Objectives: Uropathogenic Escherichia coli (UPEC) are the major cause of urinary tract infections (UTIs). Here, we determined whether sensitivity to antibiotics was related to the prevalence of iron scavenging genes, or to biofilm and hemolysis formation.

Methods: A total of 110 UPEC and 30 E.coli isolates were collected from the urine of UTI patients and feces of healthy individuals without UTI, respectively. The presence of iron receptor genes and phenotypic properties were evaluated by polymerase chain reaction and phenotypic methods, respectively. Susceptibility to routine antibiotics was evaluated using the disc diffusion method.

Results: The prevalence of iron scavenging genes ranged from 21.8% (ireA) to 84.5% (chuA) in the UPEC. Resistance to ceftazidime and cefotaxime was significantly correlated with the presence of fyuA and iutA iron genes. Biofilm production was significantly associated with the prevalence of fyuA and hma iron genes. A higher degree of antibiotic resistance was exhibited by isolates that produced biofilms than by their non-biofilm producing counterparts.

Conclusion: Our study clearly indicates that biofilm production is associated with antibiotic resistance, and that iron receptors and hemolysin production also contribute to reduced antibiotic sensitivity. These results further our understanding of the role that these virulence factors play during UPEC pathogenesis, which in turn may be valuable for the development of novel treatment strategies against UTIs.

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Introduction

Uropathogenic Escherichia coli (UPEC) is the most frequent cause of urinary tract infections (UTIs), and causes significant morbidity and mortality globally [1]. The UTIs include a range of disorders such as cystitis and pyelonephritis [2]. The pathogenicity of UPEC is associated with expression of several virulence factors, such as adhesion elements, flagella, toxins, capsule, serum resistance factors and iron uptake systems [3].

UPEC strains utilize different strategies to acquire iron from the host urinary tract. For example, these strains can produce iron receptors, hma and chuA, which facilitate heme uptake and transfer into the periplasm. UPEC also use iron chelators called siderophores, including salmochelin, aerobactin, enterobactin, and yersiniabactin [4,5]. Furthermore, outer membrane iron receptors confer important properties on UPEC such as colonization, biofilm production and formation of Intracellular Bacterial Communication of UPEC that are likely responsible for recurrent UTIs and increased resistance to antibiotics [6].

Other pro-virulence characteristics, such as biofilm production, also facilitate colonization of the urinary tract...
by UPEC. Biofilm formation attenuates both the activity of antimicrobial agents and the host immune response, which contributes to persistence of UPEC in the urinary tract and the consequent severe symptoms and antibiotic resistance [7].

Knowledge of the constellation of iron acquisition genes or factors correlated with biofilm production among UPEC strains is potentially valuable in developing strategies for treatment or prevention of UTIs. To date, several studies have reported the epidemiology, virulence factors and antibiotic resistance profiles associated with UPEC strains isolated from Iranian patients [8,9]. However, little is known regarding the distribution of genes encoding iron acquisition systems and their correlation with antibiotic resistance or biofilm and hemolysin formation among the UPEC strains. The objective of this study was therefore to determine the pattern of iron acquisition genes including fyuA, iroN, iutA, iha, ireA, chuA, and hma in UPEC isolated from Iranian patients and commensal E coli strains. Additionally, we investigated whether iron scavenging gene pattern was related to biofilm formation and hemolysis activity, as well as the susceptibility of the UPEC and commensal isolates to antibiotics.

Materials and Methods

1. Bacterial collection and identification

In this study, a total of 200 samples from patients with UTI symptoms were collected from different hospitals in Tehran, Iran. One hundred and ten E coli were isolated from these samples, which were mainly obtained from cystitis and pyelonephritis patients. All the E coli were isolated from women patients aged between 20 to 60 years, with a mean age of 39.5 years old. Furthermore, 30 commensal E coli were isolated from fecal samples of healthy women without UTI symptoms. The isolates were identified by conventional bacteriological tests. After identification, all isolates were kept frozen at –80°C in 20% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA) until further use. The work was done in accordance with the Ethical Principles for Medical Research Involving Human Subjects outlined in the Helsinki Declaration of 1975 (revised in 2008), and approved from the institutional review board of Pasteur Institute of Iran (No. IR.pII. REC.1394.45).

