Journal of Applied Biological Sciences 8 (3): 79-84, 2014
ISSN: 1307-1130, E-ISSN: 2146-0108, www.nobel.gen.tr

Uropathogenic *E.coli* PapG Protein: The Expression and Immune Characterization in Balb/C Mice

Fatemeh ASHRAFI [1] Jalil Fallah MEHRABADI [2*] Seyed Davar SIADAT [3]
Mohammad Reza AGHASADEGHI[4]

[1] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2] Department of Biotechnology and Biosciences, Malek-Ashtar University of Technology, Tehran, Iran
[3] Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran
[4] Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

*Corresponding author:
Email: Jalil.Fallah@gmail.com

Received: October 05, 2014
Accepted: November 18, 2014

Abstract

Urinary tract infection (UTI) is one of the most complications in human caused by uropathogenic Escherichia coli (UPEC) and there is no effective vaccine against UTI. In this study the PapG protein was expressed and monitored for immunoassay. The pEXA/PapG vector was digested with NdeI/EcoRI enzymes, and the gene segment was sub-cloned into pET21a vector. After transformation to *E. coli* strain Origami, PapG was expressed under the 1 mM IPTG. Protein expression was detected by SDS-PAGE and western blotting. Protein purification was carried out using Ni-NTA column. Experimental mice (n=18) were divided into three groups. The groups 1 to 3 were immunized three times with 2 weeks interval with the PapG protein with freund's adjuvant, alum adjuvanted protein and PBS, as a control. Two weeks after the last injection, lymphocyte proliferation assessed with Brdu method, secretion of IL-4 and IFN-γ cytokines were quantified by ELISA kit. In addition, total antibody, IgG1 and IgG2a subtypes were assayed with ELISA. The results indicated that PapG, as a vaccine candidate, significantly increase lymphocyte proliferation and Th1 cytokine pattern versus control group. Humoral immune responses induced with both IgG1 and IgG2a classes. Although PapG, as a vaccine candidate, strongly induced cellular and humoral immune responses, more studies and experimental bacterial challenges remained to be clarified.

Keywords: Uropathogenic *E.coli*, PapG, expression, immunization, immune responses

INTRODUCTION

Urinary tract infection (UTI) remains one of the well-known infections in humans especially in young women [1]. The bacteria responsible for this infection, UPEC, often originate from feces. After overcoming the host's immune responses, the bacteria were colonized in the lower urinary tract, establishing infection in the urinary tract that can progress to pyelonephritis [2-4] . The infection caused by UPEC is usually self-limiting and rarely spread beyond the urethra. However, UPEC bacteria invade to bladder epithelial cells by intracellular amplification, and upon exiting the cell's surface, invade to other epithelial cells in the tissues of the bladder, establishing a persistent infection [5, 6]. Due to urinary flow, mucus and secretory IgA secretion as well as bactericidal property of urine epithelial cells, the urinary tract is normally sterile [7, 8]. However, some strains of E coli bacteria can grow in the urinary tract because they share several virulence factors such as adhesion molecules and toxins [9].The early step in the colonization of UPEC strains on the mucosal surfaces of the host is dependent on some adhesion molecules such as pili S, adhesion family of Dr, pili p and pili type I [10]. Them, type I and p Pili in the adhesion is considered to be more important [11-14]. PapG, as a critical adhesion molecule, is known to be pyelonephritis-associated pili [15, 16].Current treatment of acute urinary tract infection is limited due to the increasing rate of antibiotic resistance strains as well as adverse effects of vaccines[9, 17]. Vaccine as a most economic method for prevention and controlling infection is considered in public health. Herein, many vaccines were used for prevention of infection diseases in human and also in livestock and poultry Industry and resulted to the control of some infection diseases [18, 19]. Because of origination of antibiotic resistance strains of UPEC, it is seemed that preparation of a vaccine against urinary tract infection would be useful for controlling the infection [20, 21]. Several clinical trials of vaccine candidates have currently been tested against urinary tract infection in human [22-25], no suitable results have been found so far. Therefore, targeting critical molecules in the adhesion process such as PapG as a vaccine candidate can lead to an increase in vaccines efficacy [26].

In the present study, the recombinant PapG protein was produced in E.coli BL21 DE3 and immune responses against the molecule was evaluated in the murine model.

