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**Running title**: Virulence and MDR among UPEC
SUMMARY

To determine the resistance patterns of uropathogenic *Escherichia coli* (UPEC) isolates and to investigate the frequency of several virulence genes, *fimH*, *papA*, *hlyD*, *cnf-1*, *sitA* and *tsh*, among different phylogenetic groups of UPEC isolates. A total of 85 *E. coli* isolates were recovered from urine samples from outpatients with clinical diagnosis of uncomplicated urinary tract infections. Molecular approach and antimicrobial resistance pattern were done by PCR and disc diffusion method. The frequency of virulence factor genes detected by PCR was: *fimH* (34.1%), *papA* (9.4%), *hlyD* (21.2%), *cnf-1* (3.5%), *sitA* (15.3%) and *tsh* (27.1%). The results revealed that isolates were resistant to SXT (74.1%), CTX (68.2%) and AMC (94.1%) and relatively less resistance to N (56.5%). According to the results, larger investigations are suggested to be done in future to exactly determine whether SXT, CTX and AMC are appropriate antibiotics for treatment of UPEC infections in southern Iran or not. Although these results have demonstrated that *fimH* is the most frequent virulence genes among UPEC, but high prevalence of isolates which do not encoded *fimH* (75.9%) and also relatively low frequency of isolates carrying other virulence genes need further investigations to clarify the role of other potential virulence factors in pathogenesis of these isolates.

*E. coli* strains isolated from urinary tract are known as uropathogenic *Escherichia coli* (UPEC), which causes 80-90% of community acquired urinary tract infection (UTI) and also 50% of nosocomial UTI (1). Most individuals suffering from this infection are young women which half of them experience UTI by age 30. There are some reports that men and children are also exposed to this infection (2-4). UTI is the cause of about 8 million physician visit and costs about 1.6 billion Dollars annually for the health care system in United States (5). Virulence
factors involved in UPEC infection include fimbrial and non-fimbrial adhesins, toxins, iron acquisition factors, LPS and capsule which facilitate colonization of bacteria in urinary tract and its invasion to the host cells (6).

The evidences have indicated that common antibiotic therapies are not so promising to prevent UTIs because persistent and recurrent infections are occurred in the bladder (5). Since antibiotic resistance genes are located on mobile genetic elements, distribution of them expedites in the community. Thus, high degree of resistance to various classes of antibiotics has been observed among UPEC strains and is accompanied with great concern for treatment of UTI (7). As the antibiotic resistance patterns of UPEC are different among various countries, so, there is a need to investigate susceptible antibiotic profiling for treatment of UPEC in this country (8). The aims of this work were to investigate susceptibility of UPEC isolates to four commonly used antibiotics and to determine the prevalence of *E. coli* virulence genes including toxins (*hlyD*, *cnf1*), adhesins (*papA, fimH*), iron transport (*sitA*) and hemagglutinin sensitive temperature (*tsh*) along with the association of different phylogenetic group.

During January 2012 to December 2012, clinically diagnosed patients for UTI were selected from different regions of southern Iran referred to Shiraz hospitals. The criteria included urge to urinate frequently, sharp pain or burning sensation in urethra when urine was released and pyuria. Midstream urine samples were collected in sterile bottles. Patients were selected from both gender and different ages with appropriate informed consent. *E. coli* isolates were identified based on standard biochemical methods previously described (9).

The susceptibilities of all *E. coli* isolates to selected antimicrobials were determined by disk diffusion method (10). Commercial antimicrobial disks used in this study were
trimethoprim-sulfamethoxazole (25 µg), cefotaxime (30 µg), amoxicillin-clavulanic acid (10 µg) and neomycin (30 µg). *E. coli* strain (ATCC 25922) was used as a control strain.

Bacterial DNA extraction was carried out by boiling method. PCR assays for investigating the presence of *hlyD*, *cnf-1*, *tsh*, *papA*, *fimH* and *sitA* genes in *E. coli* isolates were carried out using MJ Mini thermal cycler (Bio-Rad, USA) and specific primers previously used (11). PCR mixture for all reactions contained 2.5 µL 10X PCR buffer, 0.75 µL dNTP (containing 0.2mM of each), 0.2 µL Taq DNA polymerase (5 u/µL), 1 µL of each primer (20pmol), 0.75 µL 50mM MgCl₂ (CinnaGen, Iran) and 3 µL of template DNA. All amplification procedures were carried out separately except for *papA* and *fimH*, which were amplified by using a duplex PCR assay. Amplification reactions for *hlyD*, *cnf-1*, *papA* and *fimH* were performed as it was done previously (11). PCR condition for detection of *tsh* was as follow: 5 min at 95°C, 30 cycles of 30 sec at 94°C, 45 sec at 55°C, 45 sec at 72°C, and a final extension of 10 min at 72°C. PCR products were transferred and electrophoresed on 2 % agarose gels contained ethidium bromide (CinnaGen, Iran) and visualized by UV trans-illuminator.

