars represent mean ± S.D. from 10 mice per groups at serum dilution1:800.

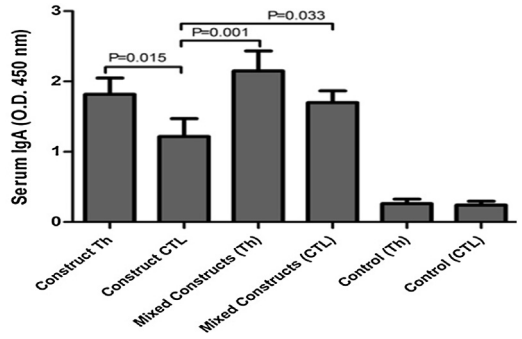

Fig. 4. Evaluation of IgA responses in mice.
Serum IgA antibody response after vaccination in different groups was shown. Mice were immunized with Th and CTL purified proteins. The control group was injected with PBS. P values were calculated between groups received expressed proteins and a P value below 0.05 were considered as significant. The results are the average of three independent experiments. Bars represent mean ± S.D. from 10 mice per groups at serum dilution1:800.

3.2. Expression of candidate vaccine

Multi-epitope Th and CTL constructs were cloned into the pET28a vector. The constructs were expressed and the expression of constructed proteins (Th and CTL) was confirmed by western blotting (Fig. 2a and b). The constructs alone were successfully expressed in *E. coli BL21/plysS* and purified using a nickel chromatography column.

3.3. Antibody production

After immunization, the induced responses were assessed by different dilutions of IgG; total serum (1:50–1:6000) was obtained 2 weeks after the last injection. As shown in Fig. 3a, serum dilutions differentiated between the IgG levels in mice groups (Th and CTL). It was also observed that the IgG response by Th vaccine candidate was higher than CTL construct ($p < 0.001$). In addition, total IgG responses with Mixed Th and CTL constructs was significant comparing with controls (Th or CTL coated plates) ($p < 0.01$) and even the plate was coated with Th though CTL construct as shown in Fig. 3a.

A significant increase can be detected in IgG1 and IgG2 responses of Th ($p < 0.0001$) and CTL groups ($p < 0.001$) as compared with controls (Th or CTL coated plates) (Fig. 3b). However, a significant difference was observed between the IgG1 and IgG2 responses of the mixed Th (Th coated plate) and mixed CTL (CTL coated plate) groups ($p < 0.001$).

IgA antibody was measured in serum samples 2 weeks after the third vaccination. As shown in Fig. 4, in all vaccine combinations, significant responses were induced in Th group ($p < 0.015$) and mixed Th ($p < 0.001$) as compared to the control group. The induced mucosal immune responses were measured in the urine samples after the last immunization.

Considering the role of mucosal responses in the UTIs prevention, total IgG, IgG1, IgG2 and IgA antibody responses were significant in all groups.

3.4. Cytokines immune response

IFN-γ, IL-17 and IL-4 were assessed in all groups. High levels of IL-4, IL-17 and IFNγ were produced in all the immunized groups compared to the controls (Fig. 5). Mice vaccinated with constructs showed cytokines production. While no significant difference was observed in production of IL-4 and IL-17 between the groups, there was a significant release of IFN-γ in the mixed CTL group ($p < 0.009$) compared to CTL group ($p < 0.039$).

3.5. Immune protection and challenge in bladder and kidney

The efficacy of the induced immune responses in bladder and kidney infection was evaluated by the mice groups challenged by standard strains of UPEC, *P. mirabilis*, and *K. pneumonia* infection. As Fig. 6 suggests, the bacterial load in the bladders and kidneys of all groups showed approximately 10–100 folds decreased compared to the controls ($p < 0.05$).

4. Discussion

As a common cause of UTI, *E. coli* is responsible for more than 85 % of UTIs. Their incidence rank differs country by country; however roughly 130–175 million patients are diagnosed with UTI worldwide [27]. Approximately, half of women experience UTI, 25 % of them will suffer recurrent UTI [27]. UTIs can also lead to pyelonephritis, bacteremia, and sepsis. Antibiotic is generally the first and effective step in treatment of infection. However, increase in antibiotic resistance has resulted in recurring infections and