MATERIALS AND METHODS

Construction of a recombinant PapG expression vector

The synthesized PapG gene in pEXA vector at NdeI/EcoRI restriction enzyme sites was purchased from Bioneer Company (Bioneer, Korea). The plasmid was digested with NdeI and EcoRI (Fermentase, Lituania), and the PapG gene segment was ligated into the pET21a vector. To confirm whether pET21a vector contained the PapG gene segment, the recombinant plasmid was double digested with NdeI/EcoRI enzymes and electrophoresis carried out in a 1% agarose gel. Finally, the fidelity of the pET21a/PapG vector was confirmed by gene sequencing. (Macrogene Institute, South Korea).

The expression of the recombinant PapG protein

PET21a/PapG vector was transformed into the *E. coli* strain origami using a standard calcium chloride protocol. To express the recombinant PapG protein, the bacteria containing the plasmid were induced 4 hours using 1 mM IPTG. Furthermore, the protein expression was confirmed by SDS-page.

Western blot analysis of recombinant PapG

After IPTG-induction of the cells to express the recombinant PapG, SDS-page was performed to separate the protein bands according to the molecular weights. Subsequently, proteins from an SDS-PAGE gel were electrophoretically transferred to the nitrocellulose membrane in Tris-glycin buffer containing 20% methanol. The membrane was blocked by 3% TBS-BSA containing tween-20 and then washed three times with TBS-T, and incubated with the 6 His-tag antibody HRP conjugate (Roche, Germany) for 2 hours. Afterwards, the membrane was washed again and incubated with of 3, 3-diaminobenzidine (DAB) substrate solution containing 1% H_2O_2. When a deep brown band appeared, the membrane was washed with distilled water to stop the enzymatic reaction.

Purification of the PapG protein

Ni-NTA column (Qiagen, USA) was used for mass purification. The pellet of *E.coli* cells expressing the recombinant proteins was suspended in buffer A (20mM Tris-Hcl, 0.5M NaCl, 0.3% Triton X100, 1mM PMSF, 10mM imidazole, pH 8.0), and sonicated in 10 cycles of 40 seconds at 4°C by a sonicator (MSE, UK). The suspension was centrifuged (12000rpm, 20min, 4°C), the sample was loaded on a Ni-NTA column and purification was performed according to the manufacturer's standard protocol. Protein refolding was carried out by urea. After dialysis of purified samples, the concentration of each protein was detected through the Bradford method.

Endotoxin analysis of the recombinant PapG protein

The endotoxin level of purified PapG was detected by the limulous amoebocyte lysate test (LAL test) according to the manufacturer's standard protocol (Thermoscientific, USA).

Mice

Six-to-eight weeks inbred female BALB/c mice were purchased from Pasteur Institute of Iran (Karaj, Iran). Mice were housed for 1 week 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 hours. All mouse experiments were performed in agreement with the Animal Care and Use Protocol of Islamic Azad University.

Experimental groups and immunization

Experimental mice (n=18) were divided into 3 groups (n=6). The groups 1 to 3 were subcutaneously immunized three times with 2 weeks interval with 20 µg of the PapG protein adjuvanted with complete freund's adjuvant, 20 µg of the PapG protein adjuvanted with alum and PBS, as a control group.

Lymphocyte proliferation assay

Two weeks after the last shooting of the vaccine, the spleens of mice were re-suspended in cold PBS containing 5% FBS. RBCs were lysed with lysis buffer and, after centrifugation, the pellet was re-suspended and adjusted to 3×10^6 cells/ml in RPMI 1640 (Gibco, Germany) supplemented with 10% FBS, 4 mM/L-glutamine, 1mM sodium pyruvate, 50µm 2ME, 100µg/ml streptomycin and 100 IU/ml penicillin. 100 µl of the cell suspension was then dispensed into each well of 96-well flat-bottom culture plates in triplicate and stimulated with 10 µg/ml of the PapG protein. Phytohemagglutinin-A (5 µg/ml, Gibco), unstimulated wells and complete culture medium were used as a positive control, the negative controls and a blank, respectively. Three days later, 100 µl of 5-bromo-2-deoxy-uridine labeling solution was added to each well and incubation continued for 18 hrs. Subsequently, the plates were centrifuged, and culture medium was completely removed and dried at 60 ° C for 30 minutes. Then, 100 µl of the anti-Brdu antibody was added to each well for 2 hrs, the plates were washed five times with PBS, 100 µl of the TMB substrate was added to each well in the dark condition and the reaction was stopped by adding 100 µl of 2N H_2SO_4. Absorbance was measured using a spectrophotometric plate reader at 450/630 nm. Stimulation Index (SI) was calculated according to the following formula: OD of stimulated wells/OD of un-stimulated wells.

