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Prevalence and Evaluation of Toxin Genes among Uropathogenic *Escherichia coli* Clinical Isolates by Duplex PCR

Javad Saraylu [1], Jalil Fallah Mehrabadi [1], Abbas Ali Imani Fooladi [2], Aylar Sabbaghi [3], Hedrosha Molla Aghamirzaei [3], Mohsen Hasankhani [4]*

[1] *Department of Microbiology, Faculty of Basic Sciences, Minoodasht Branch, Islamic Azad University, Minoodasht, IR Iran*
[2] *Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, IR Iran*
[3] *R & D Laboratory of Molecular Biology, Gene Fanavaran Company, Tehran, IR Iran*
[4] *Clinical Pathology Laboratory, Omid Hospital, Abhar, IR Iran*

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ABSTRACT

Background: One of the most common infections in human is urinary tract infection (UTI) and Uropathogenic *Escherichia coli* is one of its major causative agents. UTI is extremely common among young women. Children under age 5 are also highly at risk. Considering the prevalence of this disease, it is necessary to design an appropriate diagnostic method for its effective diagnosis. The aim of present study was to identify the prevalence of two virulence genes (*sat* and *vat*) among Uropathogenic *E. coli* isolates.

Methods: Urine samples were taken from 350 patients with urinary tract infection. The samples were cultured on EMB agar and Blood agar. The suspected *E. coli* colonies were isolated and confirmed by biochemical tests. The genomic DNA was extracted from 297 isolated *E. coli* and target genes were amplified by PCR. The amplicons were sequenced and analyzed with ClustalW software. Moreover, data analysis was performed by using SPSS software. Subsequently, Duplex PCR was optimized for simultaneous detection of two genes.

Results: The prevalence of *sat* and *vat* genes were 75 (n: 225) and 36 (n: 106) percent, respectively. In addition, less than 4% (n: 11) of clinical isolates comprised two genes.

Conclusion: According to the conducted research, molecular identification of Uropathogenic *E .coli* strains according to detection of *sat* gene is potentially an appropriate method and could be noted for diagnosis.

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* *Corresponding Author:* Jalil Fallah Mehrabadi, PhD., Department of Microbiology, Faculty of Basic Sciences, Minoodasht Branch, Islamic Azad University, Minoodasht, IR Iran. *Tel:* +98 21 44404055, *Fax:* +98 21 44452017, *E-mail:* jalil.fallah@gmail.com

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infectious diseases encountered in clinical practice and account for significant morbidity and high medical costs. *Escherichia coli* is the most predominant pathogen causing 80-90% of community-acquired UTIs and 30-50% of nosocomially-acquired UTIs (1). The *E. coli* pathotypes that cause extraintestinal infections are collectively called Extraintestinal Pathogenic *E. coli* (ExPEC) which comprise various virulence factors and could not be determined by conventional diagnostic methods (2). One of these pathotypes is Uropathogenic *Escherichia coli* (UPEC), the most common etiological agent of community-acquired UTIs (3).

Antibiotics usually are effective in treating acute infections and are the primary means of prophylaxis for recurrent UTI patients; however, their value is being lessened by the emergence of increasing numbers of drug-resistant bacteria. Consequently, it is important that alternative prevention strategies be developed, and one approach being actively explored is immunization of susceptible individuals to increase natural immunity against infection (4). The components of the vaccine should necessarily reflect the antigenic profile of bacteria responsible for the largest number of UTIs, which are various strains of *E. coli*, *Klebsiella*, and *Proteus* species (5). UPEC isolates typically carry large blocks of genes, called pathogenicity-associated islands which are not found in fecal isolates. Some of these strains also secrete specific proteins that may contribute to pathogenesis of UTI. Among these proteins are the well-characterized hemolysins and moreover, described secreted autotransporter toxins: *Sat* (6) and *Vat* (7). The virulence factors among UTI agents are really different and clinical isolates could not be detected as UPEC by using traditional methods. Also, some UPEC strains have ascending properties after colonization in bladder and can affect the kidney tissue. Therefore, using molecular detection methods is critical for identification of UPEC strains. The aim of this study was to assess the prevalence of *sat* and *vat* virulence genes among *E. coli* strains.

