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Original Article

Cloning of Protective and Broadly Conserved Vaccine Antigens from the Genome Of Extraintestinal Pathogenic Escherichia coli into pET28a Vector

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Abstract

Urinary tract infections are one of the most common infectious diseases that lead to significant health problems in the world. Urinary tract infections are referred to any infection in any part of the renal system. Uropathogenic Escherichia coli, Proteus mirabilis, and Klebsiella are main organisms that are involved in these infections. After identifying pathogenic epitopes in these microorganisms with a similarity upper than 80%, sequences of synthetic gene was provided by bioinformatics techniques and ordered from Thermo Fisher Scientific Company. PCR amplification of this gene was performed by specific primers designed for this purpose. Construction of gene was performed by overlap PCR. The synthetic gene was cloned into pET28a vector. Our gene was amplified in E. coli Top10 tested.

To confirm cloning, three methods including colony PCR, digestion and sequencing were used. First, two techniques were performed using horizontal electrophoresis, and also the synthetic gene showed significant homology with the sequence (100% Identified) in third technique that was sequencing. Sequencing of this gene showed that fusion was constructed correctly. Determination of biochemical properties such as 3D structure, Ramachandran and comparison of Non-redundant Set of PDB structure was done by bioinformatic software and had exact and expectable results.

A large part of the health system in the world is occupied by a urinary tract infection and governments spend a huge amount of money for the treatment and recovery of patients with these infections. On the other hands, antibiotic resistance in the not-far future will be a disaster for medical societies. This is the most important reason for the emergence of vaccine production against urinary tract infections.

Keywords: urinary tract infection, Escherichia coli, cellular immune system, cloning

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INTRODUCTION

Urinary tract infection (UTI) is one of the most common infections that cause significant health problems in the world and is referred to any infection of the urinary tract (Ragnarsdottir, et al. 2008). UTIs account for about 7 million clinic visits annually, with a cost exceeding $3.5 billion and 250,000 hospitalizations in United States (Langermann, et al. 2000; Dhakal, et al. 2008). Approximately, 40% of women experience UTIs at least once in their life time (Wright, et al. 2005). Urinary tract infections are a significant cause of morbidity in older men, infant boys and females of all ages. Several risk factors are related to cystitis, including female gender, a prior UTI, sexual activity, vaginal infection, diabetes, obesity and genetic susceptibility. Clinically, Urinary tract infections are categorized as complicated or uncomplicated (Lane, et al. 2007). Uncomplicated UTI has not any structural or functional abnormality of the urinary tract. Uncomplicated UTI is the commonest type of infection. Complicated UTI appears in the presence of an abnormal urinary tract or another factor that increases susceptibility to infection. Bladder outflow obstruction, neuropathic bladder, prostatic disease, urethral structure, multiple sclerosis and diabetes mellitus are some of the common causes of complicated urinary tract infection. Since the urinary tract infection in men often occurs in the elderly and infants in association with urologic abnormalities, it conventionally has been considered complicated UTI. In the United States, indwelling catheters are the cause of 80% of complicated UTIs. UTI is classified as lower UTI (urethritis and cystitis) or upper UTI (pyelonephritis and perinephric abscess) (Armbruster, et al. 2017). UTIs encompass infections of the urethra, bladder, ureters, and kidney. Most of the UTIs occur in the bladder (Cystitis) (Armbruster, et al. 2017, Fusco, et al. 2017). UTI can lead to serious conditions such as bacteremia and renal scarring (Jain, 2017). Patients with UTI are commonly treated with antibiotics; these antibiotics can result in long-term alteration of the normal microbiota of the vagina and gastrointestinal tract and in the development of multidrug-resistant microorganisms (Jain 2017, Yu; et al. 2017). Furthermore, the prevalence of antimicrobial resistance in patients with UTI is increasing in recent decades (Dhakal, Kulesus et al.). These are some of the reasons for the importance of developing an effective vaccine against UTIs. Escherichia coli, Klebsiella pneumonia, and Proteus mirabilis are the most common uropathogens in both the community and hospitals (Priyanka, 2017), whereas, Staphylococcus saprophyticus, Enterococcus faecalis, Staphylococcus aureus and Pseudomonas aeruginosa each are the cause of less than 11% of cases (Das, et al. 2006) (Table 1).