IFN-γ and IL-4 Cytokines ELISA

Two weeks after the final immunization, a total number of 3×10^6 splenocytes were seeded into each well of a 24-well plate and stimulated with 10 µg/ml of PapG protein and incubated at 37°C in 5% CO_2. Three days later, supernatants were collected and IFN-γ and IL-4 cytokines were assessed through commercial ELISA Kits (Mabtech, Sweden) according to the manufacturer's instructions. The quantity of cytokines was expressed as pg/ml according to the related standard curve.

Total antibody ELISA and determination of IgG1, IgG2a subclasses

Sera of experimental mice were collected and total antibodies were determined by an optimized ELISA method. Briefly, 100 µl of 10 µg/ml of the PapG antigen in PBS buffer were added to 96-well ELISA Maxisorp plates (Nunc, Naperville, IL), and incubated overnight at 4 ˚C. The wells washed three times with PBS containing 0.05% Tween 20 (washing buffer) and blocked for 1 hour at 37 ˚C with 5% skimmed milk in PBS (blocking buffer). The plates were washed with washing buffer, and 100µl of 1/50 to 1/6400 diluted sera were added to individual wells and

incubated at 37 ˚C for 2 hours. The wells were washed five times with washing buffer, and incubated for 2 hours with 100 µl of 1/10000 dilution of HRP-conjugated anti mouse (Sigma, USA). The wells were washed six times and incubated in the dark for 30 minutes with 100 µl of TMB substrate. Finally, the reaction was stopped with adding 100 µl of 2N H2SO4, and the color density was measured at $A_{450/630}$ nm with an ELISA plate reader. In addition, specific IgG1and IgG2a subclasses were assessed at dilution of 1/50 of experimental sera with goat anti-mouse IgG1and IgG2a secondary antibodies (Sigma, USA) according to the manufacture's protocol.

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 annova test and SPSS v18 software. In all of the cases, *P* values < 0.05 were considered to be statistically significant.

RESULTS

Construction of the PapG expression vector

The constructed pET21a/PapG vector was double digested with NdeI/BamHI restriction enzymes. The results of agarose gel electrophoresis of digested plasmid showed a band 629 bp for the PapG gene segment. The results of sequencing also confirmed the gene segment in the pET21a vector (data not shown) (figure 1).

Figure 1. Enzymatic digestion of pET21a/PapG expression vector.

Expression of the PapG protein in the E. coli Origami strain

The *E.coli* strain origami, as a host, was used to express the recombinant PapG protein. After transformation and expression under 1 mM IPTG induction, SDS-page analysis showed over expression of about 24 KD (figure 2). In addition, western blotting analysis with the anti His-tag antibody indicated a band about 24 KD for PapG protein (figure 3).

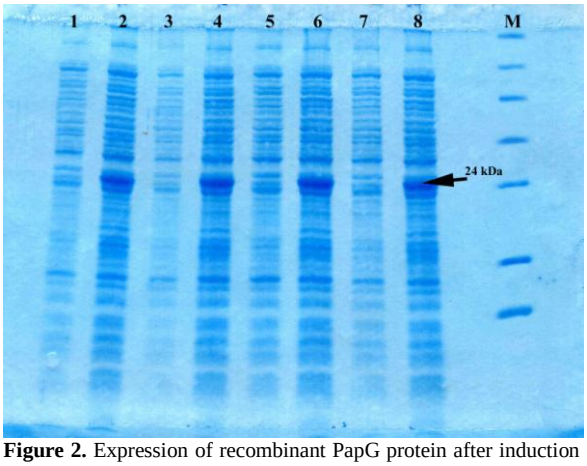

Figure 2. Expression of recombinant PapG protein after induction with IPTG.