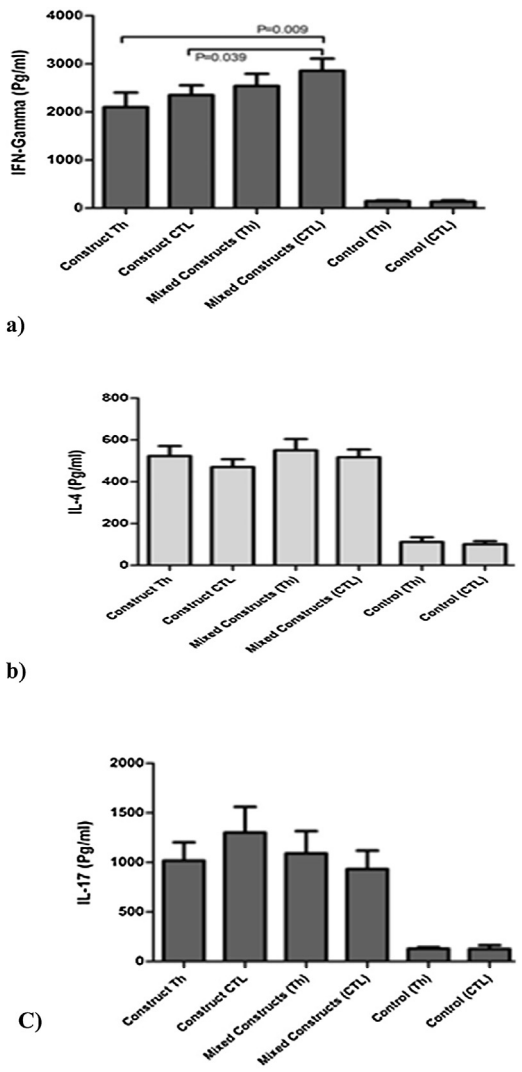

Fig. 5. a) IFN-γ, b) IL-4 and c) IL-17 assessment.
The cytokine response was evaluated in the immunized mice. Splenocytes of each mice group were stimulated and cultured in the presence of expressed proteins. The supernatant of splenocytes cells were collected. Then splenocytes secretion was analyzed with ELISA for a) IFN-γ, b) IL-4 and c) IL-17. The control group was injected with PBS. P values were calculated between groups received expressed proteins and a P value below 0.05 were considered as significant. Results are the mean stimulation index ± S.D. of mice per group from three independent experiments.

alteration of normal gut flora. For more than a century, the purpose of vaccine usage against UTI was basically therapeutic rather than prophylactic. Different types of classical UTI vaccines such as attenuated, inactivated, subunit, toxoid and conjugate have been used so far. However, a limited number of modern vaccines have been tested in humans, and only one is currently commercially available [28]. To the best of our knowledge, there are commercial vaccines such as **Uro-Vaxom** (OM Pharma, consisting of 18 uropathogenic strains), **Solco-Urovac** (a mixture of heat-killed uropathogenic *E. coli;* comprising 6 strains), **Urvakol** and **Urostim** (which contain *E. coli, P. mirabilis* and *E. faecalis* strains but Urvakol also includes a *Pseudomonas aeruginosa* strain, whereas Urostim contains *K. pneumonia)*. Both **Uro-Vaxom** and **Solco-Urovac** vaccines were tested on humans.

Basically, there are two types of UTI vaccines; "whole cell" and "specific-antigen" vaccines. The former includes whole bacteria (either live attenuated or inactivated) or bacterial lysates while the latter encompasses one or more antigens (subunit, toxoid, or conjugate vaccines).

E. coli-induced UTI infections are an important part of vaccine controlling infection. *E. coli* and *K. pneumoniae* are the predominant bacteria involved in recurrent UTI. One of the most effective approaches to prevent UTIs is to design a potent immunogenic vaccine which could be well tolerated and reduce the frequency of UTI recurrence.

Isolated antigens typically do not elicit powerful or long-lasting immune responses; thus their inflammatory response must be increased by enhancing immunogenicity [29]. Moreover, the anticipated UPEC virulence factors were found in about 50 % or fewer of all isolates which would make a multi-subunit vaccine as the most powerful one. Bioinformatics have been also applied to predict B- and T-cell epitopes of antigens to increase the protective immunity, due to the possible role of B-cell (humoral response) and T-cell (cellular response) in protection against UTIs [30].

