Molecular Immunology 96 (2018) 88–97

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

A novel multi-peptide subunit vaccine admixed with AddaVax adjuvant produces significant immunogenicity and protection against *Proteus mirabilis* urinary tract infection in mice model

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ARTICLE INFO

Keywords:
UTI
Proteus mirabilis
AddaVax
Multi-peptide vaccine
Immune responses

ABSTRACT

Proteus mirabilis is a common pathogen in urinary tract infections (UTIs). There is no vaccine against *P. mirabilis*, thus a novel multi-peptide vaccine of MrpA, UcaA and Pta factors of *P. mirabilis* we designed and a mice model was used to evaluate its efficacy in combination with AddaVax adjuvant. According to the bioinformatics studies, 7 fragments of MrpA (31–75, 112–146), UcaA (68–117, 132–156) and Pta (210–265, 340–400, 496–570) with B and T cell epitope regions were selected for fusion construction. Mice subcutaneously vaccinated with the fusion MrpA.Pta.UcaA induced a significant increase in serum and mucosal IgG and IgA responses. The fusion also showed a significant induction in cellular responses (Th1 and Th2). The addition of AddaVax to fusion and the mixture of MrpA, UcaA, and Pta (MUP) improved the humoral and cellular responses, especially the IgG2a and IFN-γ (Th1 responses) levels. Fusion with and without AddaVax and MUP + AddaVax could maintain significant humoral responses until 6 months after the first vaccine dose. All vaccine combinations with and without adjuvant showed high effectiveness in the protection of the bladder and kidney against experimental UTI; this could be attributed to the significant humoral and cellular responses. The present study suggests that the AddaVax-based vaccine formulations especially the fusion Pta.MrpA.UcaA admixed with AddaVax as potential vaccine candidates for protection against *P. mirabilis*. Furthermore, AddaVax could be considered as an effective adjuvant in designing other vaccines against UTI pathogens.

1. Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases that occur in both community and hospital environments. *Proteus mirabilis* is recognized as a common pathogen in the catheter-associated UTIs (CAUTIs), nosocomial UTIs, and complicated UTIs (Adams-Sapper et al., 2012; Zunino et al., 2007). Among the known virulence factors of *P. mirabilis*, the mannose-resistant *Proteus*-like fimbriae (MR/P) and uroepithelial cell adhesin (UCA, newly renamed as non-agglutinating fimbriae) play important roles in the binding and colonization of the urinary epithelial cells. MrpA and UcaA constitute the major subunits of MR/P and UCA fimbriae, respectively in *P. mirabilis* (Pellegrino et al., 2003; Scavone et al., 2015). Furthermore, another virulence factor of *P. mirabilis* which is characterized by its cytotoxic role in the formation of pore in the epithelial cells of the host is Proteus toxic agglutinin (Pta) (Alamuri et al., 2009).

The majority of UTIs are always treated with the administration of antibiotics, but the emergence of multi-drug resistant strains is becoming a major problem in the treatment of UTIs. Furthermore, the deficient response to antibiotics in complicated UTIs, recurrent UTIs, and the ability of *P. mirabilis* to cause acute pyelonephritis, stone formation, bacteremia, and sepsis are other major challenges (Armbruster and Mobley, 2012; Scavone et al., 2011). These challenges show the need to work on the development of vaccines for the prevention of UTIs

Abbreviations: UTI, urinary tract infection; CAUTI, catheter-associated urinary tract infection; MR/P, mannose-resistant *Proteus*-like fimbriae; UCA, uroepithelial cell adhesin; Pta, proteus toxic agglutinin; MUP, MrpA UcaA and Pta; GPGPG, Gly-Pro-Gly-Pro-Gly; MHC, major histocompatibility molecules; PCR, Polymerase Chain Reaction; IPTG, isopropyl-beta-thio galactopyranoside; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; LPS, lipopolysaccharide; LAL, Limulus Amebocyte Lysate; S.C, subcutaneous; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; HRP, horseradish peroxidase; TMB, 3,3′,5,5′tetramethylbenzidine; IFN-γ, interferon-γ; IL-4, interleukin-4; CMI, cell mediated immunity; I.N, intranasal; APC, antigen presenting cell; CT, cholera toxin

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https://doi.org/10.1016/j.molimm.2018.03.001
Received 17 October 2017; Received in revised form 23 February 2018; Accepted 2 March 2018
Available online 09 March 2018
0161-5890/

caused by *P. mirabilis* (Alamuri et al., 2009).

The pathogenesis of bacteria does not rely on a particular virulence factor, thus in most cases, vaccination with an antigen alone could not elicit a protective immune response (Skwarczynski and Toth, 2016). In this regard, to induce considerable immune responses, the use of multi-subunit or multi-peptide approaches is suggested. A multi-epitope or peptide-based vaccine has the advantages of delivering high doses of antigens for developing the immune responses while avoiding potentially hazardous and undesirable side effects for killed and attenuated vaccines (Li et al., 2012; Purcell et al., 2007).

To date, flagellin, killed *P. mirabilis* strain, purified MR/P fimbriae, recombinant MrpH and Pta, MrpA expressed in *Lactococcus lactis* and *Salmonella typhimurium*, and a complex of recombinant MrpA, UcaA and PmfA were previously considered as vaccine candidates against *P. mirabilis* (Alamuri et al., 2009; Habibi et al., 2015a; Li et al., 2004; Pellegrino et al., 2003; Scavone et al., 2007; Scavone et al., 2004; Scavone et al., 2011). Limitations such as the phase variation of MR/P (and MrpH) and flagellin suggest that these proteins have been used in combination with other proteins (Alamuri et al., 2009). Presently, no multi-epitope or peptide based vaccine has been developed against *P. mirabilis* (Scavone et al., 2004), whereas *P. mirabilis* strains contain different virulence factors which could be applied in a multi-peptide vaccine. These selected peptides should preferentially be surface exposed, be highly antigenic, have important role in pathogenicity, and contain conserved sequences among *P. mirabilis* strains (Haugen et al., 2007; Wieser et al., 2010). The highly conserved nature of MrpA, UcaA and Pta components and their important roles in cystitis and pyelonephritis make them promising candidates against UTI by *P. mirabilis* (Pellegrino et al., 2003; Scavone et al., 2015).

Thus, the aim of the present study was to design a novel multi-peptide vaccine containing both T and B-cell epitopes of MrpA, UcaA and Pta proteins of *P. mirabilis*. Then, the immunogenicity and efficacy of the induced humoral and cellular responses by the constructed fusion protein with and without AddaVax adjuvant was evaluated in the mice model.

2. Materials and methods

2.1. Sequence, databases and construct design

The full-length protein sequences of MrpA (GenBank: CAA83633.1), UcaA (GenBank: CAA54703.1) and Pta passenger domain (GenBank: CAR44626.1) of *P. mirabilis* HI4320 strain were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/protein/). BLASTP was used to recognize common and conserved antigenic fragments among these sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The location of signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP) and PREDTMBB was used to determine the extracellular regions of Pta passenger domain (http://bioinformatics.biol.uoa.gr/PRED-TMBB/). All the protein sequences were analyzed to identify the best fragments containing combined B and T cell epitopes. The selected antigenic fragments were fused together by a repeat of the GPGPG (Gly-Pro-Gly-Pro-Gly) linker. Finally, the fused antigen was characterized using the VaxiJen 2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html).