2. Biofilm production assay

The ability of the UPEC and commensal isolates to produce biofilm was evaluated according to a previous protocol [10]. Briefly, overnight cultures were diluted and cultured in 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany). After incubation at 37°C for 48 hours, biofilms were stained using crystal violet solution (Merck, Darmstadt, Germany). The crystal violet dye was washed, 33% acetic acid (Merck) added and absorbance was measured at optical density 590 nm with an enzyme-linked immunosorbent assay reader. For analysis of the results, isolates were classified into four categories according to their adherence capabilities, namely, non-adherent (–), weakly (+), moderately (++) and strongly (+++) adherent. E coli ATCC 25922 was used as a positive control.

3. Hemolysis production assay

Production of hemolysin by the isolates was evaluated using a culture method. The isolates were inoculated on 5% blood agar plates (Merck), incubated overnight at 37°C and hemolysis was detected by the presence of a zone of complete lysis of the erythrocytes (red blood cells) around the colonies.

4. Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (Merck) using commercial antibiotic discs (MAST Group Ltd., Merseyside, United Kingdom) following the standard disc diffusion method; results were interpreted as described in the Clinical & Laboratory Standards Institute (CLSI) recommendations [11]. The antibiotics used were ceftazidime (30 µg), cefotaxime (30 µg), trimethoprim–sulfamethoxazole (SXT) (25 µg), norfloxacin (10 µg), amikacin (30 µg), imipenem (10 µg), and nitrofurantoin (300 µg). E coli ATCC 25922 was used as control.

5. Extraction of total DNA

For DNA extraction, the isolates were grown overnight in Luria-Bertani medium (Merck). The isolates were pelleted and genomic DNA was extracted using a phenol chloroform method according to previously published protocols [12]. The quality and quantity of the extracted genomic DNA was evaluated using a spectrophotometer and electrophoresis on an agarose gel (Sigma- Aldrich).

6. Detection of virulence iron genes by polymerase chain reaction (PCR)

All isolates were tested for the presence of fyuA, iroN, iutA, iha, ireA, chuA, and hma genes that encode either siderophores or heme iron receptors. Table 1 shows the primers (Genfanavaran, Tehran, Iran) used for the detection of these genes. The amplification conditions were as follows: denaturation at 94°C for 3 minutes; 30 cycles of denaturation at 94°C for 1 minutes, annealing at specific temperature (Table 1) for 1 minutes, and extension at 72°C for 3 minutes; and a final extension at 72°C for 5 minutes. Finally, the PCR products were stained with ethidium bromide and analyzed
by running them on 1–2% agarose gels.

7. Statistical analysis

Comparison of gene frequencies in different groups was measured by using a two-tailed Fisher's exact test. Correlations between the presences of genes were measured by using Fisher's exact test (two-tailed). Chi-square and Fisher's exact tests were performed for the analysis of associations using Statistix 7.0 for Windows (Analytical Software, Tallahassee, FL, USA). Associations among genes were considered significant when p-values were < 0.05, in which case odds ratios and their 95% confidence intervals were calculated using the same software.

Results

1. Bacterial isolates and their epidemiological data

In total, 110 E coli isolates from patients with symptomatic UTIs (76.4% for cystitis and 23.6% for pyelonephritis) and 30 E coli isolates from stool samples of healthy women were enrolled in this study. The incidence of UTI in female patients in the age groups of 20–35, 36–50, and 51–65 years old was 44.5%, 24.5%, and 31%, respectively. Among the UPEC obtained, most of them (52.7%) were from inpatients, and were isolated from the following wards: urology 13.6%, women 11.8%, intensive care units (ICUs) 9.1%, infectious 7.3%, emergency 7.3%, and others 3.6%.