Table 1 Bacteria causing urinary tract infection according to community acquired

Types	Percent
Escherichia coli	77%
Proteus mirabilis	4%
Klebsiella pneumoniae	4%
Enterococcus faecalis	4%
Others	11%

After identifying pathogenic genome in major microorganisms involved in UTIs (Escherichia coli, Proteus mirabilis, and Klebsiella Pneumoniae), sequences of our synthetic gene was provided by bioinformatic techniques and ordered from Generay Biotechnology Company (Shanghai, China). In this study, we cloned and characterized the recombinant gene that is designed from common pathogens that have a homology of upper than 80% in three major uropathogens included Uropathogenic Escherichia coli, Proteus mirabilis, and Klebsiella Pneumoniae.

MATERIALS AND METHODS

I. Materials

The enzymes and kits used for the various experiments in this study were purchased from Thermo Fisher Scientific except for the DNA extraction kit, which was from Roche Applied Science Kit (Roche).

II.Design of the synthetic gene

Designing the synthetic gene was done with the Bioinformatics software and considered most stable secondary structure. This gene has uropathogens with more than 80% homology in three major microorganisms included Uropathogenic Escherichia coli (UPEC), Proteus mirabilis and Klebsiella Pneumoniae that involved in urinary tract infections.

III. Digestion of Gene and pGH vector

A plasmid vector (pGH) (Figure 1) was digested with the HindIII restriction enzyme (Fermentas, Lithuania) and ligated at 22ºC for 70 min by T4 DNA ligase (Fermentas, Lithuania) followed by inactivation at 80ºC for 20 min. The result of digestion was identified by the 1% agarose gel electrophoresis.

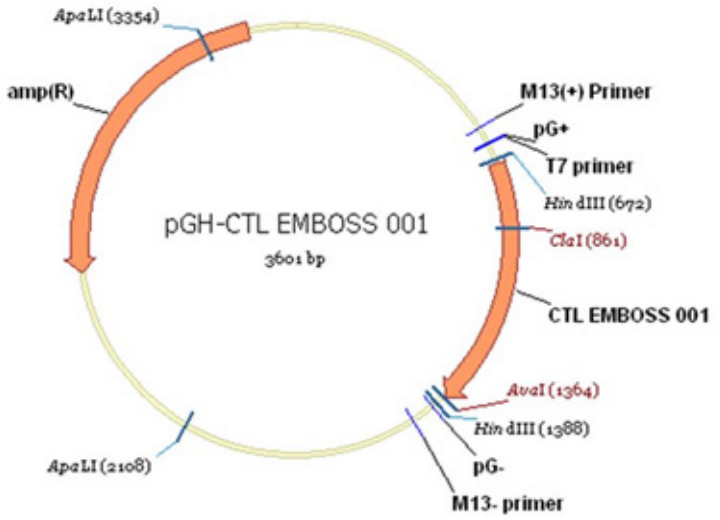


Figure 1: Constructed Map of pGH vector with synthetic gene.

IV. Clone Preparing

Escherichia coli (TOP10 strain) was transformed with the plasmid according to Instruction. Bacterial clones containing the recombinant plasmid were cultured on ampicillin embedded LB agar. The plasmids were isolated and the presence of the desired fragment was examined by digestion assay according to the manufacturer's protocol.

V. Vector and DNA Extraction

E. coli TOP10 was grown in 5 ml of Luria Bertani (LB) broth medium. Vector pET28a and DNA were extracted using Roche Applied Science Kit (Roche) as suggested by the producer.

VI. PCR Amplification and purification

PCR amplification of the gene was performed by forward and reverse primer designed for conserved 3' and 5' (Figure 2) end of the gene. PCR reactions were performed by Eppendorf thermo-cycler; PCRs were carried out in 50 μl

Figure 2: Digestion of Plasmid pGH vector.