2.2. Prediction of B- and T- cell epitopes for fusion construction

Protein sequences of MrpA, UcaA and Pta passenger domain were used to predict linear B-cell epitopes (20mers) using ABCpred (http://www.imtech.res.in/raghava/abcpred/), BCPred (http://ailab.ist.psu.edu/bcpreds/predict.html) (EL-Manzalawy et al., 2008) and BepiPred (http://www.cbs.dtu.dk/services/Bepipred/) servers. The B cell epitopes were predicted using three servers with cutoff score > 0.8. Propred-1 (47 MHC Class-I alleles) (http://www.imtech.res.in/raghava/propred1/) (Singh and Raghava, 2003), Propred (51 MHC Class-II alleles) (http://www.imtech.res.in/raghava/propred/) (Singh and Raghava, 2001) and MHCPred (http://www.ddg-pharmfac.net/mhcpred/MHCPred/) (Guan et al., 2003) servers were used to identify T-cell epitopes containing B-cell epitope sequences, which could interact with both major histocompatibility molecules (MHC) class I & II with the highest score (more than 15) and further analyzed with VaxiJen (threshold > 0.4). Among the different haplotypes of MHC in mice, those containing H2d haplotypes were selected. These alleles contained I-Ad and I-Ed of class II as well as H2-Kd, H2-Ld, H2-Dd of class I MHC (Hood et al., 1983). The sequences of peptides binding to various mice MHC alleles were obtained from the RANKPEP (http://bio.dfci.harvard.edu/RANKPEP/) and SYFPEITHI databases. In RANKPEP, MHC-II restricted epitopes with high score were predicted at 4–6% binding threshold. In RANKPEP, MHC-I restricted epitopes with significant score were predicted at 2–3% binding threshold. Score > 20 was assumed as strong binding in the SYFPEITHI database.

2.3. Cloning, expression, and protein purification

The designed fusion gene consisting of *mrpA*, *ucaA* and *pta* epitopes was codon optimized and synthesized by Biomatik Company (Canada) and then cloned within the expression vector pET28a (Novagen, USA). Furthermore, the cloning of full-length *mrpA* (175 aa) and *ucaA* (180 aa) and also the passenger domain of the *pta* (524 aa) was performed in accordance with previous protocols (Asadi et al., 2012). Briefly, these genes in *P. mirabilis* HI4320 strain were amplified using Polymerase Chain Reaction (PCR), having primers designed with the restriction enzymes *Nco*I and *Hind*III. Thereafter, the digested fragments were cloned into the digested pET28a vector and transformed into the competent *E. coli* BL21/plysS host (Invitrogen, USA). Positive clones were confirmed by PCR, double digestion and sequencing. Synthesis of recombinant proteins fusion, MrpA, UcaA, and Pta alone was induced by adding different concentrations of isopropyl-beta-thio galactopyranoside (IPTG) in different concentrations (Asadi et al., 2012; Habibi et al., 2017). 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 (Sigma, USA). Briefly, after separation of recombinant proteins on a SDS-PAGE gel and their blotting into a nitrocellulose membrane, the membranes were blocked and the conjugated His-specific antibody was added in 1:1000 dilution to develop by DAB + H_2O_2 substrate (Asadi et al., 2012).

In the next stage, the expressed proteins were purified by His tag affinity chromatography on Ni-NTA column (Qiagen) with a denaturation system and by applying triton X-114 for removing the lipopolysaccharide (LPS) of the recombinant proteins (Habibi et al., 2015b). 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.

2.4. Animals

Female BALB/c mice of 6–8 weeks old were purchased from the Pasteur Institute of Iran and all the animal tests were performed according to the European Communities Council directive of 24 November 1986 (86/609/EEC).

2.5. Subcutaneous immunization of mice

The mice were divided into five groups of each 15. The injections were given three times within 2-week intervals (on days 0, 14, and 28) from a subcutaneous (S.C.) route with 50 µg of fusion protein alone, fusion protein admixed with AddaVax (50 µg protein + 50 µl adjuvant),

and a combination of purified proteins MrpA, Pta and Uca with AddaVax (17.5 µg of each protein + 50 µl adjuvant) (Habibi et al., 2015a). AddaVax was prepared following the manufacturer's instructions (InvivoGen). The control groups (negative controls) were injected with AddaVax or PBS alone. Sera were collected after each injection until 180 days to determine the humoral responses. The vaccinated mice were consequently used for cytokine and challenge experiments.

2.6. Antibody assay in the immunized mice

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). Briefly, the ELISA 96-well plates (Greiner, Germany) were coated with purified proteins MrpA, UcaA, and Pta (10 µg/ml in PBS) and incubated overnight. The boundless positions in plates were blocked with 3% bovine serum albumin (BSA) and the plates were incubated with twofold serial dilutions of immune serum (1:50–1:6400) and mucosal samples (undiluted, 1:5 and 1:10) in 1% BSA. Thereafter, HPR-conjugated goat anti-mouse IgG, IgG1, IgG2a and IgA (Sigma, USA) were used as secondary antibodies. The plates were incubated with the TMB substrate to visualize antibody reactivity at 450 nm using an ELISA reader (Habibi et al., 2015a; Li et al., 2004).

2.7. Cytokine assay in the vaccinated mice

Six mice from each group were sacrificed two weeks after the last vaccine dose 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. Briefly, the spleen cells (3×10^5 cell/well) were cultured in 24-well microtiter plates (Greiner, Germany) under sterile conditions and incubated with and without 10 µg/ml of filtered proteins MrpA, UcaA, and Pta to collect the supernatants after 72 h of incubation. Finally, the cytokine levels in the supernatants were measured using the Mouse DuoSet ELISA kit following the manufacturer's instructions (R&D Systems, USA) (Habibi et al., 2015a).

2.8. Urinary tract infection model in mice

The protection effectiveness of induced immune responses was assayed in the vaccinated groups using the bladder challenge model. This model was optimized in our animal facility with naïve mice to achieve the best time period for the challenge assay. Briefly, in this ascending UTI model, six mice in each vaccinated group were anesthetized; their bladders were emptied with gentle pressure and challenged transurethrally with 1×10^8 cfu/ml of *P. mirabilis* HI4320 strain using sterile polyethylene catheter (Habibi et al., 2015a). One week after inoculation of *P. mirabilis*, the mice were sacrificed and their homogenized bladders and kidneys were cultured in different dilutions to determine the bacterial loads in these plates.

2.9. Statistical analysis

Statistical analysis of the antibody and cellular immune responses were performed using One Way Analysis of Variance (ANOVA), Student *t*-test, and Tukey HSD tests. Challenge results were analyzed in different groups with Version 6 Prism (GraphPad) program, which compare the median results obtained in different groups with the Kruskal-Wallis test (Dunn's multiple comparison tests). In all statistical analyses, the value of $p < 0.05$ was considered as the statistical index.