In our study, the selected common antigen epitopes in virulence pathogenic *E. coli* were compared with the highly conserved epitopes of *P. mirabilis* and *K. pneumoniae* strains. Linkers and carrier adjuvants were selected for multi-domain epitope antigens and their accurate function [31–35]. Adjuvant linkers were used to enhance the immune response and shift the responses to the desired direction as an internal adjuvant [36]. Induction ability of both humoral (Th2) and cellular (Th1) responses were assessed against Th and CTL constructs first computationally and then experimentally. In this study, the multi-epitope vaccine candidates were designed for the most UTI infection coverage. Furthermore, carrier linker adjuvants were also employed as an internal adjuvant for the first time.

The candidate vaccine in this study decreased the renal bacteria load and disease severity in mice after their challenging with a UPEC, *P. mirabilis*, *K. pneumoniae* standard strains. Vaccination managed to protect both the bladder and the kidneys after challenge.

In this experiment, it was found that the designed (Th and CTL) constructs immunization induced IgG1 (Th2) and IgG2a (Th1) responses without using common adjuvants. High levels of IgG1 and IgG2a (the long-lasting antibodies) induction was also observed. Moreover, as IgA is the most effective means of inducing mucosal immunity, it is known to be protective against invasive infection of the gut. In this experiment, antigen-specific IgA responses and protection were also assessed in the UTI mice model. In the present study, the multi-epitope vaccine constructs induced IFN-γ and IL-4 production which are indicators of Th1 and Th2 responses, respectively. A mixture of Th1 and Th2 responses could be also due to the recruitment of the antigen presenting cells (APCs), T CD4+ cells and CD8+ which resulted in high levels of Th1 and Th2 cytokines production. A significant protection was also observed in bladder and kidneys of the models during their challenging with vaccine constructs. The highest protection was detected against UPEC, *P. mirabilis*, *K. pneumoniae* strains in the bladder and kidney. Such significant protection could be attributed to the high mucosal and systemic immune responses. The protection efficacy was lower in kidneys of vaccinated mice compared to their bladder which may be due to the decrease in IL-17 levels or other immunological mechanisms.

Most of tested UTI vaccines use UPEC strains as uropathogenic *E. coli* is responsible for approximately 85 % of uncomplicated UTIs [37]. Development of new and effective therapies is of high priority in UTI research; as such therapies could positively affect the quality of life of millions of individuals and decrease the overall use of antibiotics. The significant contribution of this study is that the new candidate vaccine will likely eliminate the recurrent UTI in patients. In the absence of vaccination, UTI infections pathogens