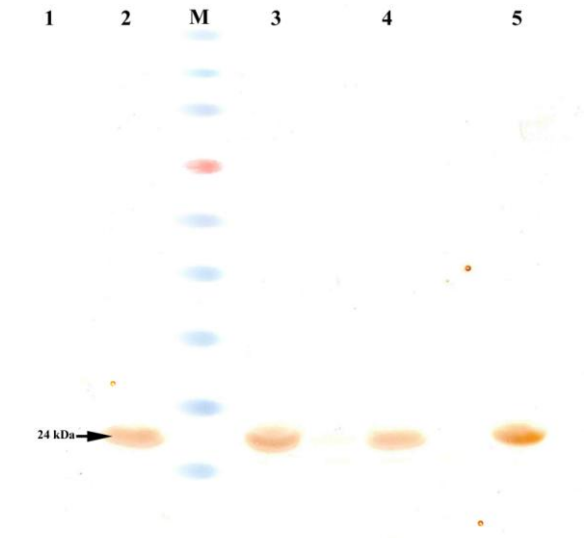

Figure 3. Western blot analysis of PapG protein.

Purification of the recombinant PapG protein

The Ni-NTA column was used to purify the recombinant PapG protein. The purification results showed that after purification of the PapG protein and SDS-page analysis, a 24 KD band for PapG was observed (figure 4).

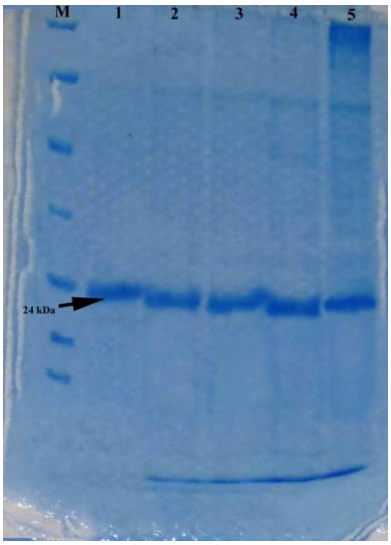

Figure 4. Purification of PapG protein using Ni-NTA column and analysis of the purification with SDS-page.

Endotoxin level in the purified PapG protein

The results of endotoxin levels in the purified recombinant PapG proteins using LAL test showed that the endotoxin level was less than 0.07 EU/ml.

Lymphocyte proliferation

The results of lymphocyte proliferation indicated that the PapG protein adjuvanted with alum and freund's resulted in an increase in lymphocyte proliferation as compared to control group (p=0.0001, p= 0.0708 respectively). In addition, the PapG/alum immunization of mice led to increased lymphocyte proliferation in comparison to the PapG/freund's immunized group (p=0.0011). (figure 5).

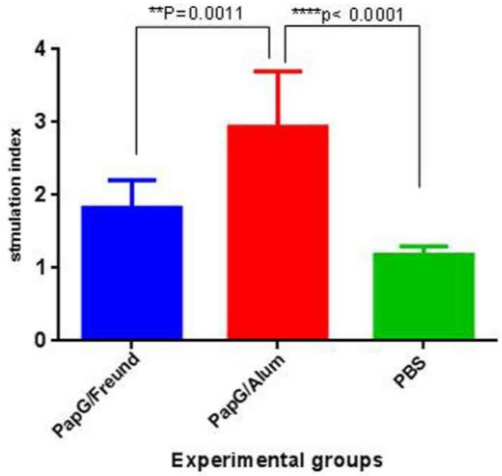

Figure 5. Lymphocyte proliferation according to stimulation index.

IL-4 and IFN-γ cytokine assay

The results of the IL-4 cytokine analysis showed that immunization with the candidate vaccine formulated with freund's and alum adjuvants increased the IL-4 cytokine compared to the control group (p= 0.0182, p= 0.1282 respectively). No significant difference was observed between alum and freund's adjuvanted groups in the induction of the IL-4 cytokine (figure 6).

Assessment of the IFN-γ cytokine in the experimental groups showed that immunization with PapG/freund's and PapG/alum increased the IFN-γ cytokine as compared to the control group (p=0.0008, p=0.005 respectively). Analysis of the IFN-γ cytokine release in the PapG/freund's group when compared to the PapG/Alum group, indicated no significant difference (p=0.7069) (figure 6).