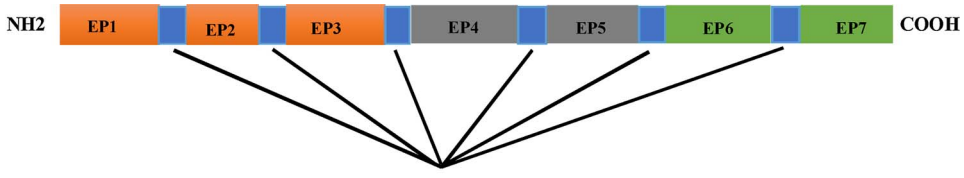
3. Results

3.1. Defining the B-cell and T-cell epitopes of proteins

MrpA, UcaA and N-terminal passenger domain of the Pta protein were selected for the construction of a fusion gene. The topology of the Pta passenger domain was predicted using PRED-TMBB. Graphical position of the transmembrane ß strands and the location of the periplasmic and extracellular loops are mentioned in Fig. S1 in the Supplementary Material. Then, B-cell epitopes in each antigen were predicted by ABCpred, BCPred and BepiPred servers. Generally, 20-mer linear epitopes with scores > 0.8 in ABCpred and BCpreds were selected from MrpA, UcaA and Pta antigens and these data are shown in Table 1 in the Supplementary Material. To screen the T-cell epitopes, Propred-I, Propred, and MHCPred were used and data about the T-cell predictions of antigens are shown in Table 2 in the Supplementary Material. Finally, after analyzing the results, 7 fragments were selected including: MrpA (31–75, 112–146), UcaA (68–117, 132–152), and Pta passenger domain (210–265, 340–400, 496–570) with B and T cell epitope regions expected to be strong antigenic fragments. These fragments were sequentially connected by the GPGPG linker to form a fusion protein, which contained 373 amino acids. A schematic diagram of the protein domain structures with linkers is shown in Fig. 1. Furthermore, the composition of the designed multi-peptide vaccine candidate along with their various parameters is shown in Table 1.

3.2. Cloning, expression, and protein purification

In this study, a fusion gene of the selected epitopes of MrpA, UcaA and Pta was constructed and cloned into the pET28a vector. Subsequently, *mrpA*, *ucaA* and *pta* genes were cloned successfully into the vector and confirmed by different methods. Fusion *pta.mrpA.ucaA* and the genes alone were successfully expressed in *E. coli* BL21/plysS and purified using a nickel chromatography column. As shown in



EP1: Epitope of Pta passenger domain (AA $_{340\text{-}400}$)
EP2: Epitope of Pta passenger domain (AA $_{210\text{-}265}$)
EP3: Epitope of Pta passenger domain (AA $_{496\text{-}570}$)
EP4: Epitope of MrpA (AA $_{31\text{-}75}$)
EP5: Epitope of MrpA (AA $_{112\text{-}146}$)
EP6: Epitope of UcaA (AA $_{68\text{-}117}$)
EP7: Epitope of UcaA (AA $_{132\text{-}152}$)

Fig. 1. Schematic representation construct consists of MrpA, UcaA and Pta epitopes of the *P. mirabilis* HI4320 strain linked together with linkers.

Table 1
Composition of the designed multi-peptide vaccine candidate along with their various parameters.

Target protein	Subfragment no.	Position (amino acid)	No. of amino acid	No. of B cell epitopes	No. of putative epitopes (Human)		No. of putative epitopes (mice)		VaxiJen scores
					MHC-I	MHC-II	MHC-I	MHC-II	
MrpA	1	31–75	45	4	4	3	1	5	0.4559
	2	112–146	35	1	3	3	1	1	
UcaA	1	68–117	50	2	1	2	1	1	1.105
	2	132–152	21	1	1	1	1	1	1.3424
Pta	1	210–265	56	4	3	5	2	4	0.8244
	2	340–400	61	1	2	9	1	5	0.5327
	3	496–570	75	4	3	7	2	7	0.7046

Fig. 2A, the SDS-PAGE results of the fusion, MrpA, UcaA, and Pta purified proteins showed that the expressed proteins had molecular weights of approximately 44, 18, 22, and 60 kDa confirmed by the use of Western blot (Fig. 2B). The endotoxin level of the purified and dialyzed proteins was less than 1 EU/ml measured using the LAL test.

3.3. Assessment of total IgG and longevity of responses

At first, the induced IgG responses were assayed in different dilutions of serum (1:50–1:6400) obtained 2-weeks after the last vaccine dose. As shown in Fig. 3A–C, lower serum dilutions could not differentiate between the IgG levels of mice groups, while the dilution of 1:800 could differentiate between the IgG levels of the mice groups. It was observed that IgG responses against Pta (A), MrpA (B) and UcaA (C) in all vaccine combinations with and without AddaVax were significantly enhanced as compared to the control mice which received the AddaVax or PBS ($p < 0.001$). Furthermore, the addition of AddaVax to fusion Pta.MrpA.UcaA ($p < 0.05$) or the mixture of MrpA, UcaA and Pta (MUP) ($p < 0.01$) resulted in a significant increase of IgG responses than fusion protein alone (Fig. 3). However, there was no significant difference between the IgG responses of the Fusion + AddaVax and MUP + AddaVax groups ($p > 0.05$).

The longevity results of humoral responses showed that anti-Pta, MrpA and UcaA responses were increased after the first vaccine dose in mice received the fusion, fusion + AddaVax and MUP + AddaVax until day 110, and the immune responses were maintained in a steady state until day 180 after the first immunization (Fig. 4A–C). Furthermore, a significant difference was observed between the induced responses by the vaccine formulations with control groups after the second vaccine dose, which was maintained throughout the study ($p < 0.001$). The significant difference between IgG responses of the Fusion alone with Fusion + AddaVax and MUP + AddaVax groups was found after the third vaccination until day 180 ($p < 0.05$).

3.4. Measurement of isotype antibodies in serum

The isotype antibodies IgG1, IgG2a and IgA were measured in serum 2-weeks after the third vaccination. As shown in Fig. 5A–C, all vaccine combinations with and without adjuvant were able to induce significant IgG1 and IgG2a responses against antigens Pta (A), MrpA (B) and UcaA (C) as compared to the control mice ($p < 0.01$). The addition of AddaVax to the vaccine combinations of fusion alone ($p < 0.01$) and the mixture of Pta, MrpA and UcaA significantly boosted the IgG2a responses ($p < 0.02$), whereas AddaVax could not significantly enhance the IgG1 responses of these combinations ($p > 0.05$) (Fig. 5A–C). The IgG1/IgG2a ratio in mice inoculated with AddaVax formulations were significantly reduced as compared to mice immunized without AddaVax ($p < 0.05$) (Fig. 5A–C).

All the vaccine combinations developed a significant anti-IgA serum response as compared to mice injected with AddaVax or PBS ($p < 0.001$) (Fig. 5D). Similar to the IgG1 responses, AddaVax could significantly improve the IgA responses of fusion and MUP vaccine combinations ($p < 0.01$).

3.5. Mucosal responses in the mice groups

According to the role of mucosal responses in the prevention of UTIs, the induced mucosal responses were measured in the urine samples obtained after the last immunization dose. As shown in Table 2, fusion with and without AddaVax and MUP + AddaVax significantly showed higher mucosal IgG and IgA levels compared to the control mice ($p < 0.05$). AddaVax was also observed to significantly increase the mucosal IgG responses of all antigens than the fusion alone ($p < 0.05$), whereas AddaVax was only able to significantly increase the mucosal anti-UcaA IgA of mice vaccinated with the MUP mixture than mice immunized with fusion alone ($p = 0.045$) (Table 2). Furthermore, there was no significant difference in the induced IgG or IgA mucosal responses between fusion + AddaVax and MUP + AddaVax vaccine combinations ($p > 0.05$).