In order to identify significant relationships between phylogenetic groups of *E. coli* isolates and presence of virulence genes and correlation among *E. coli* virulence genes, Fisher’s exact tests (SPSS version 19.0; SPSS Inc., Chicago) was performed. The *P* values less than 0.05 were considered statistically significant.

A total of 85 UPEC isolates were identified from patients using conventional biochemical identification. According to previously published report, 56 (65.9%) of these isolates belonged to phylogenetic group A, 15 (17.6%) belonged to phylogenetic group B2 and 14 (16.5%) of the isolates were found to belong to group D (9).
According to the present study the prevalence of resistant isolates against amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole, cefotaxime and neomycin was 94.1%, 74.1%, 68.2% and 56.5% respectively. In addition, 36.4% of UPEC isolates exhibited multidrug resistance properties. The incidence of antibiotic resistance within the phylogenetic groups was summarized in Table 1. In contrast to WHO recommendation for UTIs treatment with trimethoprim-sulfamethoxazole, many studies have declared SXT as an inappropriate antibiotic for treatment of UTI (12-15). Abass et al. have indicated that UPEC strains not only displayed high resistance to SXT (72%), but also showed 100% resistance against amoxicillin-clavulanic acid which is in accordance with the present results (13). In India, Murugan et al. have demonstrated a significant resistance (74%) of E. coli isolates against cefotaxime in patients with UTI which is comparable to our findings (16). Ramos et al. have shown higher resistance of UPEC isolates from Iran and Vietnam against AMC comparing to three other countries. In addition, this report showed the high prevalence (44%) of multidrug resistant UPEC isolated from Iran which is close to our observations (14). In one study, it is indicated that the frequency of UPEC strains resistant to Neomycin (37.8%) were less than other antibiotics (17). According to the present study, due to high-level resistance of isolates against AMC, SXT and CTX, it is concluded that these antibiotics are relatively ineffective for UTI treatment in Southern Iran. However, neomycin could be considered as efficient treatment for UTIs.

Presence of mentioned virulence genes in total 85 UPEC isolates was investigated by PCR assays. It is noteworthy to mention that 35 (41.1%) of these strains did not encode any of these virulence genes. The prevalence of the virulence genes among the phylogenetic groups of the isolates has been shown in Fig. 1. Of the UPEC adhesins, FimH at the type 1 fimbria tip has a crucial role in UPEC colonization in bladder which is required for initiation of UTI (4).
According to present observations, $\textit{fimH}$ had the highest frequency (34.1%) among virulence genes; while, $\textit{cnf-1}$ had the lowest one (3.5%). Several studies showing $\textit{fimH}$ is the most prevalent among these virulence factors and it has been detected in 67%, 86% and 87% of UPEC isolates in three separate studies performed in Iran (18-21). Although the present results reconfirmed that $\textit{fimH}$ is the most frequent virulence gene among UPEC; but, the frequency of UPEC encoding $\textit{fimH}$ dramatically differed from the most of the above mentioned reports. These various findings may result from difference in UPEC isolates derived from various populations living in different geographical regions. Moreover, our findings bring up a challenging question about mechanism of pathogenesis in 75.9% of our UPEC isolates which did not encode $\textit{fimH}$. It seems that further investigations are essential in order to clarify the pathogenesis mechanism and detect other potential virulence factors in UPEC which do not encode $\textit{fimH}$. Major subunit of p-fimbriae is encoded by $\textit{papA}$ gene in UPEC. This fimbria is involved in colonization of UPEC in urinary tracts and development of pyelonephritis (6, 22). The results showed the presence of $\textit{papA}$ gene in 9.4% of UPEC isolates. Fascinatingly, all the isolates encoding $\textit{papA}$ carried $\textit{fimH}$ gene and our statistical examinations revealed a significant association between presence of $\textit{papA}$ and $\textit{fimH}$ genes ($p<0.05$) which worth to be investigated in more depth in the future. However, this study is not in accordance with others’ which have been determined $\textit{pap}$ in UPEC isolates in Iran (30% and 30.2%), Tunisia (41%) and China (54%) (12, 19, 21, 22). Also, the statistical analysis revealed the significant relationships between the presence of $\textit{papA}$ with $\textit{tsh}$ and $\textit{sitA}$ genes ($p<0.05$). The gene $\textit{hlyD}$ is participated in transport of hemolysin; therefore, involved in UPEC mediated hemolysis (6). Cytotoxic necrotizing factor-1, a Rho GTPase encoded by $\textit{cnf-1}$, is involved in invasion to the host cells (4). In this report, it was indicated that $\textit{hlyD}$ and $\textit{cnf-1}$ genes are encoded by 21.2% and 3.5% of UPEC isolates respectively and none of
the isolates carried both of these genes. Interestingly, there are several contradictory published works about the presence of these two toxin related genes. Both toxin genes, *hly* and *cnf-I*, were detected by Karimian et al. in 50.4% of UPEC in Tehran, Iran (20). However, Tarchouna et al. reported the prevalence of *hly* and *cnf* in 19% and 3% of isolates respectively which were almost similar to the present findings (22). It is noteworthy that statistical analysis has been determined the significant correlation between the presence of *hlyD* with *papA*, *sitA* and *tsh* (*p*<0.05). Also, it has to be mentioned that due to low prevalence of *cnf-I*, its presence was not statistically correlate with the other virulence genes. *SitA* is one of the iron acquisition related genes in *E. coli* which encodes a component of ABC iron transport system (23). The findings indicated that *sitA* is present in 15.3% of UPEC isolates. To the best of our knowledge, there is no published data about the presence of *sitA* in UPEC in Iran. This gene was detected previously in about 77% of UPEC causing recurrent UTI in China and in 85.5% of UPEC in United States (15, 24). In the study performed by Luo et al. (*sitA*=77%) UPEC phylogenetic group D was the most prevalent followed by groups B2 and A respectively (24). However, our findings indicated that the frequency of phylogenetic group A was more than two others. Then, the different findings about prevalence of *sitA* in UPEC obtained in our study and the one mentioned may be a result of this significant difference in the phylogenetic groups. Temperature-sensitive hemagglutinin, encoded by *tsh*, is an autotransporter protein which is involved in UPEC pathogenesis (25). This gene was found in 27.1% of our studied UPEC strains. The presence of this gene was formerly reported in 39.5% of UPEC isolates (11). However, one previously published researche in Iran declared the absence of *tsh* encoding UPEC in its study population (18). Finding *tsh* in 27.1% of the samples and significant correlation of its presence with *papA* and *hlyD* is one of the hallmarks of our study. Overall, these contradictions among data collected in different studies may come from the
fact that the specimens in these various studies were obtained from different geographical
regions of the world. Even in studies performed in Iran samples were collected from cities which
are hundreds to thousand kilometers faraway from each other and from different populations
vary in their lifestyle. In recent years, multidrug resistance among UPEC strains has been
increased and these strains have become a public health concern in several countries (4).
Therefore, research on developing new therapies for UPEC derived UTI is certainly one priority
for future studies. One of these new therapies has been developed by Totsika et al. who
introduced an inhibitor of FimH as a possible agent especially for treatment of multidrug
resistant UPEC infections (15).