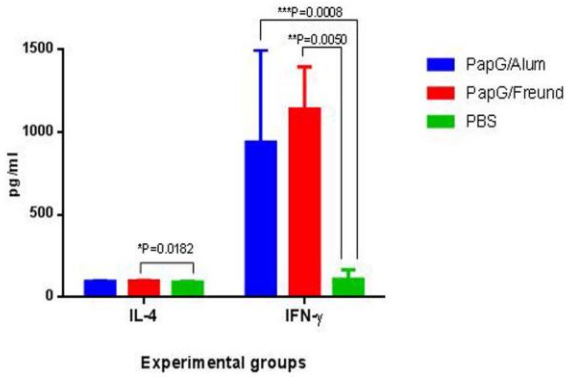

Figure 6. Interleukin-4 and IFN-γ cytokines in the experimental groups.

Total antibody

The results of total antibody indicated that immunization with PapG/Alum increased the total IgG responses compared to the control group at dilutions of 50,100 and 200 (p<0.0394). In addition, PapG/freund's group increased the total antibody responses compared to the control group at dilutions of 50, 100, 200, 400 and 800 (p<0.0188).No significant differences were observed between freund's and alum-adjuvanted groups in the induction of the total IgG response (figure 7).

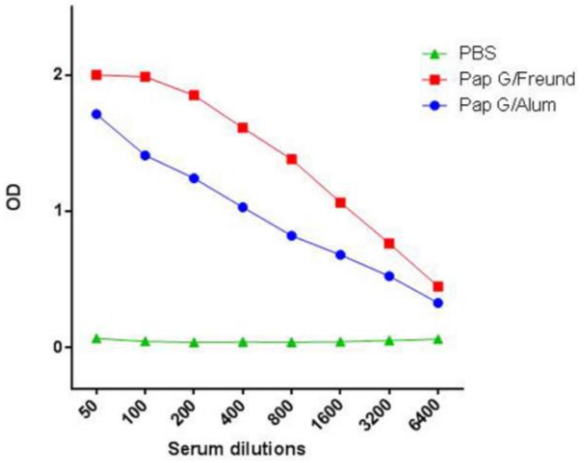

Figure 7. Total antibody responses after immunization course.

IgG1 and IgG2a isotypes

Assessment of a specific IgG1 isotype showed that immunization with PapG/Alum and PapG/freund's increased the IgG1 level versus control group (p< 0.0001). Immunization with PapG/alum increased the IgG1 level in comparison to the PapG/freund's but no significantly difference (p= 0.9838) (figure 8). The IgG2a isotype responses indicated that immunization with PapG/alum as well as PapG/freund's increased IgG2a when compared to the control group (p= 0.0374). No significant differences were observed in the IgG2a level between PapG/alum and PapG/freund's immunized groups (p= 0.8604) (figure 8).

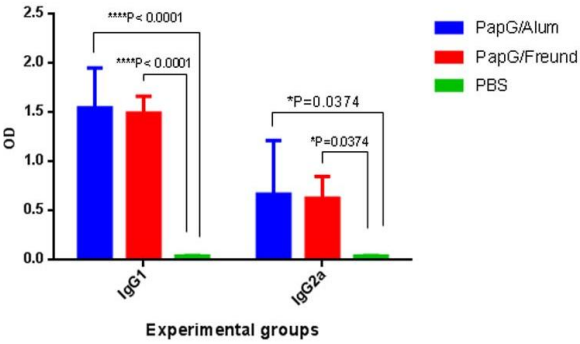

Figure 8. Specific IgG1 and IgG2a in the experimental groups.

DISCUSSION

Vaccination is the most economic and safe method for prevention and even therapy of the infectious diseases[17]. *E. coli* is the most important agent of urinary tract infection in human [14, 27, 28]. On the other hand, antibiotic therapy was encountered to origination of antibiotic resistant strains. Therefore, a vaccine is the best choice for fighting

against UTI [26, 28, 29]. Attachment of UPEC to the bladder is an important stage in the colonization and pathogenesis of the disease. In this regard, targeting the attachment and abrogation stage may be resulted in the control and prevention of the infection. Nowadays, vaccine strategies against UTI focused on the inhibition of bacterial binding molecules as targets [29-31].