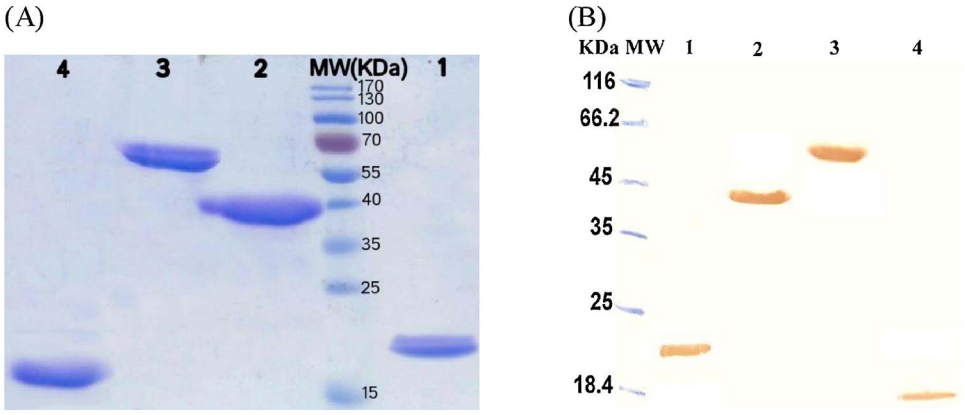


Fig. 2. Evaluation of the purified proteins. The recombinant proteins MrpA, UcaA, Pta and fusion were purified using the nickel resins and evaluated by (A) SDS-PAGE and (B) Western blot using the His-specific monoclonal antibody. Lane 1: UcaA; Lane 2: Fusion; Lane 3: Pta; Lane 4: MrpA; and MW: protein marker (prestained and non-prestained for SDS-PAGE and Western blot, respectively).

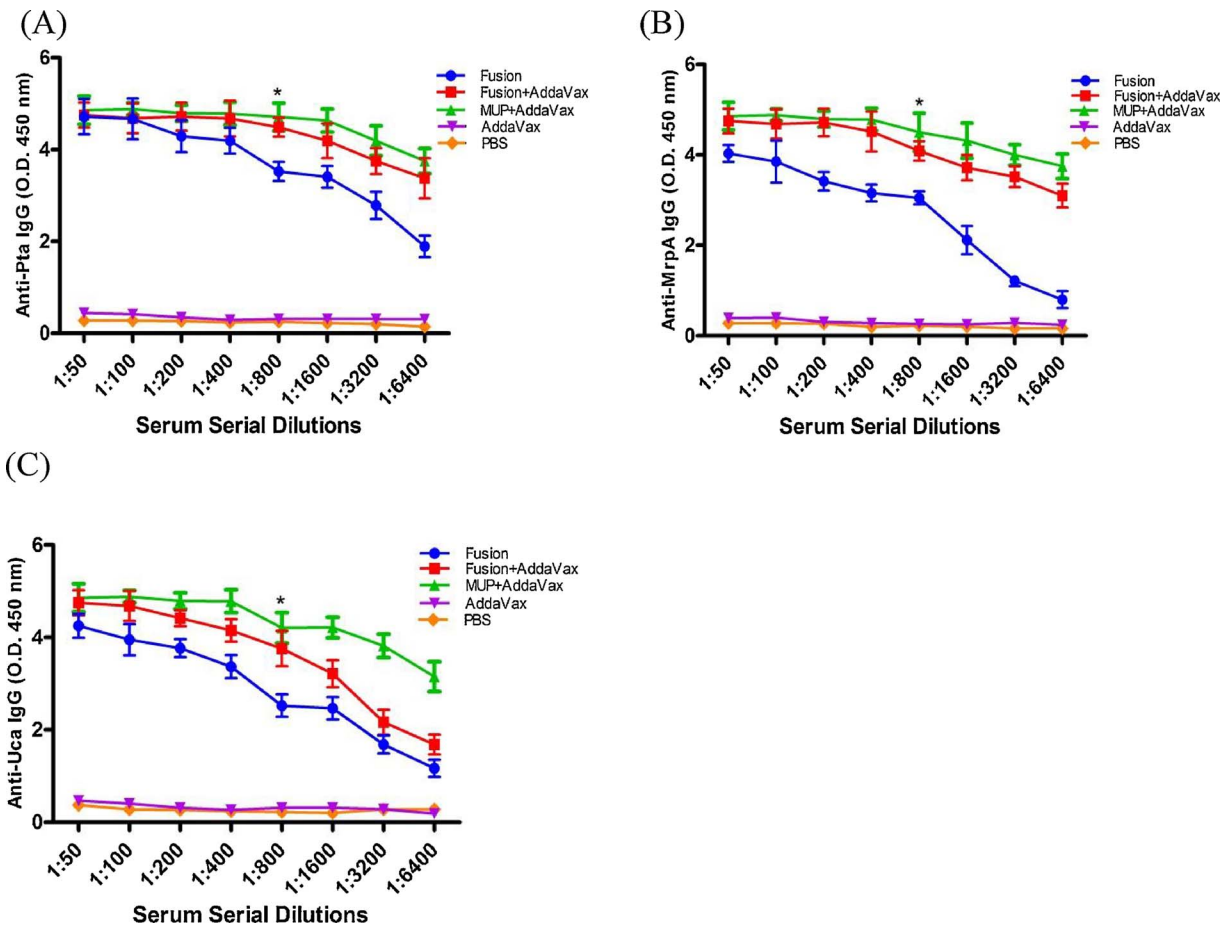


Fig. 3. Measurement of the serum IgG responses. Mice were immunized with the fusion, fusion + AddaVax and the mixture of MrpA, UcaA and Pta (MUP) with AddaVax and IgG responses were measured 2-weeks after the third vaccine dose. The control groups received the AddaVax or PBS alone. Results are mean and standard errors of three independent experiments at different serum dilutions (1:50 to 1:6400). The single asterisks show statistical significance of IgG responses of all groups over the control mice, and AddaVax combinations than fusion alone in serum dilution 1:800.

3.6. Cytokine responses in the immunized mice

In this study, the measurement of cytokines levels was done to determine the cell mediated immunity (CMI) induced by the vaccine combinations. The secretion of IFN-γ, IL-4 and IL-17 in the splenocytes of all mice groups stimulated with Pta, MrpA and UcaA was increased compared to the control mice ($p < 0.05$) (Fig. 6). The findings showed that a higher level of IFN-γ production in mice groups received MUP + AddaVax and fusion + AddaVax compared to mice injected with fusion alone ($p < 0.05$), whereas the difference between the IFN-γ levels of mice groups MUP + AddaVax and fusion + AddaVax was not statistically significant ($p > 0.05$) (Fig. 6A). Although, AddaVax was found to enhance the IL-4 secretion of the vaccine combinations, these increases were not statistically significant as compared to the fusion alone ($p > 0.05$) (Fig. 6B). Interestingly, immunization with fusion + AddaVax combination resulted in significantly higher IL-17 levels compared to the fusion protein alone ($p < 0.001$) and MUP + AddaVax group ($p < 0.01$). In addition, there was no significant difference between the levels of IL-17 in comparison of MUP + AddaVax group with fusion alone ($p > 0.05$) (Fig. 6C). The addition of AddaVax to the fusion alone group could not significantly enhance the IFN-γ/IL-4 ratio ($p > 0.05$). Also, no significant difference was observed between the IFN-γ/IL-4 ratio in Pta or UcaA-stimulated splenocytes of mice vaccinated with fusion alone and MUP + AddaVax ($p > 0.05$) (Fig. 6A), whereas the ratio in MrpA-stimulated splenocytes of mice immunized with MUP + AddaVax significantly reduced compared to mice injected with fusion protein alone ($p < 0.05$).