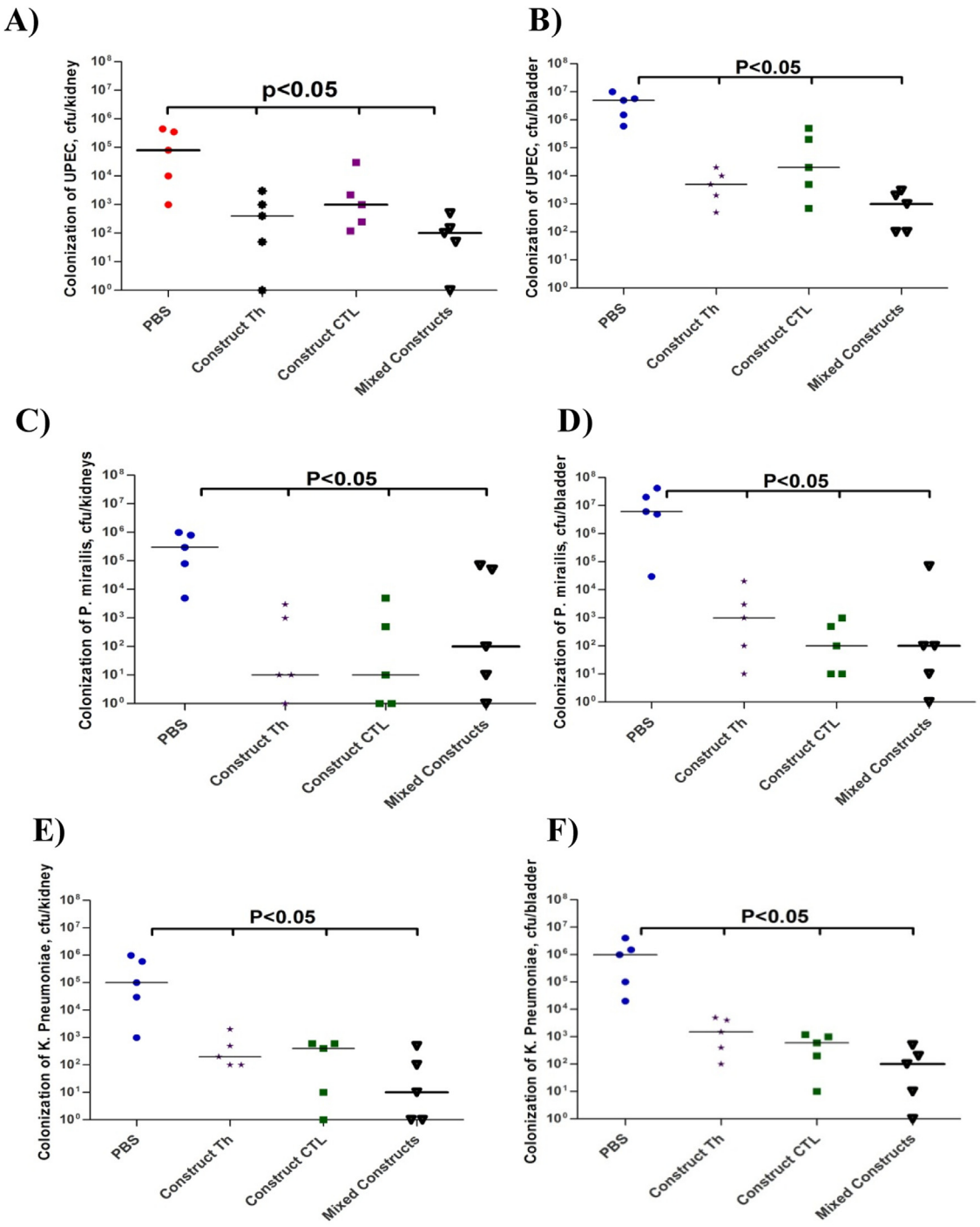

Fig. 6. Evaluation the efficacy of immune responses against UTI.
Immune responses in bladder and kidney infection were evaluated by challenge of the immunized mice by standard strains of UPEC (A, B), *Proteus mirabilis* (C, D) and *Klebsiella pneumonia* (E, F). Two weeks after the last vaccine dose, the bladders of mice (n = 6) were infected with standard strains. One week after challenge, the levels of standard strains in the kidneys (A), (C), (E) and bladders (B), (D), (F) of mice were determined. Solid lines indicate median of the colonization levels. Statistical significance of the differences between mice groups were determined by kruskal-wallis analysis (Dunn's multiple comparison test) and are shown by P value levels. $P < 0.05$ was considered significant.

were able to predominate in the urogenital niche of the subjects, vaccination however overcame this issue.

Moreover, protection was enhanced by substitution of internal adjuvants (*i.e.* addition of cholera toxin adjuvant).

Further randomized controlled trials are necessary to determine the true clinical benefit of candidate vaccine. Recurrent UTI treatment is not adequately addressed by antibiotics; therefore, new vaccines with choice of internal adjuvant can resolve the limitations of vaccine efficacy and success.

5. Conclusions

The present study reports development of a novel multi-epitope candidate vaccine based on the common virulence epitope

antigens of *E. coli*. The efficacy of the mentioned vaccine was significantly shown. Furthermore, the findings of the present study suggest that internal carrier adjuvants could be also used as an alternative for traditional adjuvants especially for vaccine against UTI pathogens. Further clinical trials are required to present the candidate vaccine as a potential powerful candidate against UTI infections.

UTIs are generally induced by exogenous organisms including extraintestinal pathogenic *Escherichia coli* (ExPEC), *Proteus mirabilis* and *Klebsiella pneumonia,* which are closely related strains. These organisms are able to colonize the urinary tract and cause UTIs. In this study, a cross-reactive multi-epitope vaccine was designed by two constructs to stimulate the immune system ($CD8^+$ and $CD4^+$ T cells) against ExPEC, *Proteus mirabilis* and *Klebsiella pneumonia* strains.

We have developed a potential vaccine through *in silico* strategy, using anti-virulent compounds to make a multi-epitope vaccine. The designed constructs managed to induce significant immunity and protection against UTI infection. The candidate vaccine reported here is able to reduce bacterial burdens in a murine model of UTI. The candidate vaccine could provide a potential multi-epitope vaccine to prevent from bacterial pathogenesis and resistant infections.

Contribution to authorship

Study design: MO, SB; recruitment and enrolment of participants: MO; acquisition of data: MO, MRK; bioinformatics: MJ, biostatistical analyses: MRAK; study supervision: SB; drafting of the manuscript: JK, ZH.

Declaration of Competing Interest

The authors reported no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00564.

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