2. The prevalence of biofilm and hemolysis among the isolates

Eighty-five percent of the UPEC isolates had biofilm formation capacity. Among these isolates, 26%, 44%, and 30% of them were strong, moderate and weak biofilm producers. Half of the 30 commensal isolates could also be classed as weak biofilm producers.

Hemolysin activity was observed among 44.5% and 6.7% of the UPEC and commensal isolates, respectively; this difference was statistically significant (p < 0.001). Although hemolysin production was higher in pyelonephritis UPEC isolates than in cystitis isolates, the difference was not statistically significant (p > 0.05). In addition, UPEC isolates with biofilm-forming capacity showed significantly greater hemolysin activity (p < 0.05), while there was no significant difference between the prevalence of biofilm and hemolysin among the commensal isolates (p > 0.05).

3. Pattern of antimicrobial resistance among the isolates

Resistance patterns of UPEC and commensal isolates are presented in Table 2. The UPEC and commensal isolates showed maximum resistance to ceftazidime (59.1%), and trimethoprim-sulfamethoxazole (33.3%), respectively. Although antibiotic resistance among the UPEC isolates occurred at a higher rate than in the commensal isolates, only the resistance to ceftazidime, cefotaxime and norfloxacin was statistically significant (p < 0.001). Multi-drug resistance, which is defined as resistance to 3 or more classes or sub-classes of antibiotics [13], was most commonly observed in UPEC isolates (32.7%).
compared with commensal isolates (6.6%).

Although the overall antibiotic resistance rate among the UPEC isolated from inpatients was higher than that observed in outpatients \((p = 0.033)\), only resistance to ceftazidime and SXT was significantly more prevalent in the former group \((p = 0.001\) and \(0.023\), respectively). There was also a significant correlation between higher levels of resistance to amikacin \((p = 0.042)\) in patients with pyelonephritis compared to those with cystitis.

### 4. The correlation between antibiotic resistance, biofilm and hemolysis formation

The relationship between antimicrobial resistance, biofilm and hemolysis production in UPEC is shown in Table 3. Among biofilm producer isolates, maximum resistance was observed to ceftazidime \((63.4\%)\) followed by cefotaxime \((49.5\%),\) norfloxacin \((45.2\%)\) and SXT \((39.8\%)\). There was a significant correlation between the intensity of biofilm formation and resistance to ceftazidime and norfloxacin \((p < 0.05)\). Moreover, isolates that produced hemolysin were more sensitive to cefotaxime than were non-hemolysin producers. No relationship was found between resistance to antibiotics with biofilm formation and hemolysis activity in the commensal isolates \((p > 0.05)\).

### 5. Prevalence of iron uptake genes among the tested isolates

The frequencies of the iron receptor genes are reported in Table 4, and ranged from 21.8\% \(\text{ireA}\) to 84.5\% \(\text{chuA}\) in the UPEC isolates. All the genes exhibited higher frequencies in the UPEC than in the commensal isolates, but no significant difference was observed between the prevalence of iron genes \(\text{ireA}\) and \(\text{iha}\) in the UPEC and in commensal isolates.

As mentioned in Table 5, \(\text{fyuA}, \text{iutA}\) and \(\text{chuA}\) genes presented more frequently in cystitis and pyelonephritis isolates than in commensal isolates, while there was no significant difference in the prevalence of these genes among cystitis and pyelonephritis isolates \((p > 0.05)\). The \(\text{iroN}\) and \(\text{iha}\) genes were significantly more frequent among the cystitis isolates than pyelonephritis and fecal isolates \((p < 0.05)\). Furthermore, \(\text{hma}\) was more frequently associated with pyelonephritis than