3.7. Results of bladder challenge in the mice model

In mice, the efficacy of protective immune responses against experimental UTI was evaluated by bladder challenge. After optimization of challenge with *P. mirabilis* HI4320 strain, the naïve mice were observed to be colonized with about 10^7 and 10^5 cfu of bacteria in the bladder and kidneys, respectively after 1 week post bladder challenge. It was also observed that all vaccine combinations with and without AddaVax reduced the load of *P. mirabilis* to about 10^3–10^6 CFU in both the bladders and kidneys of mice as compared to the control mice (Fig. 7). The addition of AddaVax to fusion vaccine formulation decreased the mean of bacteria from 219 to 119 in the bladder and from 1425 to 52 in kidneys, but these reductions were not statistically significant ($p > 0.05$) (Fig. 7). Furthermore, no significant difference was observed between the mean and median of bacteria recovered from the bladders and kidneys of mice vaccinated with MUP + AddaVax as compared to the fusion alone and fusion + AddaVax ($p > 0.05$) (Fig. 7).

4. Discussion

Proteus mirabilis strains cause UTIs, particularly in patients who are obligated to use urinary catheters during their treatment period (Jacobsen and Shirtliff, 2011; Scavone et al., 2011). Despite the importance of *P. mirabilis* and emergence of multi-drug resistant strains, there are several vaccine candidates based on the monovalent factors or fusion proteins tested by our research team (Habibi et al., 2015a; Habibi et al., 2015b) and by others (Alamuri et al., 2009; Li et al., 2004;

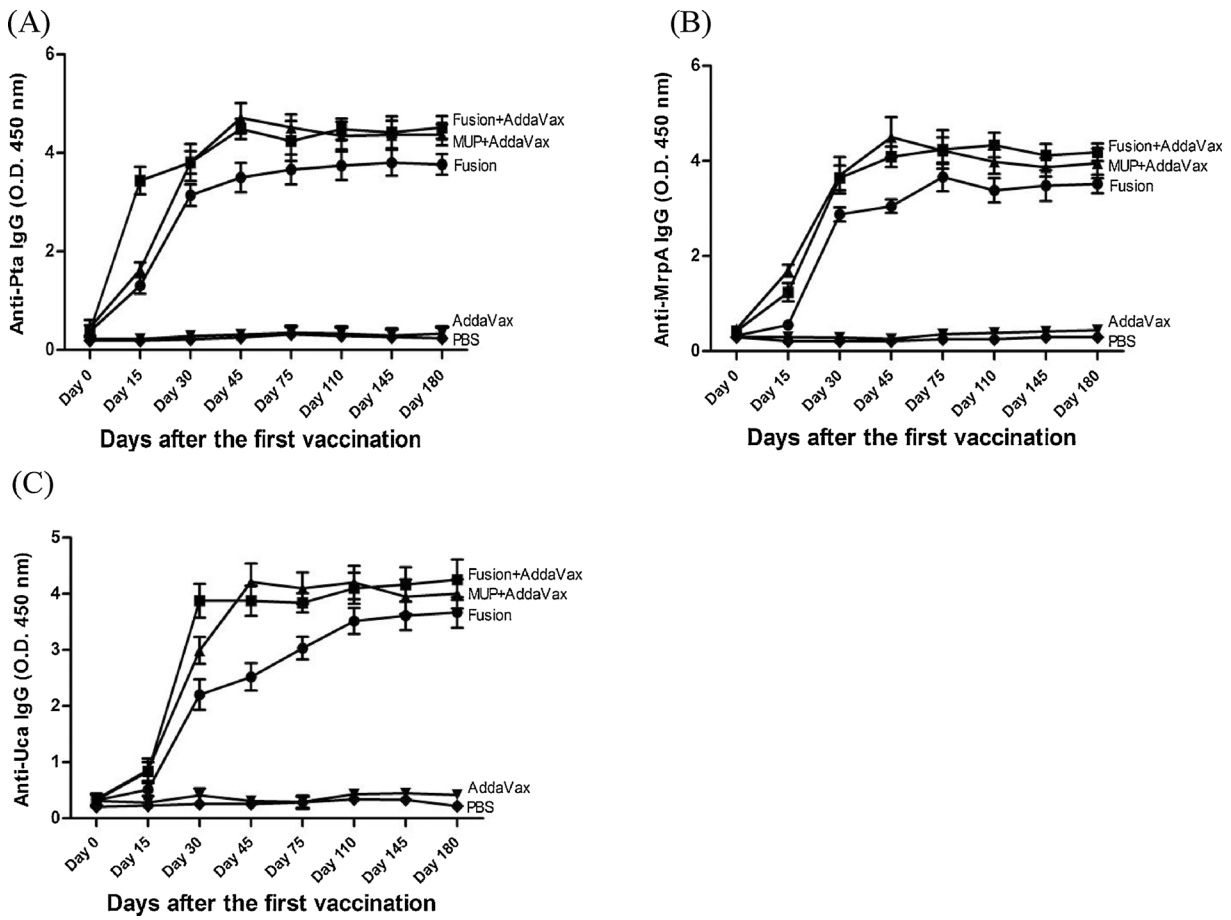


Fig. 4. Evaluation of the longevity of serum IgG responses. Mice were immunized and the longevity of (A) anti-Pta, (B) MrpA and (C) UcaA responses was measured. Results are expressed as mean and standard errors of three independent experiments from 3 mice per groups at serum dilution 1:800.

Scavone et al., 2007; Scavone et al., 2004; Scavone et al., 2011) against *P. mirabilis* UTIs in mice model. These researches are ongoing to achieve effective vaccine combinations against *P. mirabilis* UTIs. Since, the importance of virulence factors MrpA, UcaA, and Pta among *P. mirabilis* strains and also their consideration as promising vaccine candidates, a novel multi-peptide vaccine of these factors was designed against *P. mirabilis* that might be more likely to develop as an effective vaccine against *P. mirabilis* UTIs (Nielubowicz et al., 2008; Wieser et al., 2010). Different bioinformatics approaches have been employed for the prediction of B- and T- cell epitopes of MrpA, UcaA and Pta antigens in a fusion form to increase the chance of inducing protective immunity against *P. mirabilis*, because of the possible role of B-cell (humoral response) and T-cell (cellular response) in protection against UTIs (Thumbikat et al., 2009). The selected epitopes were found to be highly conserved among different *P. mirabilis* strains. Following the importance of linkers for attaching the multi-domain proteins and accurate function (Arai et al., 2001), these selected domains were fused together by a linker such that the epitopes could function independently and the formation of new epitopes could be avoided (Yano et al., 2005).