Lane 1: Molecular weight marker (1 kb Ladder DNA). Lane 2, 3 and 4: digested plasmid vector with HindIII

volume containing 3μl of DNA template, 2μl of forwarding primer (Nco1), 2μl of reverse primer (Xba1), 25 μl Master Mixture and 18 μl DDW (Deionized Water). The PCR condition for amplification of the synthetic gene included initial denaturation for 5 min at 95.0 ˚C, and then 20 cycles, each 1 min at 95 ˚C, 1 min at 60 ˚C and a final extension at 72 ˚C for 1 min. 5 μl of samples were subjected to electrophoresis on a 1% agarose gel to confirm the presence of the amplified products.

VII. Double Digestion of Gene and pET28a vector

DNA and Plasmid vector (pET28a vector) were digested with the restriction enzymes (Fermentas, Lithuania) and ligated at 22ºC for 70 min by T4 DNA ligase (Fermentas, Lithuania) followed by inactivation at 80ºC for 20 min. The result of digestion was identified by the 1% agarose gel electrophoresis.

VIII. Ligation of the synthetic gene into the pET28a vector

5 μl of PCR products of the synthetic gene was purified by the use of gel extraction kit (Roche) and ligated into 10 μl of pET28a vector for regulated, secreted expression of recombinant proteins containing C-terminal 6xHis tags in E. coli (purchased from Invitrogen; catalog no. V450-01) 35 μl in the 67 mM Tris-HCl, pH 8.0, 10 mM MgCl2, heated at 100°C for 2 min and cooled to room temperature for an hour. 1 μl T4 DNA ligase (purchased from

Table 2: content of forward and reverse primers which were designed for 3' and 5' end of synthetic gene.

Forward primer for NcoI	3' CATGCCATGGCAGGTACACCACAG 5'
Reverse primer for Xba1 enzyme	3' GCTCTAGAACGCAAGCAGCATGATG 5'

Fermentas) was used for the ligation at 14°C for 16 h. Finally, plasmids were transformed into the competent E. coli Top10 cells.

The Mac Conkey and agarose plates containing 100 μg/μl kanamycin were used for selection of transformed colonies. The white colonies were selected and following an overnight cultivation, they were subjected to plasmid extraction and PCR. In order to verify the fidelity of the cloned fragments, the selected recombinant plasmids were subjected to sequencing (MWG service).

IX. Identification of the recombinant colony with digestion

Competent E. coli Top10 cells prepared by the DMSO method (Chung, et al. 1989) were transformed with the ligation mixture. Recombinant colonies were evaluated in terms of size by the 1% agarose electrophoresis after digestion with the restriction enzymes (Fermentas, Lithuania), followed by inactivation at 80ºC for 20 min.

X. Identification of the recombinant colony with PCR

5 μl of samples were subjected to electrophoresis on a 1% agarose gel to confirm the presence of the amplified products. PCR was carried out in 50 μl volume containing 3μl of DNA template, 2μl of forwarding primer (Nco1), 2μl of reverse primer (Xba1), 25 μl master mixture and 18 μl deionized water. The PCR condition for amplification of the synthetic gene was similar to earlier one (Table 3). 5 μl of samples were subjected to electrophoresis on a 1% agarose gel to confirm the presence of the amplified products.

Table 3: PCRs were done under this program.

Steps	**Time**	Temperature
Initial denaturation	5 min	95°C
Denaturation	1 min	95°C
Annealing	1 min	60°C
Extension	1 min	72°C
Repeat	30 time	-
Final extension	10 min	72°C

XI. N-terminal amino acid sequence analysis

Sequential Edman degradation for sequencing surveys was ordered to Gene Fanavaran Company.

XII. Determination of biochemical properties of synthetic gene

Determination of biochemical properties of synthetic gene was done by bioinformatic

tools included 3D structure by ExPASy server; SWISS-MODEL Workspace (https://www.expasy.org/proteomics), ramachandran plot by QMEAN (https://swissmodel.expasy.org/qmean) and comparison of Non-redundant Set of PDB structure by PDBePISA (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html).

RESULT

Digestion of Plasmid vector (pGH) (Figure 1) was identified by the 1% agarose gel electrophoresis presented in Figure 2. Extracted vector and DNA were identified by

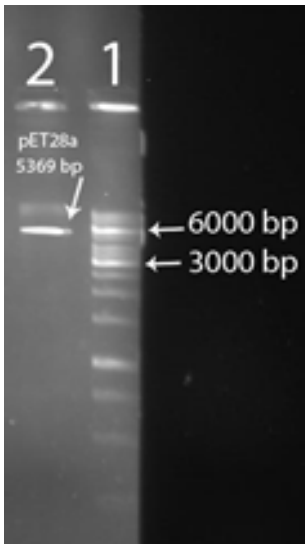


Figure 3 Extracted vector. Lane 1: Molecular weight marker (1 kb Ladder DNA). Lane 2: extraction of vector pET28a.