### Table 3. Relationship between antimicrobial resistance and virulence factor genes in UPEC.

| Virulence marker | Antibiotics (%R) |           |           |           |           |           |
|------------------|------------------|-----------|-----------|-----------|-----------|------------|
|                  | CAZ              | CTX       | SXT       | NOR       | AMK       | NIT        |
| Biofilm formation|                  |           |           |           |           |            |
| Strong           | 87.5             | 58.3      | 41.7      | 58.3      | 8.3       | 4.2        |
| Moderate         | 58.5             | 51.2      | 36.6      | 46.3      | 26.8      | 7.3        |
| Weak             | 50               | 39.3      | 42.9      | 32.1      | 14.3      | 3.6        |
| Negative         | 41.2             | 47.1      | 82.4      | 17.6      | 23.5      | 11.8       |
| p                | 0.01             | NS        | 0.01      | 0.042     | NS        | NS         |
| Hemolysin activity|                 |           |           |           |           |            |
| Positive         | 63.3             | 36.7      | 38.8      | 40.8      | 20.4      | 10.2       |
| Negative         | 57.4             | 59        | 52.2      | 41        | 18        | 6.6        |
| p                | NS               | 0.023     | NS        | NS        | NS        | NS         |

UPEC = uropathogenic Escherichia coli; CAZ = ceftazidime; CTX = cefotaxime; SXT = trimethoprim -sulfamethoxazole; NOR = norfloxacin; AMK = amikacin; NIT = nitrofurantoin; NS = not significant.

### Table 4. Distribution of iron acquisition genes among UPEC and commensal isolates.

| Virulence gene | Prevalence of gene | p       |
|----------------|--------------------|---------|
|                | UPEC \((n = 110)\) | Commensal \((n = 30)\) |
| \(\text{fyuA}\) | 88 (80.0)          | 12 (40.0) | < 0.001 |
| \(\text{iroN}\) | 60 (54.5)          | 8 (26.7)  | 0.006   |
| \(\text{iutA}\) | 73 (66.4)          | 8 (26.7)  | < 0.001 |
| \(\text{iha}\)  | 52 (47.3)          | 9 (30.0)  | NS      |
| \(\text{ireA}\) | 24 (21.8)          | 2 (6.7)   | NS      |
| \(\text{chuA}\) | 93 (84.5)          | 10 (33.3) | < 0.001 |
| \(\text{hma}\)  | 65 (59.1)          | 5 (16.7)  | < 0.001 |

Data are presented as \(n (\%)\) of isolates. UPEC = uropathogenic Escherichia coli; NS = not significant. \(p\)-values (by Fisher’s exact test) are shown where \(p < 0.05\).
cystitis and fecal isolates \((p < 0.05)\). \(i\text{reA}\) was the only iron receptor gene that was not found more frequently in cystitis or pyelonephritis cases compared with fecal isolates \((p > 0.05)\) (Table 5).

### 6. Association between virulence iron genes in UPEC isolates

We performed pairwise comparisons of virulence iron genes against each other, and identified a wide variety of distinctive associations. We observed that \(i\text{roN}\) was associated positively with the presence of \(i\text{ha}\) and \(i\text{reA}\), and negatively with the presence of \(f\text{yuA}\), \(i\text{utA}\), \(c\text{huA}\) and \(h\text{ma}\) iron genes. We also found a negative association between the presence of the \(i\text{utA}\) gene and the \(f\text{yuA}\), \(i\text{roN}\) and \(i\text{ha}\) iron scavenging genes.

### 7. Relationship between the presence of iron genes and biofilm formation

The statistical analysis of biofilm formation and the presence of iron virulence genes are shown in Table 6. According to these results, biofilm production was significantly associated with the prevalence of \(f\text{yuA}\) and \(h\text{ma}\) virulence genes \((p = 0.006\) and 0.014, respectively), but these findings were not observed for other iron genes. Our results also showed that \(f\text{yuA}\) and \(i\text{reA}\) had the highest and lowest prevalence among the strong biofilm producer isolates, respectively. In our study, there was no statistically significant correlation between the presence of iron genes and biofilm formation in commensal isolates \((p > 0.05)\).