Despite the advantages of peptide-based vaccines, the use of adjuvants is often required to enhance the immune response against antigens and shift the responses to a desired direction (Skwarczynski and Toth, 2016). AddaVax adjuvant is a squalene-based oil-in-water nano emulsion with a formulation similar to the MF59 adjuvant (Calabro et al., 2013). Little studies about the efficacy of AddaVax showed its ability to induce both humoral (Th2) and cellular (Th1) responses against antigens. Thus, AddaVax was used as an adjuvant to increase the chance of activation of both B and T cells in eradicating the superficial and intracellular reservoirs of *P. mirabilis*. Furthermore, to the best of our knowledge this is the first report in which AddaVax has been used in combination with a vaccine candidate against UTI.

According to our previous experiments (Asadi Karam et al., 2013; Habibi et al., 2015a; Karam et al., 2013; Karam et al., 2016) and the fact that the multi-peptide vaccine candidate was designed for the first time, its effectiveness in S.C. route of immunization was evaluated. Furthermore, by using AddaVax as a systemic adjuvant in previous studies (Mitchell et al., 2016; Smet et al., 2016), it was rational to use the S.C. route in this study.

In this study, it was found that antigens Pta, MrpA and UcA in fusion and non-fusion form could elicit serum IgA, IgG1 (Th2) and IgG2a (Th1) responses without the use of adjuvant. In other studies (Pellegrino et al., 2003; Scavone et al., 2004), the S.C. or intranasal (I.N.) administration of MrpA and UcaA alone resulted in the production of serum IgG, whereas the serum IgA was only induced in the I.N. route. A comparison of the vaccine formulations with the combinations in our recent studies (Habibi et al., 2015a; Habibi et al., 2015b) showed the higher potential of the present candidates in inducing systemic humoral responses compared to previous candidates. The data also showed that the use of AddaVax could improve the serum IgA, IgG and IgG2a responses of antigens in both fusion and combined forms. Smet et al. (2016) also showed the potential of AddaVax in the induction of high titers of both IgG1 and IgG2a against the synthetic mycolates from *Mycobacterium tuberculosis* antigens.

As the importance of long-lasting antibodies and generation of long-lived B cell memory in prevention of recurrent UTIs (Brumbaugh et al., 2013), the longevity of humoral responses was assessed until 6 months when all vaccine combinations could maintain high levels of the IgG

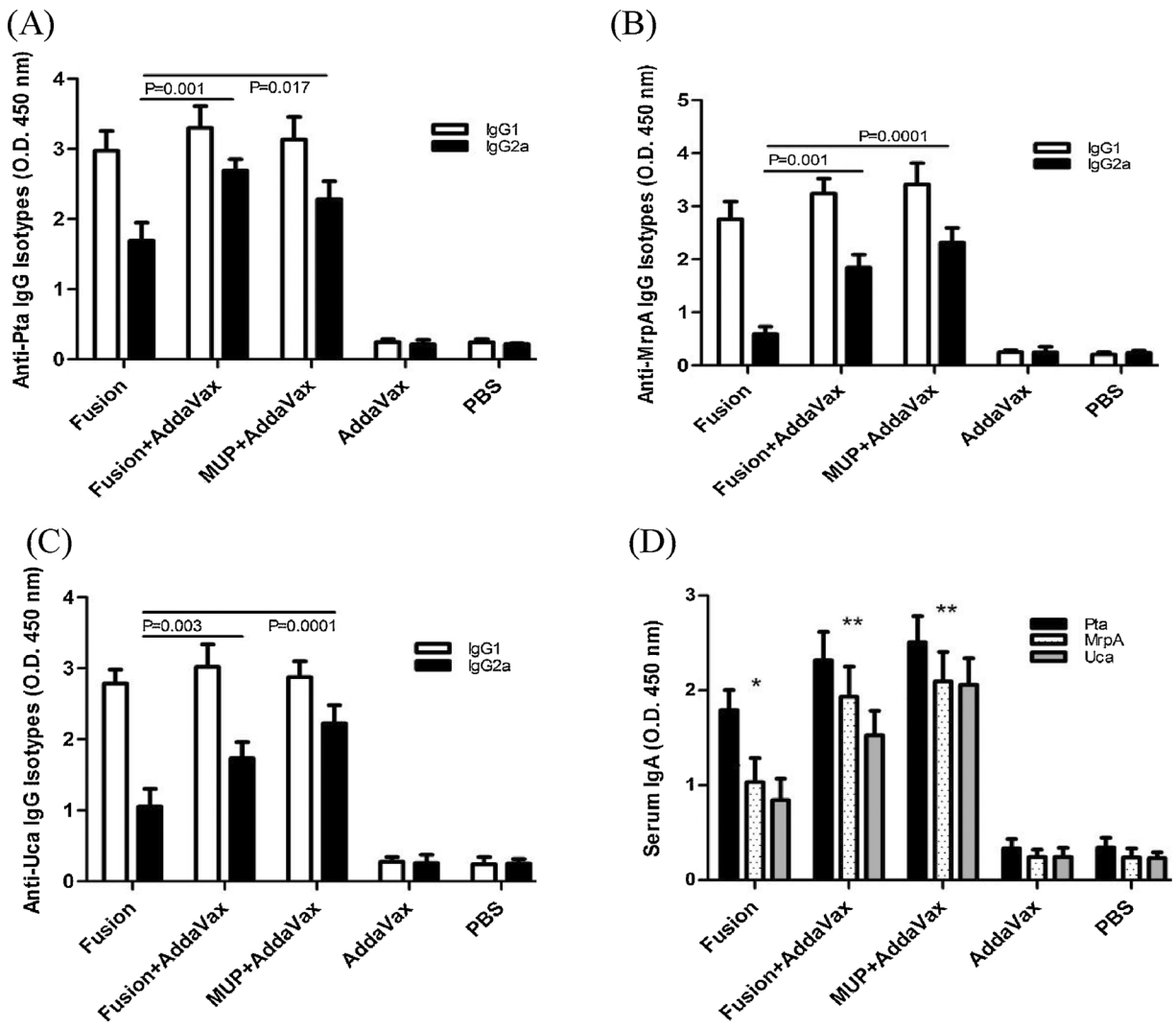


Fig. 5. Evaluation of isotype antibodies in the mice sera. The mice were immunized and (A) anti-Pta IgG1 and IgG2a, (B) anti-MrpA IgG1 and IgG2a, (C) anti-UcaA IgG1 and IgG2a and (D) IgA responses were assessed in the immunized mice. Single asterisk shows statistical significance of IgA responses over the control mice, and double asterisks show statistical significance of IgA over Fusion and control groups. The results are the average of three independent experiments. Bars represent mean ± S.D. from 12 mice per groups at serum dilution 1:800.

responses and this finding was one of the most important advantages of these designed vaccines. The IgG responses in mice vaccinated with fusion without adjuvant were observed to be maintained until the end of this assay, whereas in our recent study (Habibi et al., 2015a), anti-MrpH responses in fusion MrpH.FimH tend to decline 12 weeks after the first vaccine dose. This point shows the advantage of the designed multi-peptide vaccine than the previous candidates.