Pili type P of UPEC is one of the important agents of the urinary tract infection [9-12]. According to the various reports, the binding process in uropathogenic *Escherichia coli* is facilitated through type 1 and P fimbriae; P fimbria causes the ascending infections in the urinary tract. Ascending of the urinary tract infection from the lower parts to the upper urinary tract system provides the progression of infection to the kidney, establishing pyelonephritis [14, 16]. Considering the mechanism of pyelonephritis establishment and the critical role of PapG in the formation of pyelonephritis, it can be considered as a suitable vaccine candidate for UTI. In this study, the recombinant PapG protein was expressed in E.coli. Herein, the PapG gene segment was cloned into the PET21a expressing vector and then expressed in E. coli strain origami, as a host. In the following, the recombinant protein PapG was purified and used as a vaccine candidate in immunoassay using alum as well as freund's adjuvants. Results of lymphocyte proliferation showed that immunization of mice with PapG/alum as well as freund's adjuvants increase lymphocyte proliferation when compared to the control group. Lymphocyte proliferation as a cellular immunity marker [32, 33], showed the ability of PapG protein in the induction of cellular immunity.

Several studies showed that cellular immune responses play important roles in the clearance of infection in UTI [34-37] and PapG and, as a vaccine candidate, can successfully induce cellular immune responses. Cytokine analysis showed that PapG, as a vaccine, in combination with both alum and freund's adjuvants could strongly induce IFN-γ secretion and polarize the Th1 pattern. A study of Carson et al. in immunodeficient mice showed that the Th1 immune platform and cellular immunity play important roles in the resistance to UTI [36]. The results of cytokine assay confirmed the ability of PapG protein to induce Th1 cytokine profile, and the possible potency of this vaccine to induce the resistance to the infection. Considering the involvement of antibodies in the resistance to UTI, total antibodies were then evaluated. The results of total antibodies showed that immunization with PapG/alum and freund's adjuvants significantly increased total antibodies as compared to the control group. Various studies showed the role of humoral immune responses in the resistance to the infection [34, 38, 39]. In fact, antibodies masking the adhesion molecules on UPEC are able to inhibit the attachment to the host cells and abrogate colonization and subsequently the infection [38] and PapG, as an adhesion molecule, can induce strong antibody responses with increased IgG1 and IgG2a classes. Taken together, in the preset study we have shown the potency of the PapG molecule as an immunogen in the induction of humoral and cellular immune responses. However, in the near future, the PapG vaccine using the surface display strategy would be evaluated in the experimental UTI challenges.

Acknowledgements

The authors would like to thank Mr. Mohammad Reza maasoumian from Department of Biochemistry of Malek-Ashtar University, Mr. Hamid Reza Moozarmpour, Mr. Mohammad Sadegh Beikverdi and Ms. Soophie Olfat from Lister research Group for their technical helps.

REFERENCES

[1] **Gompelman M, Rozemeijer W, Kortmann W., 2014**.A life-threatening complication of an ordinary urinary tract infection? The Netherlands journal of medicine,72(9):502-5.

[2] **Tanushi H, Kvist M, Sparrelid E., 2014.** Detection of healthcare-associated urinary tract infection in Swedish electronic health records. Studies in health technology and informatics,207:330-9.

[3] **Gupta S, Kapur S, Padmavathi D., 2014.** Comparative prevalence of antimicrobial resistance in community-acquired urinary tract infection cases from representative States of northern and southern India. Journal of clinical and diagnostic research : JCDR,8(9):Dc09-12.

[4] **Cheng WY, Lin YH, Lai CC, Chao CM., 2014.** Impact of catheter-associated urinary tract infection bundle on other health care-associated infections. American journal of infection control.

[5] **Ali AB, Bagnis CI., 2014.** [Recurrent urinary tract infection]. La Revue du praticien,64(7):969-71.

[6] **Nienhouse V, Gao X, Dong Q, Nelson DE, Toh E, McKinley K, et al., 2014.** Interplay between Bladder Microbiota and Urinary Antimicrobial Peptides: Mechanisms for Human Urinary Tract Infection Risk and Symptom Severity. PLoS ONE,9(12):e114185.

[7] **Uehling DT, Hopkins WJ, Dahmer LA, Balish E., 1994.** Phase I clinical trial of vaginal mucosal immunization for recurrent urinary tract infection. The Journal of urology,152(6 Pt 2):2308-11.