Methods

Collection of samples and E. coli isolates identification

Three hundred and fifty urine samples were collected from three hospitals in Tehran, Minoodasht and Abhar cities during six months period (May to October 2011). All samples were cultured on Blood agar and MacConkey agar at the same hospital lab. The 297 *E. coli* isolates were confirmed using standard biochemical tests for *E. coli* (IMViC reactions; *E. coli* isolates are identified by positive indole and methyl red tests, and negative Voges-Proskauer and citrate utilization tests) (8).

Storage of E. coli isolates

After identification, each *E. coli* isolate was inoculated into 5 ml of Luria-Bertani broth and incubated, with shaking, at 37°C overnight. Isolates were stored as a mixture of the broth culture and glycerol (1:1 ratio, by volume) at -70°C until PCR assays were performed.

Primer designing

All sequences of *sat* and *vat* genes available in GenBank database were aligned using MEGA 4 program. The conserved sequences with a high degree of homology were selected and used to design primers via the Gene Runner software (Hastings software, Inc.). The designed primers were subsequently tested in silico against submitted sequences in a BLAST analysis. The primer sequences and expected sizes of amplicons for each PCR assay are described in Table 1.

Genomic DNA extraction, PCR mixtures and conditions

Genomic DNA was extracted from *E. coli* strains using the extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. PCR was performed to amplify the *sat* and *vat* genes using primers described in Table 1. Each reaction contained 2.5 µl buffer (10X), 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTP (10 mM), 1 µl (50 pmol) each for the forward and reverse primers, 1 µl Taq DNA polymerase (1U/µl), 1 µl template DNA (200 ng), and 16 µl H_2O in a final volume of 25 µl. All reaction mixtures were overlaid with 30 µl of mineral oil. The PCR was carried out under the following conditions: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min. The final elongation step was conducted at 72°C for 5 min. The PCR amplicons were subsequently resolved by electrophoresis on a 1% agarose gel. The amplicon with the expected size was excised from the gel using DNA extraction kit (Fermentas, Germany) and sent to Macrogen Company, South Korea for sequencing. The sequences were then aligned with the known *sat* and *vat* sequences in the NCBI database. Duplex PCR reactions were performed with the simultaneous addition of primers for the two genes in the same mixture.

Table 1. Primers used for amplification in this study

Target genes	Primer sequences (5`to 3` as synthesized)	Expected amplicon size (bp)
sat	F: CTACAGCTTGATCACCTATGGC	410
	R: CTCCCTGGTATTTCTTTGTGG	
vat	F: TTCACGGTACTGTTGTTCGC	217
	R: CAGATAACTCCAGCGTCACG	

Results

Three hundred and fifty (350) urine clinical samples were collected and 297 *E. coli* isolates were identified among them. The uniplex PCR of *sat* and *vat* genes was performed for 297 *E. coli*, and the results showed 225 strains (75%) encompass *sat* gene and 106 strains (36%) comprise *vat* gene. Moreover, the Duplex PCR detected 11 strains (3.7%) which contained two genes.

Discussion

Uropathogenic Escherichia coli is the most common cause of urinary tract infection. Although UPEC has no uniquely defining features, certain serotypes and electrophoretic types are predominate.

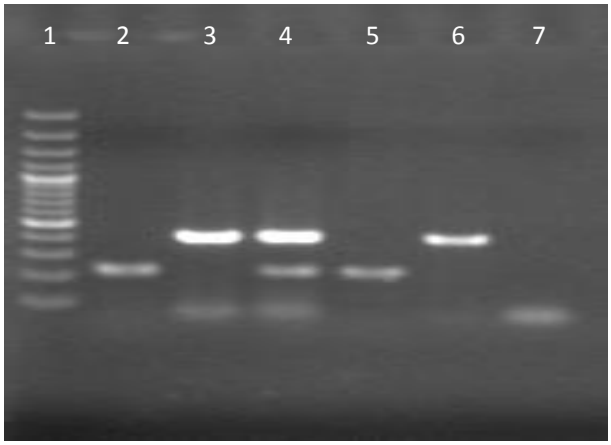

Figure 1. PCR (uni and Duplex PCR) of *sat* and *vat* genes in *E. coli* isolated from urine samples.
Lane 1, Ladder 100bp;
Lane 2 and 5, *vat* amplicon (217 bp);
Lane 3 and 6, *sat* amplicon (410 bp);
Lane 4, Duplex PCR of *sat* and *vat* amplicons;
Lane 7, Negative control.