The present study and our recent findings (Habibi et al., 2015a; Karam et al., 2016) demonstrated that systemic immunization of mice could induce mucosal responses and adjuvant improved these responses. Pellegrino et al. (2003) could not detect the anti-IgG or IgA mucosal responses in S.C. immunization of mice with MrpA or UcaA, whereas Scavone et al. (2004) showed the ability of MrpA or UcaA in the induction of mucosal responses in I.N. route.

In the present study, the vaccine combinations induced the production of both IFN-γ and IL-4 as the indication of Th1 and Th2 responses, respectively. A mixture of Th1/2 response could be due to the recruitment of the antigen presenting cells (APCs) and T $CD4^+$ cells which resulted in high levels of Th1 and Th2 cytokines production. Scavone et al. (2007) also reported that MrpA expressed by *L. lactis* produced a significant IFN-γ levels that was several folds less than the vaccine formulations of the present study. Furthermore, the ability of the fusion and MUP combination in inducing the IFN-γ was significantly higher than our previous candidates against *P. mirabilis* (Habibi et al., 2015a) and it showed the other advantage of the present vaccine formulations than that of the previous vaccine candidates.

The findings of the present study similar to the report of Wu et al. (2015) showed that AddaVax induced a mixture of Th1 (IgG2a and IFN-γ) and Th2 (IgG1 and IL-4) responses against antigens. The comparison of the MPL-vaccine formulations in our recent studies with the AddaVax combinations in the present study showed the ability of AddaVax in inducing cytokines Th1 and Th2 when compared with MPL adjuvant (Habibi et al., 2015a; Habibi et al., 2015b). Based on the IgG1/IgG2a ratio, it can be concluded that AddaVax tends to shift the immune responses preferentially towards Th1 direction which has the advantage to eradicate the intracellular reservoirs of *P. mirabilis*. Also, other studies have shown the potential of AddaVax in shifting the responses toward Th1 or Th2 direction (Mitchell et al., 2016; Umthong et al., 2015; Wei et al., 2017; Wu et al., 2015). Wei et al. (2017) and Umthong et al. (2015) showed that AddaVax generated the Th1 responses against antigens from *Ascaris suum* and Leptospirosis, respectively. The potential of AddaVax in eliciting the Th2-type IgG1 antibody response was observed against antigens of *Mycoplasma hyopneumoniae* (de Oliveira et al., 2017) and *Plasmodium falciparum* (Mitchell et al., 2016). These

Table 2
Mesurement of the mucosal responses in the immunized mice. Two weeks after the third vaccine dose, (A) IgG and (B) IgA levels were evaluated in the urine samples. Single asterisks show statistical significance of IgG and IgA over the control mice, and double asterisks indicate statistical significance of IgG and IgA over fusion group and control mice in dilution 1:5 of urine. The results are the average of O.D. three independent experiments from 12 mice per groups at different dilutions of urine.

(A)

	Anti-Pta IgG			Anti-MrpA IgG			Anti-Uca IgG		
Groups	undiluted	1:5	1:10	undiluted	1:5	1:10	undiluted	1:5	1:10
Fusion	1.35	1.3*	0.65	1	0.8*	0.5	1.1	0.63*	0.43
Fusion + AddaVax	2.5	2.32**	1.5	1.3	1.4**	0.7	1.31	1.12**	0.71
MUP + AddaVax	2.1	1.92**	0.9	1.45	1.61**	0.9	1.48	1.41**	0.79
AddaVax	0.95	0.46	0.41	0.91	0.4	0.35	0.73	0.36	0.32
PBS	0.85	0.33	0.28	0.75	0.31	0.28	0.82	0.28	0.26

(B)

	Anti-Pta IgA			Anti-MrpA IgA			Anti-Uca IgA		
Groups	undiluted	1:5	1:10	undiluted	1:5	1:10	undiluted	1:5	1:10
Fusion	1.74	1.45*	0.63	1.66	1.3*	0.75	1.47	1.32*	0.67
Fusion + AddaVax	1.55	1.34*	0.57	1.7	1.23*	0.83	1.83	1.42*	0.81
MUP + AddaVax	1.2	1.16*	0.6	1.57	1.53*	1.11	1.96	1.74**	1.15
AddaVax	0.88	0.38	0.32	0.75	0.41	0.38	0.82	0.4	0.38
PBS	0.85	0.29	0.27	0.69	0.31	0.3	0.75	0.39	0.38