[8] **James-Ellison MY, Roberts R, Verrier-Jones K, Williams JD, Topley N., 1997.** Mucosal immunity in the urinary tract: changes in sIgA, FSC and total IgA with age and in urinary tract infection. Clinical nephrology,48(2):69-78.

[9] **Yasmeen Kausar SKC, Shobha D Nadagir,LH Halesh and M R Chandrasekhar., 2009.** Virulence factors, Serotypes and Antimicrobial Suspectibility Pattern of Escherichia coli in Urinary Tract Infections. Al Ame en J Med S c i,2)1:47-5.

[10] **Piatek R, Zalewska-Piatek B, Dzierzbicka K, Makowiec S, Pilipczuk J, Szemiako K, et al., 2013.** Pilicides inhibit the FGL chaperone/usher assisted biogenesis of the Dr fimbrial polyadhesin from uropathogenic Escherichia coli. BMC microbiology,13:131.

[11] **Johnson JR, Russo TA, Brown JJ, Stapleton A., 1998.** papG Alleles of Escherichia coli Strains Causing First-Episode or Recurrent Acute Cystitis in Adult Women. Journal of Infectious Diseases,177(1):97-101.

[12] **Johanson I, Lindstedt R, Svanborg C., 1992.** Roles of the pap- and prs-encoded adhesins in Escherichia coli adherence to human uroepithelial cells. Infection and Immunity,60(8):3416-22.

[13] **Al-Mayahie SMG., 2013.** Vaginal Colonization by papG Allele II(+) Escherichia coli Isolates from Pregnant and Nonpregnant Women as Predisposing Factor to Pyelonephritis. Infectious Diseases in Obstetrics and Gynecology,2013.

[14] **Snyder JA, Lloyd AL, Lockatell CV, Johnson DE, Mobley HLT., 2006.** Role of Phase Variation of Type 1 Fimbriae in a Uropathogenic Escherichia coli Cystitis

Isolate during Urinary Tract Infection. Infection and Immunity,74(2):1387-93.

[15] **Dodson KW, Pinkner JS, Rose T, Magnusson G, Hultgren SJ, Waksman G., 2001.** Structural Basis of the Interaction of the Pyelonephritic E. coli Adhesin to Its Human Kidney Receptor. Cell,105(6):733-43.

[16] **Lane MC, Mobley HLT., 2007**. Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic Escherichia coli (UPEC) in the mammalian kidney. Kidney Int,72(1):19.25-

[17] **Liu MA., 2003.** DNA vaccines: a review. Journal of Internal Medicine,253(4):402-10.

[18] **Steinhoff MC., 2013.** Assessments of vaccines for prenatal immunization. Vaccine,31 Suppl 4:D27-30.

[19] **Barbosa C, Smith EA, Hoerger TJ, Fenlon N, Schillie SF, Bradley C, et al., 2014.** Cost-effectiveness analysis of the national Perinatal Hepatitis B Prevention Program. Pediatrics,133(2):243-53.

[20] **Wang Y, Zhao S, Han L, Guo X, Chen M, Ni Y, et al., 2014.** Drug resistance and virulence of uropathogenic Escherichia coli from Shanghai, China. The Journal of antibiotics.

[21] **Ali I, Kumar N, Ahmed S, Dasti JI**., **2014.** Antibiotic resistance in uropathogenic e. Coli strains isolated from non-hospitalized patients in pakistan. Journal of clinical and diagnostic research : JCDR. 20:(9)8;14Dc01-4.

[22] **Naber KG, Cho YH, Matsumoto T, Schaeffer AJ., 2009.** Immunoactive prophylaxis of recurrent urinary tract infections: a meta-analysis. Int J Antimicrob Agents,33(2):111-9.

[23] **Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Criado FJ, Miron-Canelo JA, Gil-Vicente A, Nieto-Huertos A, et al., 2013.** Evaluation of a therapeutic vaccine for the prevention of recurrent urinary tract infections versus prophylactic treatment with antibiotics. International urogynecology journal,24(1):127-34.

[24] **Lenk VS, Dorsch B., 2009**. [Vaccinations in recurrent urinary tract infections--an observation study relating to health economy]. Aktuelle Urologie,40(6):360-5.