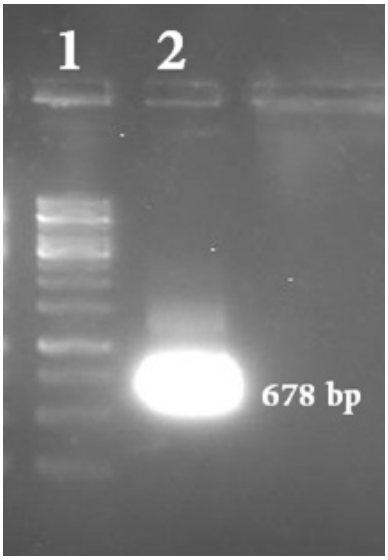


Figure 4 DNA extractions. Lane 1: Molecular weight marker (1 kb Ladder DNA). lane 2: DNA extraction with lenght of 678 bp.

the 1% agarose gel electrophoresis (Figure 3: extraction of vector and Figure 4: DNA extraction). The PCR conditions were optimized for amplification of our synthetic gene. The synthetic gene was present in all of the E. coli templates. Electrophoresis of PCR products showed that the

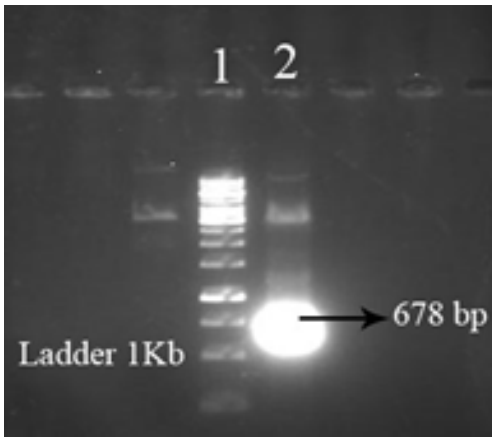


Figure 5: PCR amplification of synthetic gene.
Lane 1: Molecular weight marker (1 kb Ladder DNA); Lane 2: PCR amplification of synthetic gene with length of 678 bp.

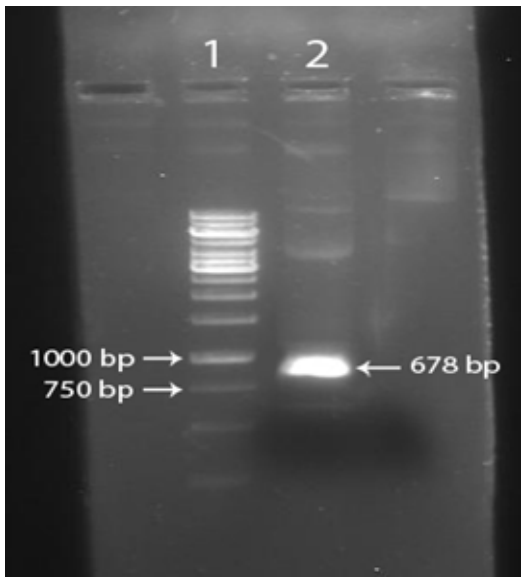


Figure 6: PCR purified DNA. **Lane 1: Molecular weight marker (1 kb Ladder DNA)**. **Lane 2: PCR purified DNA**.

length of PCR fragment of synthetic gene was approximately 678 bp (Figure 5). After amplification of synthetic gene and digestion of eluted fragment by the HindIII enzyme, it was cloned in pET28a and transformed into the competent cells (Figure 6).
Confirmation of cloning of the gene by digestion with NcoI and XbaI restriction enzymes has been shown in Figure 7. After doing ligation of the synthetic gene into the pET28a vector, 5 μl of samples were subjected to electrophoresis on a 1% agarose gel to confirm the presence of the ligated products (Figure 8). Confirmation of the recombinant colony with PCR, 7 μl of samples was subjected to electrophoresis on a 1% agarose gel, presented in Figure 9. Double digestion of this gene showed that fusion was constructed correctly, which is presented in Figure 10. Construction of plasmid/pET28a vector is shown in Figure 11. Sequential Edman degradation was performed and identification was 99% (Figure 12).

