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Evaluation of the Effectiveness of Polyclonal Antibody Developed against a Recombinant Multi-Epitope Protein Composed of Uropathogenic *Escherichia coli* Antigens

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ABSTRACT

Introduction: Uropathogenic *Escherichia coli* (UPEC) is the main cause of urinary tract infections (UTIs). Increasing antibiotic resistance among UPEC isolates complicates UTI treatment in the future. Finding alternative approaches against UPECs seems necessary. Despite many efforts to develop a vaccine for UPEC, there is no yet effective vaccine against the bacteria. **Methods:** Designing a multi-epitope vaccine based on the main UPEC virulence factors can be an effective strategy for the prevention of UTI. In the previous study, three important proteins from UPEC strains including FimH, FyuA and CNF-1 were selected to design a multi-epitope antigen that was used for the production of polyclonal antibody in rabbits. In the present study, the collected sera were used for evaluating the sensitivity, specificity, cell adherence, and biofilm inhibition of the developed polyclonal antibody. **Results:** ELISA results showed high binding activity of the rabbit polyclonal antibody against the multi-epitope protein even in low antibody titers. In addition, the polyclonal antibody showed antigenic specificity against the multi-epitope protein and UPEC UTI89 strain. Cross-reactivity of the polyclonal antibody was observed with *Klebsiella pneumonia*. Bacterial adhesion was reduced significantly in the presence of antibody, compared to the controls. **Conclusion:** The generated polyclonal antibody significantly reduced the *in vitro* biofilm formation of UTI89 strain while did not significantly affect the biofilm degradation. These results highlight the potential of the designed multi-epitope protein as a promising vaccine candidate for the prevention of UTI caused by UPEC.

INTRODUCTION

Urinary tract infections (UTIs) are considered as important infections in humans which occur in both community and hospitals with 150 million cases every year. Uropathogenic *Escherichia coli* (UPEC) is the main cause of UTIs [1]. According to the clinical manifestations, UTIs are classified to asymptomatic bacteriuria, cystitis, pyelonephritis, and prostatitis. Lack of appropriate treatment leads to serious problems like renal failure, septicemia, and sometimes death [2]. Empiric treatment of UTIs is based on antibiotics that overuse and inappropriate use of them are associated with the emergence of resistant pathogens. According to a report, UPEC strains were 54.4% resistant to cotrimoxazole and 43.1% to broad-spectrum ciprofloxacin [3]. Increasing antimicrobial resistance and emerging multi drug resistance (MDR) strains complicate treatment of UTIs. In addition, empiric treatment with antibiotics lead to adverse events including disruption of the microbiota composition and a predisposition to certain diseases [4]. Finding alternative approaches for prevention and control of UTIs are needed to reduce the occurrence and severity of UTIs. Among them, vaccines have been considered against UTIs. Different virulence factors of UPEC have roles in UTI pathogenesis, colonization and persistence in the urinary tract [5]. As UPEC is a complicated and heterogeneous pathogen with multiple steps during the pathogenesis, designing a multi-step vaccine

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could be beneficial in inducing effective protective immunity [6]. Immunoinformatics tools with computational resources are used to identify the relevant B- and T- cell epitopes. The presence of all types of epitopes, including the B-cells, the T- helper and the cytotoxic T lymphocytes (CTLs) epitopes from different antigens as vaccine targets lead to induction of all immune responses arms with long-lasting immunity [7]. Moreover, using regions of antigen with adjuvant capacity as built-in adjuvant in multi-epitope vaccines can enhance the immunogenicity via activation of innate immune response [8]. Because UPEC is a mucosal pathogen, bacterial adherence to host cells plays a key initial step in colonization and subsequent disease progression [9]. Inhibition of urinary *E. coli* attachment to urinary epithelial cells by antibodies against bacterial surface components in human serum and urine have been evaluated in previous studies [10, 11]. Therefore, multi-epitope vaccines that induce immune responses including mucosal antibodies with inhibition of bacterial attachment to epithelial cells are suitable options for prevention of UTIs. The development of immune responses against bacterial components in serum is one of the best ways to confirm that the antigen is immunogenic [12, 13]. However, determination of the specificity and sensitivity of antibodies in serum against multi-epitope proteins is a critical step to warrant the use of those multi-epitope proteins as a vaccine candidate.

Three important antigens of UPEC strains including FimH, FyuA and CNF-1 are among the promising vaccine candidates against UPEC strains [14]. The potential of these antigens has been shown in previous studies. Therefore, they have the potential to be applied as a multi-epitope target against UPEC [15-17]. In our previous study, a multi-epitope from FimH, FyuA and CNF-1 from UPEC was designed and expressed in a prokaryotic expression system and the recombinant multi-epitope protein was used for production of polyclonal antibody in rabbits [18]. In the present study, we aimed to evaluate the sensitivity and specificity of the polyclonal antibody against the multi-epitope collected from the immunized rabbit serum using ELISA. We then assessed the potential of the polyclonal antibody to inhibit the bacterial adhesion to the bladder cell line and also to inhibit biofilm formation.