[25] **Hopkins WJ, Elkahwaji J, Beierle LM, Leverson GE, Uehling DT., 2007.** Vaginal mucosal vaccine for recurrent urinary tract infections in women: results of a phase 2 clinical trial. The Journal of urology,177(4):1349-53; quiz 591.

[26] **Roberts JA, Kaack MB, Baskin G, Chapman MR, Hunstad DA, Pinkner JS, et al., 2004.** Antibody responses and protection from pyelonephritis following vaccination with purified Escherichia coli PapDG protein. The Journal of urology,171(4):1682-5.

[27] **Johnson JR, Owens K, Gajewski A, Kuskowski MA., 2005.** Bacterial Characteristics in Relation to Clinical Source of Escherichia coli Isolates from Women with Acute Cystitis or Pyelonephritis and Uninfected Women. Journal of Clinical Microbiology,43(12):6064-72.

[28] **Hooton TM., 2001.** Recurrent urinary tract infection in women. International Journal of Antimicrobial Agents,17(4):259-68.

[29] **Asahara T, Nomoto K, Watanuki M, Yokokura T., 2001.** Antimicrobial Activity of Intraurethrally Administered Probiotic Lactobacillus casei in a Murine Model of Escherichia coli Urinary Tract Infection. Antimicrobial Agents and Chemotherapy,45(6):1751-60.

[30] **Lee J-S, Poo H, Han DP, Hong S-P, Kim K, Cho MW, et al., 2006.** Mucosal Immunization with Surface-Displayed Severe Acute Respiratory Syndrome Coronavirus Spike Protein on Lactobacillus casei Induces Neutralizing Antibodies in Mice. Journal of Virology87-4079:(8)80,

[31] **Wu C-M, Chung T-C., 2007.**Mice protected by oral immunization with Lactobacillus reuteri secreting fusion protein of Escherichia coli enterotoxin subunit protein. FEMS Immunology & Medical Microbiology,50(3):354-65.

[32] **Mahdavi M, Ebtekar M, Khorram Khorshid HR, Azadmanesh K, Hartoonian C, Hassan ZM., 2011.** ELISPOT analysis of a new CTL based DNA vaccine for HIV-1 using GM-CSF in DNA prime/peptide boost strategy: GM-CSF induced long-lived memory responses. Immunology letters,140(1-2):14-2.0

[33] **Mahdavi M, Ebtekar M, Azadmanesh K, Khorramkhorshid HR, Rahbarizadeh F, Yazdi MH, et al., 2010.** HIV-1 Gag p24-Nef fusion peptide induces cellular and humoral immune response in a mouse model. Acta virologica,54(2):131-6.

[34] **Imani Fooladi AA, Bagherpour G, Khoramabadi N, Fallah Mehrabadi J, Mahdavi M, Halabian R, et al., 2014.** Cellular immunity survey against urinary tract infection using pVAX/fimH cassette with mammalian and wild type codon usage as a DNA vaccine. Clinical and experimental vaccine research,3(2):185-93.

[35] **Thumbikat P, Waltenbaugh C, Schaeffer AJ, Klumpp DJ., 2006.** Antigen-specific responses accelerate bacterial clearance in the bladder. Journal of immunology (Baltimore, Md : 1950),176(5):3080-6.

[36] **Jones-Carson J, Balish E, Uehling DT., 1999.** Susceptibility of immunodeficient gene-knockout mice to urinary tract infection. The Journal of urology,161(1):338-41.

[37] **Wieser A, Romann E, Magistro G, Hoffmann C, Norenberg D, Weinert K, et al., 2010.** A multiepitope subunit vaccine conveys protection against extraintestinal pathogenic Escherichia coli in mice. Infect Immun,78(8):3432-42.

[38] **Asadi Karam MR, Oloomi M, Mahdavi M, Habibi M, Bouzari S., 2013.** Vaccination with recombinant FimH fused with flagellin enhances cellular and humoral immunity against urinary tract infection in mice. Vaccine,31(8):1210-6.

[39] **Asadi KM, Oloomi M, Habibi M, Bouzari S., 2012.** Cloning of fimH and fliC and expression of the fusion protein FimH/FliC from Uropathogenic Escherichia coli (UPEC) isolated in Iran. Iranian journal of microbiology,4(2):55-62.