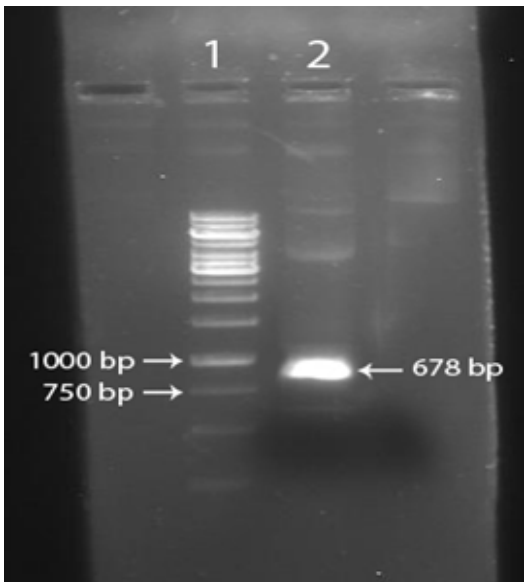


Figure 6: PCR purified DNA. Lane 1: Molecular weight marker (1 kb Ladder DNA). Lane 2: PCR purified DNA.

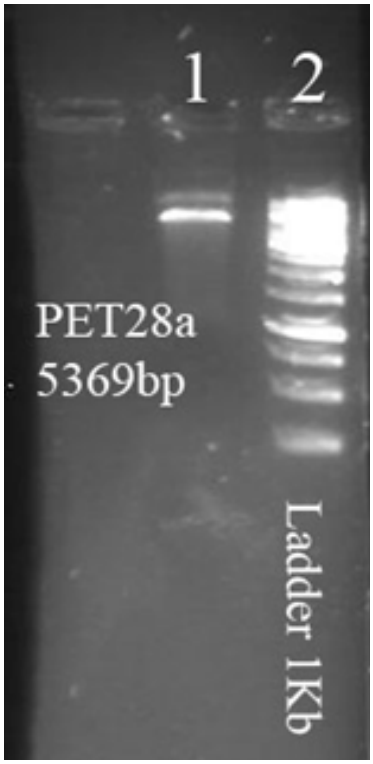


Figure 7: Double Digestion of pET28a vector. Lane 1: Molecular weight marker (1 kb Ladder DNA). Lane 2: Digestion of pET28a vector with length of 5369 bp.

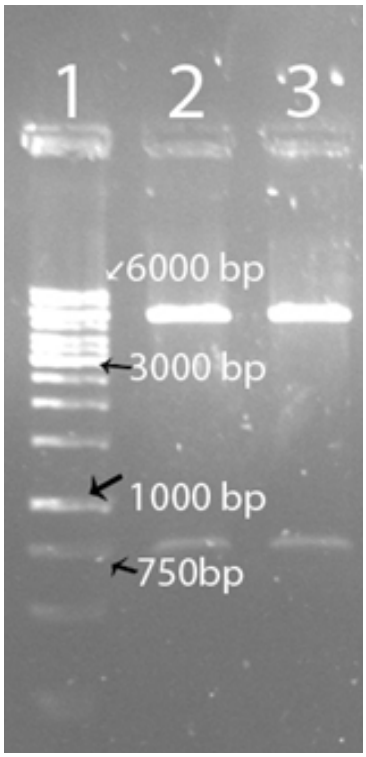


Figure 8: Ligation of synthetic gene into pET28a vector. Lane 1: Molecular weight marker (1 kb Ladder DNA). Lane 2 and 3: ligation of DNA into pEET28a vector.