Ethics Statement

The animal studies were approved by the Ethical Committee of Pasteur Institute of Iran under approval number IR.PII.REC.1394.80.

Bacterial Strain and Cultivation

UPEC strain UTI89 as a standard strain was used for evaluating the developed polyclonal antibody. Clinical isolates of *Klebsiella pneumonia*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were isolated and collected from hospitals in Tehran, *Iran.* Luria-Bertani (LB) medium (Merck, Germany) was used for cultivation of the bacterial isolates.

Immunization of Rabbits and Serum Collection

In the previous study, New Zealand white rabbits (n = 2) received the multi-epitope protein [18]. Sera of the immunized rabbits were collected 14 days after the last vaccination. The collected sera were used for the immunoassay.

Determination of Sensitivity and Specificity of the Developed Antibody

Sensitivity of the developed antibody in the sera of rabbits was measured by indirect ELISA (iELISA). In brief, ELISA plates were coated with the multi-epitope protein, washed with PBS containing Tween 20 and blocked with bovine serum albumin (BSA). Different dilutions of serum (1:250 to 1:64,000) were applied to let the serum react with the multi-epitope of each dilution. The plates incubated with anti-rabbit IgG peroxidase conjugate antibody (Sigma, USA) and then 3,3′,5,5′-tetramethylbenzidine (TMB; Pishtazteb, Iran) and 2N HCL were used to measure the optical density with a microplate reader. In addition, ELISA was employed to assess the specificity of the anti-multi-epitope antibody collected from immunized and control rabbits. In brief, *E. coli* UTI89, *K. pneumonia*, *P. mirabilis*, and *P. aeruginosa isolates* (n = 5 from each isolate) were cultured and coated onto ELISA plates. After blocking the plates with BSA (Sigma, USA), a 1:1000 dilution from rabbit serum and then HRP-conjugated goat anti-rabbit IgG (Sigma, USA) were added to measure the absorbance with microplate reader. In addition, the multi-epitope coated onto the ELISA plates was used as positive control.

Adhesion-inhibition Assay

Human bladder cell line ATCC5637 (HTB-9) (Provider?) was grown in culture flask containing RPMI 1640 (Biosera, France) and 10% fetal bovine serum (Biosera, France). To test the bacterial adhesion inhibition, method proposed by Kim et al. [19] with some modifications was applied. A concentration of UPEC equal to 0.4 (3.2×10^8 CFU/ml) was prepared. The centrifuged bacteria were re-suspended in the tissue culture medium and incubated with the immunized rabbit antisera for 30 min. The bacteria/serum mixture was added to the monolayer cells to adherence under 5% CO_2. The above-mentioned cell line treated with unimmunized rabbit antisera was used a control. The washed cells were trypsinized for preparing the serial dilutions. The serial dilutions were cultured on LB agar to count the CFU/ml of each sample. The experiment was repeated in triplicate.

Assessment of Inhibition/Degradation of Biofilms

The effect of rabbit polyclonal antibody against the multi-epitope protein on *in vitro* biofilm formation inhibition and biofilm degradation was evaluated by crystal violet method, according to the protocols described by O'Toole and Kolter [20]. Evaluation of the inhibition of biofilm formation was done by incubating UPEC strain *E. coli* UTI89 with either polyclonal antibody or pre-immune serum for 24 h at 37°C. To test the effect of the antibody on biofilm degradation of *E. coli* UTI89 strain, bacteria were cultured on plates to form biofilm. The plates were washed with water and the plates were incubated with pre-immune serum or developed polyclonal antibody (150 µl) for 24 h. After several washes, the plates were stained with 0.1% crystal violet (Merck, Germany) and dissolved in acetic acid (Merck, Germany) to quantify by spectrophotometry. *E. coli UTI89* strain in LB without any added antibody was used as positive control and LB medium without bacteria was used as negative control.

Evaluation of the Sensitivity and Specificity

Immunogenic potential of the multi-epitope was evaluated by assessing total IgG responses in iELISA using serum collected from the vaccinated and non-vaccinated rabbits. The optimal concentration of the multi-epitope protein for iELISA to evaluate the sensitivity and specificity of the recombinant protein was 1 µg/well. The results of sensitivity indicated that the antibody in the vaccinated rabbits could significantly react with the multi-epitope protein until the dilution of 1:64,000, compared to the controls (Fig. 1).