### 8. Association between antimicrobial resistance and virulence iron genes

Our data showed a significant correlation between resistance

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**Table 5. Comparison of the prevalence of iron genes among different groups of \(E\text{scherichia coli}\) strains.**

| VF    | Prevalence of VFs | p          |
|-------|-------------------|------------|
|       | Total \((n = 140)\) | Fecal \((n = 30)\) | Cystitis \((n = 84)\) | Pyelonephritis \((n = 26)\) | Fecal vs. cystitis isolates | Fecal vs. pyelonephritis isolates | Cystitis vs. pyelonephritis isolates |
|-------|-------------------|------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| \(f\text{yuA}\) | 100 (71.4) | 12 (40.0) | 65 (77.4) | 23 (88.5) | < 0.001 | < 0.001 | NS |
| \(i\text{roN}\) | 68 (48.6) | 8 (26.7) | 51 (60.7) | 9 (34.6) | 0.002 | NS | 0.025 |
| \(i\text{utA}\) | 73 (52.1) | 8 (26.7) | 56 (66.7) | 17 (65.4) | < 0.001 | 0.007 | NS |
| \(i\text{ha}\) | 61 (43.6) | 9 (30.0) | 46 (54.8) | 6 (23.1) | 0.032 | NS | 0.006 |
| \(i\text{reA}\) | 26 (18.6) | 2 (6.7) | 19 (22.6) | 5 (19.2) | NS | NS | NS |
| \(c\text{huA}\) | 103 (73.6) | 10 (33.3) | 72 (85.7) | 21 (80.8) | < 0.001 | < 0.001 | NS |
| \(h\text{ma}\) | 70 (50.0) | 5 (16.7) | 45 (53.6) | 20 (76.9) | 0.001 | < 0.001 | 0.041 |

Data are presented as \(n (\%)\) of isolates. VF = virulence factor; NS = not significant. \(p\) values (by Fisher’s exact test) are shown where \(p < 0.05\).

**Table 6. Relationship between the presence of iron genes and biofilm formation among UPEC.**

| Virulence gene | UPEC isolates \((n = 110)\) | p          |
|----------------|-------------------------------|------------|
|                | Biofilm producers \((n = 93)\) | Non-biofilm producers \((n = 17)\) |
| \(f\text{yuA}\) | 0.006 | 89.8 | 10.2 |
| Positive | 63.6 | 36.4 |
| Negative | NS |
| \(c\text{huA}\) | NS | 87.1 | 12.9 |
| Positive | 70.6 | 29.4 |
| Negative | NS |
| \(h\text{ma}\) | 0.014 | 92.3 | 7.7 |
| Positive | 73.3 | 26.7 |
| Negative | NS |
| \(i\text{roN}\) | NS | 88.3 | 11.7 |
| Positive | 80 | 20 |
| \(i\text{utA}\) | NS | 84.9 | 15.1 |
| Positive | 83.8 | 16.2 |
| Negative | NS |
| \(i\text{reA}\) | NS | 95.8 | 4.2 |
| Positive | 81.4 | 18.6 |
| Negative | NS |
| \(i\text{ha}\) | NS | 84.6 | 15.4 |
| Positive | 84.5 | 15.5 |

Data are presented as \(n (\%)\) of isolates. UPEC = uropathogenic \(E\text{scherichia coli}\); NS = not significant. \(p\)-values (by Fisher’s exact test) are shown where \(p < 0.05\).
to ceftazidime and cefotaxime with the presence of \textit{fyuA} and \textit{iutA} iron genes in the UPEC isolates ($p < 0.05$), while UPEC isolates harboring the \textit{chuA} gene were more susceptible to SXT antibiotic ($p = 0.036$).

**Discussion**

Distinct UPEC strains use different virulence factors for pathogenicity; combined with variations in antibiotic resistance and biofilm production, this complicates the treatment of UPEC-driven UTIs [14]. Thus, development of novel therapeutic or prophylactic strategies against UPEC requires a more comprehensive understanding of virulence properties, antibiotic resistance, and their relationship with each other.