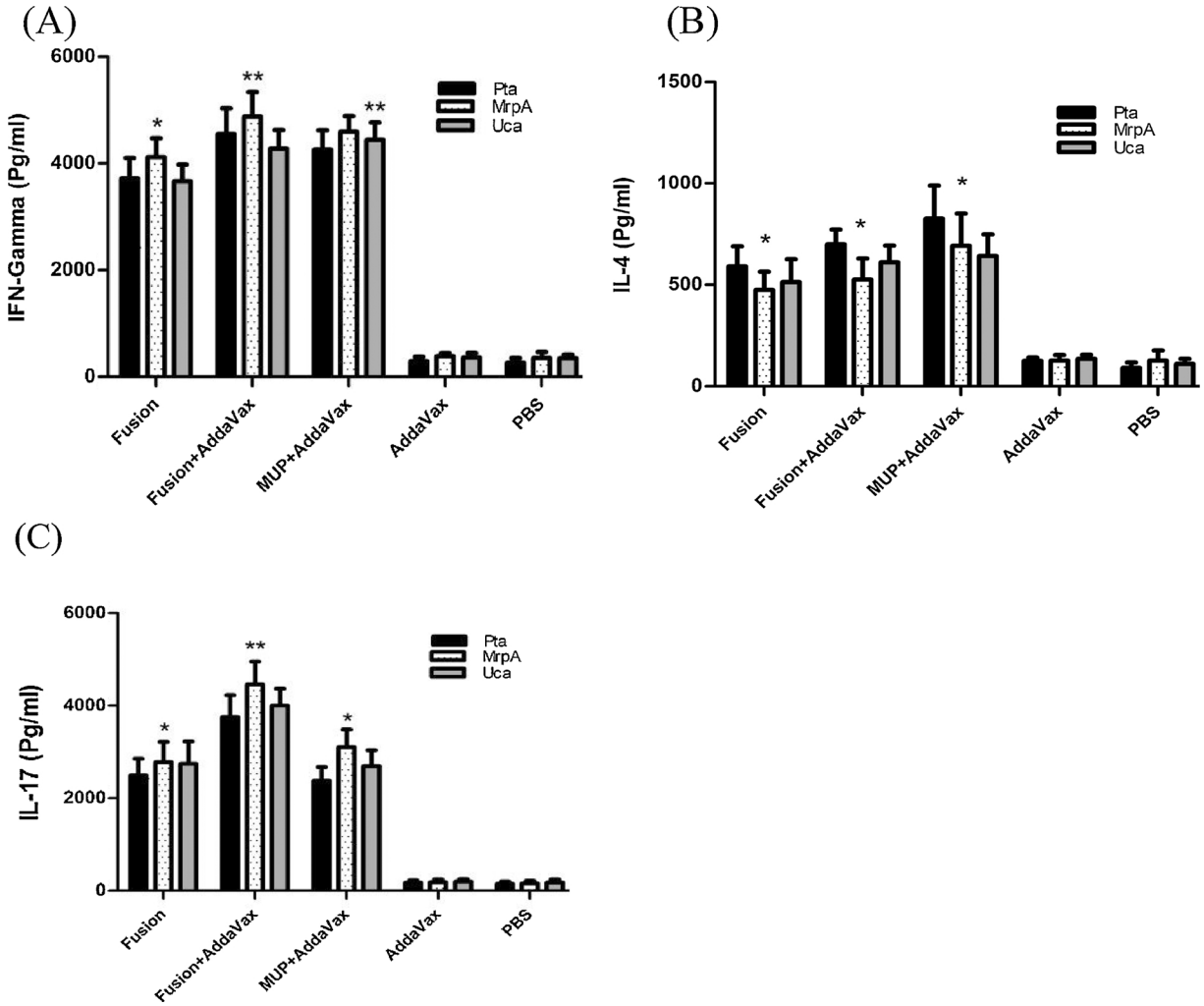


Fig. 6. Evaluation of the cytokine responses in the immunized mice. The splenocytes of each mice group were stimulated with Pta, MrpA and UcaA antigens and the supernanants were analyzed for secretion of (A) IFN-γ, (B) IL-4 and (C) IL-17 levels. The single asterisks show statistical significance of IFN-γ, IL-4 or IL-17 over control mice ($p < 0.05$). Double asterisks indicate statistical significance of IFN-γ and IL-17 levels over the non-adjuvanted combinations and control mice ($p < 0.05$). Results are the mean stimulation index ± S.D. of six mice per group from three independent experiments.

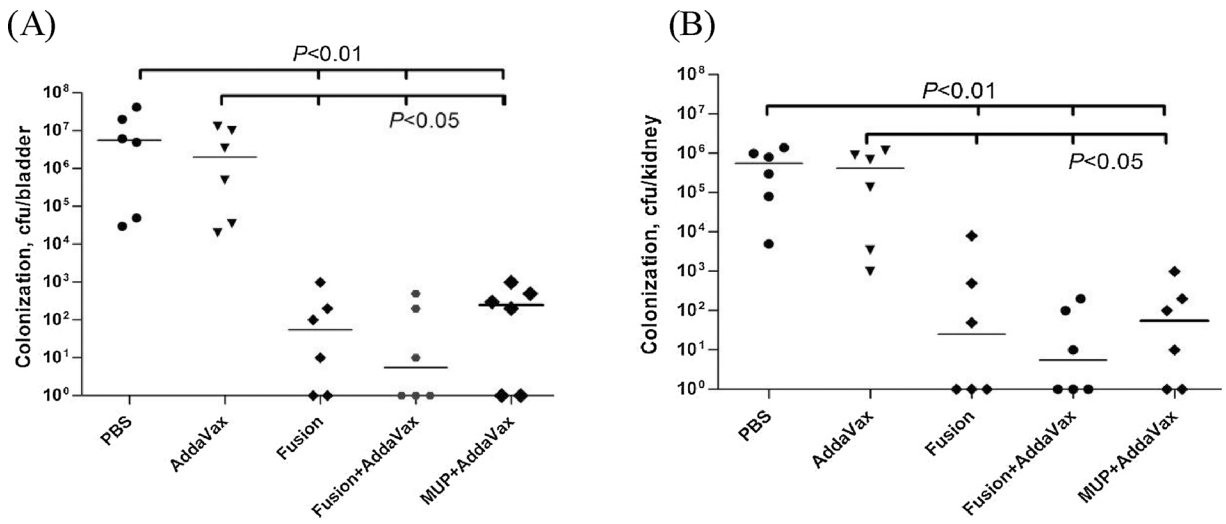


Fig. 7. Evaluation the efficacy of immune responses against UTI. Two weeks after the last vaccine dose, the bladders of mice (n = 6) were infected with *P. mirabilis* HI4320 strain. One week after challenge, the levels of *P. mirabilis* in the (A) bladders and (B) kidneys of mice were determined. Solid 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 brackets with *P* value levels. $P < 0.05$ was considered as significant.

differences in inducing the immune responses by AddaVax may be the result of the type of antigens used; the mice model, the concentration of antigen and adjuvant used as well as the route of immunization (Casella and Mitchell, 2008).

Except for IL-17 production, there was no significant difference in the immunogenicity and protection between mice vaccinated with fusion + AddaVax and MUP + AddaVax. The fusion construct was composed of limited epitopes of MrpA (80 aa), UcaA (71 aa), Pta (192 aa), and linkers (30 aa) (Fig. 1 and Table 1), whereas the MUP combination was the mixture of full-length of MrpA (175 aa), UcaA (180 aa) and the passenger domain of Pta (524 aa), thus it is notable that the selected epitopes in fusion could induce the immunogenicity and protection in the level of antigens used in the combined formulation (MUP). This noteworthy finding demonstrates if the B- and T- cells epitopes of antigens could be correctly selected, they will have the advantage over vaccines based on the full-length of antigens.

According to the challenge results, all vaccine formulations in the presence and absence of adjuvant conferred significant protection in the bladder and kidneys of mice that showed the potential of the Pta, MrpA and UcaA antigens in fusion and non-fusion forms as vaccine candidates. Pellegrino et al. (2003) reported the efficacy of candidates MrpA and UcaA in kidney protection in S.C. route, whereas Scavone et al. (2004) showed the significant protection of both bladder and kidneys in mice vaccinated I.N. with MrpA and UcaA. The protection effectiveness of MrpA expressed in *S. typhimurium* or *L. lactis* strains was also observed in mice model (Scavone et al., 2007; Scavone et al., 2011). In other study, Alamuri et al. (2009) demonstrated that immunization with Pta coupled with cholera toxin (CT) adjuvant provided significant protection against *P. mirabilis* UTI, especially in the upper urinary tract. Among the tested candidates, fusion admixed with AddaVax showed the highest protection in the bladder and kidney against *P. mirabilis*. Interestingly, the protection level of fusion + AddaVax in the present study was in the levels of protection of fusion + MPL combination in our previous study. The significant protection of mice injected with Fusion + AddaVax could be attributed to the high mucosal and systemic responses (Russo et al., 2003). It is also possible that the elevated cytokines (CMI) played a critical role in the protection of this mice group (Alteri et al., 2009). The findings also showed a direct relationship between the reduction of bacterial load within the bladders and decrease of infection in the kidneys, thus the reduction of bacterial load in bladder may result in kidney protection. Interestingly, the protection efficacy in mice vaccinated with MUP + AddaVax was lower than fusion alone, whereas this mice group revealed higher immunogenicity than mice injected with fusion alone. The reason for this discrepancy could be due to the decrease in IL-17 levels by the MUP + AddaVax or other immunological mechanisms that require further studies.

5. Conclusions

In the present study, novel multi-peptide vaccine based on the important epitopes of MrpA, UcaA and Pta was constructed and its effectiveness was evaluated with and without AddaVax as adjuvant. By comparing the results of this study with the results of previous vaccines, it was concluded that the AddaVax-based vaccine combinations had significant efficacy against *P. mirabilis*, and further studies are needed to present the promising vaccine combinations as the potential candidates against *P. mirabilis*. Furthermore, the findings of the present study suggest that AddaVax can be a good alternative for traditional adjuvants especially for vaccine candidates against UTI pathogens.

Acknowledgements

The study was supported by Pasteur Institute of Iran (Grant No: 923) and Kashan University of Medical Sciences (Grant No: 94167).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.molimm.2018.03.001.

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