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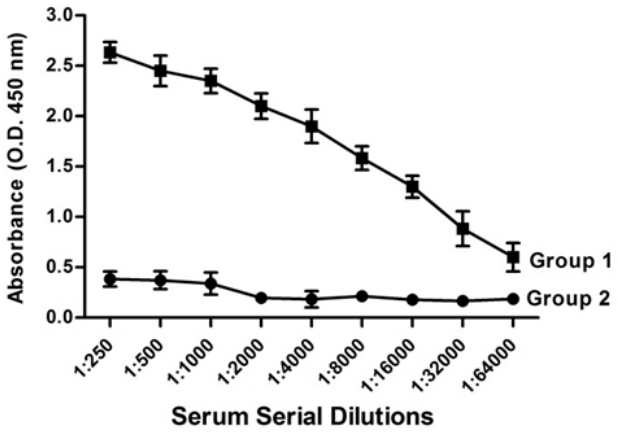

Fig. 1. Sensitivity of the antibody. Different dilutions of the serum were added to the plates coated with the multi-epitope. Group 1 is the reaction of sera from immunized rabbits with the multi-epitope. Group 2 is the reaction of sera from non-vaccinated rabbits. Bars represent mean ± Standard Deviation (SD) from 2 rabbits per groups.

The result of specificity of the antibody indicated that the polyclonal antibody has high level of binding to the multi-epitope protein and *E. coli* strain UTI89. Also, the polyclonal antibody showed some cross-reaction with *K. pneumonia* isolates, while the *P. mirabilis* and *P. aeruginosa* isolates showed no significant reaction with sera of the vaccinated rabbits (Fig. 2).

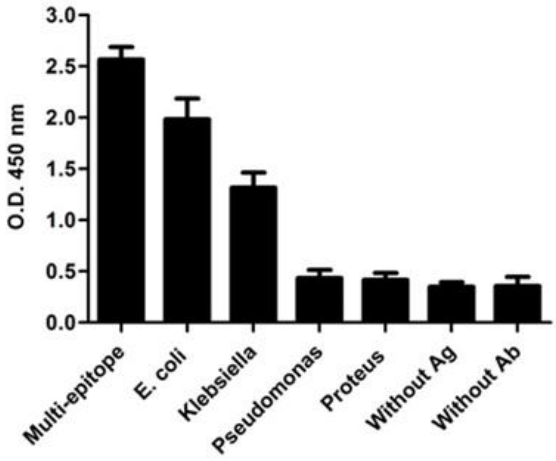

Fig. 2. Specificity of the antibody. The purified protein and also different bacterial isolates including *E. coli* UTI89, *K. pneumonia*, *P. mirabilis*, and *P. aeruginosa* were applied onto ELISA plates and the serum from the vaccinated rabbits was added to them. Wells without the multi-epitope protein and also without adding serum were used as the controls. Bars represent mean ± SD from different bacterial strains.

Adhesion and Inhibition Assay

The effect of anti-multi-epitope protein to reduce bacterial adherence to HTB-9 was evaluated. It was found that the antibody strongly reduced the adherence of UPEC to adhere to the bladder cells, compared to the unimmunized rabbit antisera which served as a negative control (Fig. 3).

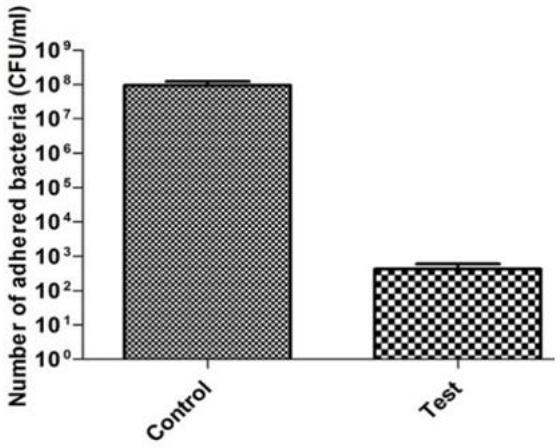

Fig. 3. Assessment of the polyclonal antibody in reduction of the bacterial adherence to HTB-9 cell line. A mixture of bacteria and serum was applied to assess the adherence to the cells. After the treatment, the supernatants were cultured to count colonies to determine the CFU/ml. The antibody significantly reduced the adherence of UPEC to HTB-9 cell line (Test). The UTI89 strain incubated with the unimmunized rabbit antisera could strongly adhere to cell line HTB-9 (Control). The experiment was repeated in triplicate.

Inhibition and Degradation of Biofilm Formation

The result of biofilm formation inhibition showed that preincubation of strain UTI89 with anti-multi-epitope antibody significantly decreased the attachment compared to controls including UTI89 strain alone and UTI89 strain incubated with preimmune serum ($P < 0.05$) (Fig. 4A). Biofilm degradation results indicated that the polyclonal antibody could reduce the biofilm biomass as compared to the controls, but this reduction was not significant ($P > 0.05$) (Fig. 4B).