Current investigation possesses some limitations which are suggested to be eliminated in
further studies. One of them is the relatively small number of samples which were investigated.
Moreover, antibiotic resistant pattern of the studied UPEC isolates would be more reliable if
presence of antibiotic resistance genes were studied by molecular approaches such as PCR. This
study has several important outcomes. First, the present study determined that high resistance to
AMC, SXT and CTX is common among investigated UPEC isolated. In addition, low rate of
resistance to neomycin has been shown amongst these isolates. However, due to the relatively
small size of our study population, we are not able to make direct conclusions according to these
findings. Then, it seems more in depth large scale studies are required to investigate antibiotic
resistance pattern among UPEC isolates in southern Iran and confirm or reject current findings.
The other noticeable outcome of current research is detection of fimH among UPEC isolates as
the most frequent virulence gene which reconfirms fimH crucial role in UPEC pathogenesis and
suggests more investigations to be performed on fimH inhibitors as potential backup therapeutics
for UPEC associated UTI. In fact, obtained data about frequency of virulence genes can be
useful in designing new targets for UPEC antibiotic independent therapies. However, the most remarkable findings achieved by detecting high prevalence of isolates which do not encode $fimH$ (75.9%) and relatively low frequency of isolates carrying other virulence genes. These significant findings suggest further investigations to be conducted in order to clarify the role of other potential virulence factors in pathogenesis of these isolates, especially for $tsh$ which was detected with significant higher frequency in this study comparing to other investigations in Iran.

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CONFLICT OF INTEREST

None to declare.

REFERENCES


Fig 1. Distribution of virulence genes detected in three phylogenetic groups of UPEC. 1, 2 and 3 represent phylogenetic groups A, B2 and D respectively.
Table 1. Distribution of antibiotics resistance genes among UPEC strains

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>N. (%) of antibiotic resistance isolates in phylogenetic groups</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Type A</td>
</tr>
<tr>
<td>N</td>
<td>29 (34.1)</td>
</tr>
<tr>
<td>SXT</td>
<td>41 (48.2)</td>
</tr>
<tr>
<td>CTX</td>
<td>39 (45.9)</td>
</tr>
<tr>
<td>AMC</td>
<td>53 (62.4)</td>
</tr>
</tbody>
</table>

N, SXT, CTX and AMC represent neomycin, trimethoprim-Sulfamethoxazole, cefotaxime and amoxicillin-clavulanic acid respectively.