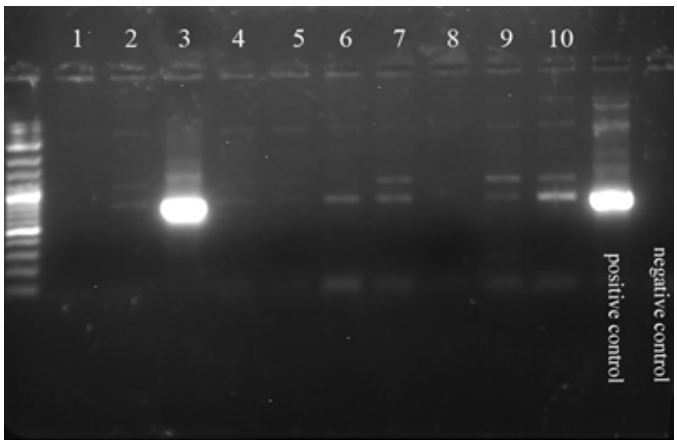


Figure 9: Confirmation of cloning with PCR. Lane 2–10: colonies after amplified by PCR. Third colony had a best result.

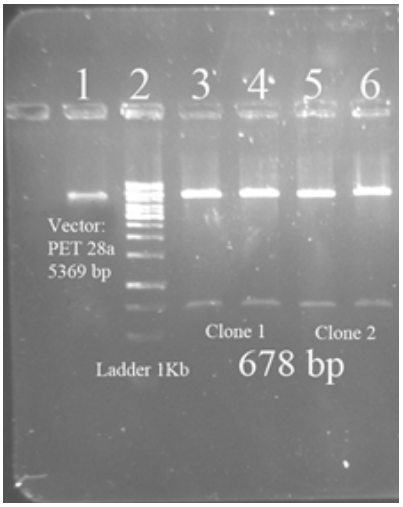


Figure 10: Confirmation of cloning with digestion with NcoI and XbaI restriction enzymes. Lane 1: pET28a Undigested. Lane 2: Molecular weight marker (MW);
Lane 3, 4, 5 and 6: Confirmation of digested pET28a-synthetic gene.

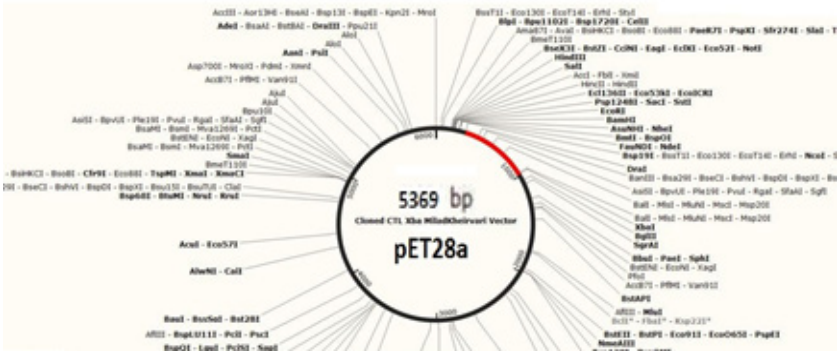
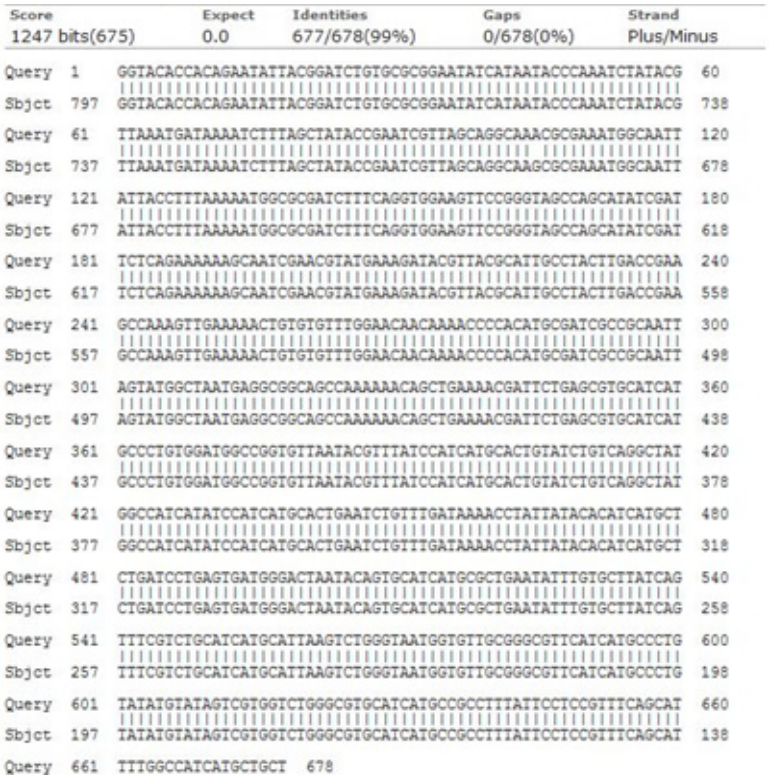