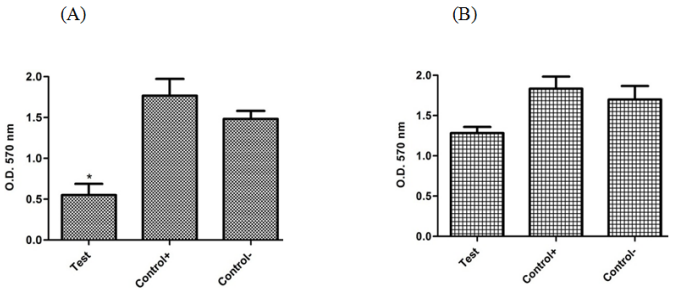

Fig. 4. Inhibition and degradation of biofilm formation. Biofilm formation inhibition and degradation of UTI89 strain was evaluated by the crystal violet method. (A) The effect of the polyclonal antibody on biofilm formation. The single asterisk indicates that the antibody significantly reduced the biofilm formation, as compared to the positive control (UTI89 strain without incubation with antibody) and the negative control (UTI89 strain incubated with pre-immune antibody). (B) The effect of polyclonal antibody on biofilm degradation of UTI89 strain. The polyclonal antibody (Test) could not significantly reduce the biofilm biomass, as compared to the controls. The results are the average of three independent experiments. Bars represent mean ± SD.

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DISCUSSION

Increased antibiotic resistance among UPEC strains complicate UTI treatment. Preventive approaches with vaccines might provide an important alternative against UTIs. Since UPEC pathogenesis is related to expression of multiple virulence factors that are necessary for urinary tract establishment, the development of a multi-epitope vaccine based on different virulence factors of UPEC may be useful [21]. Our previous study led to the designation of a multi-epitope protein, consisted of FimH, FyuA and CNF-1 proteins of UPEC which their immunogenicity was confirmed in a rabbit model [18?]. The main criteria for selection of the mentioned proteins was their critical roles in the pathogenesis of UPECs [2]. In the present study, the sensitivity and specificity of the developed rabbit polyclonal antibody against the multi-epitope protein was evaluated. Our result showed that using serial dilutions of rabbit polyclonal serum was able to react with the recombinant protein. The presence of different epitopes of the multiple proteins in a single construct is likely to increase the interaction of the polyclonal antibody with the multi-epitope protein even at low antibody titers. In addition, the polyclonal rabbit antibody had specificity for the multi-epitope protein without any reaction with *P. mirabilis* and *P. aeruginosa strains.* There was some cross reactivity with *K. pneumonia* that was probably associated with similarity between some antigenic-epitopes from *K. pneumonia* and UPEC strains. Indeed, previous studies hve also reported highly homologous amino acid sequence of FimH in *K. pneumoniae* and *E. coli* strains [22, 23].

Since adhesion is among essential pathogenicity mechanism of *E. coli* causing UTI, we investigated the impact of the polyclonal antibody against the multi-epitope protein on UPEC interaction with the human bladder cell line. A significant reduction in bacterial adherence by *E. coli* UTI89 strain to HTB9 bladder cells was observed with the polyclonal rabbit antibody compared to the pre-immune sera and without the antibody. Using several segments of FimH, FyuA and CNF-1 in the vaccine construct with attachment role to urinary epithelial cells and producing polyclonal antibody against them can justify their adhesion inhibition to HTB-9 cell line. The ability of antibodies against these antigens in reducing UPEC colonization has also been observed by previous studies [15, 24, 2].

Biofilms are involved in the majority of chronic and recurrent bacterial diseases including those affecting the urogenital tract [25]. Application of antibodies against surface components of bacteria can affect their biofilm formation [26, 27]. In the present study, it was observed that the polyclonal antibody could inhibit the biofilm formation of cultured *E. coli* UTI89 strain, while the antibody could not significantly inhibit biofilm degradation. Antibodies bound to various epitopes of proteins on the bacterial surface can possibly block the function of the components that may be crucial for biofilm formation in UPEC. The function of surface proteins including FimH and FyuA of UPEC strains in biofilm formation has also been reported in previous studies [1]. In addition, accumulation of bacteria in biofilm structures by covering antigen epitopes reduces or inhibits the effect of polyclonal antibodies in biofilm degradation.

In conclusion, this study indicated that the developed polyclonal antibody against a multi-epitope experimental vaccine, composed of FimH, FyuA and CNF-1 of UPEC strains exhibited high sensitivity and specificity for the recombinant protein. The polyclonal antibody also reduced the binding of UPEC strain to bladder cell line. Additionally, the polyclonal antibody showed high efficacy to inhibit biofilm formation in UPEC strain.

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CONFLICT OF INTERESTS

The authors declare they have no conflict of interests.

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