Many UPEC strains contain known virulence factors, including various adhesins, encapsulation, toxins, protease, and siderophore. While uropathogenic virulence factors are found less frequently among commensal strains of fecal *E. coli* than UPEC and often occur together on pathogenicity islands (PAIs), no known uropathogenic virulence factors individually or jointly identify UPEC isolates, uniquely (10).

Autotransporters are one category of secreted proteins implicated in the virulence of UPEC. Members of the serine protease autotransporters of the enterobacteriaceae (SPATE) family are proteins from *E. coli* and *Shigella* spp. which, like the immunoglobulin A1 (IgA1) proteases and Hap autotransporters of *Neisseria* and *Haemophilus* spp., possess a consensus serine protease motif. Since the description of the first SPATE, a number of investigators have described SPATE proteins in the different pathotypes of *E. coli* and in *Shigella* (11). Sat (secreted autotransporter toxin), is a 107 kDa secreted protein, that expressed significantly more often by *E. coli* strains associated with the clinical symptoms of acute pyelonephritis (68% of strains) than by fecal strains (14% of strains) (12). Ruiz and colleagues assessed nine virulence factor genes by using PCR among patients who involved cystitis or pyelonephritis and reported the relationship between prevalence of *sat* and *pap* genes (13). The same result was reported by Johnson (14) and it is a potential clue that detection of *sat* gene in UPEC pathotype is important and could be an indicator for set-up a new diagnostic method. In present survey, prevalence of *sat* gene among clinical isolates was 75% and it is the first report from our country. Moreover, it shows *sat* gene has a potential value for selecting as one of candidate antigen for designing new vaccine against UTI.

Parreira and Gyles (15) identified a gene encoding a serine protease autotransporter protein (Vat), which was responsible for the vacuolating activity of a strain of *E. coli* (Ec222) that had been isolated from a septicemic chicken. *Vat* is encoded on a PAI which is inserted adjacent to the *thrW* tRNA gene. The presence of *vat* at this position has been demonstrated for uropathogenic *E. coli* strain CFT073 and the neonatal meningitis strain *E. coli* RS218 (7). The prevalence of *vat* gene in this study was 36% and it could be considered among urine clinical isolates because *Vat* may be required for *E. coli* to enter or survive within the bloodstream and strain that have *vat* gene is more risky than other UPEC pathotype strains. Simultaneous detection of *vat* and other virulence factor genes like *sat*

and *pap* by using molecular methods ex. Duplex PCR may be informative for the best UTI treatment. Some other virulence genes have been assessed among UPEC pathotype strains. The results elucidated a relation between isolated strains and type of UTI disease. For instance, Marrs et al, reported *aer*, *kpsMT* and *papG* genes occurred significantly more often among pyelonephritic isolates than in any of the other collections. Moreover, the *fim* gene occurred in virtually all isolates from all collections (16).

Although, Zhao *et al* reported from China that O1 was the most common serogroup in the tested UPEC isolates collected from a province; *feoB* and *fimH* were the most prevalent virulence associated genes. In their study all isolates were multi-resistant to several drugs; and nitrofurantoin-resistant isolates had reduced virulence factor genes compared with susceptible strains (17). It is a surprising note and sizable result which was indicated by other studies. Drews et al reported a similar result which presented Beta-hemolysis was statistically significantly less likely in both outpatient and inpatient fluoroquinolone-resistant strains than in fluoroquinolone-susceptible isolates (17).

Conclusion

It is compulsory to evaluate the prevalence of virulence factors among clinical isolates of UTI agents for designing a molecular detection method in future.

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