Figure 11: map of pET28a with synthetic gene. This Sequence designed with special Primers for 3' and 5' end by Bioinformatic softwares included: Snap Gene, Chromas and Gene Runner.



Score	Expect	Identities	Gaps	Strand
1247 bits(675)	0.0	677/678(99%)	0/678(0%)	Plus/Minus

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  797  GGTACACCACAGAATATTACGGATCTGTGCGCGGAATATCATAATACCCAAATCTATACG  738

Query  61   TTAAATGATAAAATCTTTAGCTATACCGAATCGTTAGCAGGCAAACGCGAAATGGCAATT  120
            ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
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Query  121  ATTACCTTTAAAAATGGCGCGATCTTTCAGGTGGAAGTTCCGGGTAGCCAGCATATCGAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  677  ATTACCTTTAAAAATGGCGCGATCTTTCAGGTGGAAGTTCCGGGTAGCCAGCATATCGAT  618

Query  181  TCTCAGAAAAAAGCAATCGAACGTATGAAAGATACGTTACGCATTGCCTACTTGACCGAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  617  TCTCAGAAAAAAGCAATCGAACGTATGAAAGATACGTTACGCATTGCCTACTTGACCGAA  558

Query  241  GCCAAAGTTGAAAAAACTGTGTGTTTGGAACAACAAAACCCCACATGCGATCGCCGCAATT  300
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Query  421  GGCCATCATATCCATCATGCACTGAATCTGTTTGATAAAACCTATTATACACATCATGCT  480
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Sbjct  197  TATATGTATAGTCGTGGTCTGGGCGTGCATCATGCCGCCTTTATTCCTCCGTTTCAGCAT  138

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Figure 12: Result of sequencing analysis after blast showed 99% identification.

DISCUSSION and CONCLUSION

Urinary tract infection is one of the most common infections diagnosed in patients in Iran and the world (Prakash, 2016; Priyanka, 2017). Uropathogenic Escherichia coli (UPEC), Proteus mirabilis and Klebsiella have the most common pathogens found in urinary tract infections (Ronald, 2003, Bryce, et al. 2016). Our recombinant gene containing virulence epitopes of pathogens that has homology upper than 80%, therefore, after expression, it would stimulate cellular immune system against urinary tract infections. The emergence of antibiotic resistance in the world and ineffectiveness of available vaccines are the major causes for an increasing requirement for vaccine development against urinary tract infections (Biadglegne and Abera 2016; Walker, et al. 2016).

This work describes, for the first time, the construction of a genetically constructed recombinant protein with modern vaccine designing against urinary tract infections. Hence, further immunological studies are required for evaluation of this gene and protein as a novel and safe vaccine candidate against urinary tract infections. A large part of the health system in the world is occupied by a urinary tract infection and government spends a huge amount of money for the treatment and recovery of patients with these infections. On the other hands, antibiotic resistance in the not far future will be a disaster for medical societies. This is the most important reason for producing a vaccine against urinary tract infections. We selected the pET28a expression system for expression of this synthetic gene. The pBADgIII/A expression vector has an extremely high basal expression. In the last studies, the expression yield of recombinant proteins in pBADgIII/A was lower than the

yield of the proteins in a pETa expression system. The recombinant gene and/or protein sequence properties can influence protein expression in E .coli hosts (Francis and Page, 2010). One of the main factors that proteins fail to express in E. coli is the presence of rare codons in the gene of interest (Gomes and Mergulhão, 2017). The genes that contain a number of rare codons are more likely to be expressed at low levels (Sørensen and Mortensen, 2005). With this vision, we selected pET28a vector for the cloning process.

ACKNOWLEDGEMENT

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