In the present study, biofilm formation was observed in the majority of UPEC isolates; this is consistent with the results of Sharma et al [15] and Fattahi et al [16]. In other studies, Soto et al [17] and Neupane et al [18] reported a lower prevalence of biofilm in the UPEC isolates. In agreement with our findings, Meshram et al [19] and Soto et al [17] demonstrated that biofilm formation was higher in the UPEC than in commensal strains. Among the UPEC, biofilm production was more frequently associated with isolates that cause cystitis when compared with isolates that cause pyelonephritis ($p = 0.002$); this suggests that biofilm formation also has a role in the adhesion and establishment of UPEC in the urinary tract of cystitis patients [20].

Our observation of higher hemolytic activity in UPEC than in fecal isolates is consistent with previous studies [21,22]. In our study, hemolysin production was more frequent in pyelonephritis than cystitis cases, indicating that hemolysin may be a virulence factor important for the pathogenesis of UPEC in pyelonephritis patients. The greater propensity for biofilm formation among hemolysin producing isolates is similar to the results of Soto et al [17] and in contrary to those in the study of Marhova et al [23].

Although the spectrum and frequency of antibiotic resistance among UPEC isolates is variable [24–26], most studies report that amikacin, nitrofurantoin and imipenem are highly efficacious against such strains [27–29]. Our study revealed co-resistance to third generation cephalosporins, quinolones, and aminoglycoside among UPEC isolates; this is similar to that of the study by Karlowsky et al [30], which also reported a high degree of co-resistance to ampicillin and SXT in UPEC isolates.

Similar to other investigations, different antibiotic resistance patterns were observed in UPEC compared with commensal isolates [31,32]. We found that the resistance rate of commensal isolates to third-generation cephalosporins and amikacin was higher than was observed in reports from other countries [31–33]. The observed resistance in fecal \textit{E. coli} may be because of the extensive and long-term use of antibiotics [21,31,33]. Furthermore, the considerable number of MDR isolates among the UPEC found in Iran and other countries [28,34,35] may be the result of widespread use and misuse of antibiotics in hospitals and in the community [36].

In contrary to our findings and Saperston et al [37], others have reported higher antibiotic resistance in outpatients than in inpatients [25,38]. The antibiotic resistance in inpatients could be attributed to the higher rate of antibiotic therapy, which in turn creates selective pressure to evade antibiotic activity.

Similar to our results, previous studies found that biofilm-producing UPEC isolates had a higher degree of antibiotic resistance than non-biofilm producing isolates [39,40]. Insufficient antibiotic concentration and/or their delayed penetration into the deeper layers of biofilms are the major reasons for antibiotic resistance in biofilm structures [17,18]. Despite this, amikacin and nitrofurantoin showed high efficacy against biofilm producers, and could be considered as the antibiotics of choice for treatment of biofilm structures [40,41].

The prevalence of the majority of the iron receptor genes in UPEC is reportedly higher than that in commensal isolates [42,43]. Furthermore, and similar to our results, Alteri et al [42] and Bauer et al [44] found that novel non-hemagglutinin adhesion \textit{ihu} and iron-regulated element \textit{ireA} (a siderophore receptor) were not more frequent in UPEC than in commensal isolates. In our study, \textit{yersiniabactin} uptake receptor \textit{fyuA} and heme receptor \textit{chuA} were more frequently found in UPEC isolated from both cystitis and pyelonephritis patients. Thus, in accordance with another study [45], we suggest there is functional redundancy of \textit{fyuA} and \textit{chuA} in the UPEC isolates. We observed that the heme receptor, \textit{hma}, was present more frequently in UPEC isolates from patients with pyelonephritis rather than those with cystitis; in this regard Alteri and Hagan also indicated that UPEC unable to produce \textit{hma} have reduced fitness within the kidney during UTI [42,46]. Because the \textit{ihu} receptor confer adhesion properties, its higher prevalence in cystitis compared with pyelonephritis isolates may indicate a key role for adhesion in pathogenicity of UPEC isolates in patients with cystitis.

In accordance with our study, iron genes such as \textit{fyuA} and \textit{hma} (and neither aerobactin, \textit{iutA} or \textit{aer}, nor enterobactin genes) are reportedly upregulated in biofilm-producing UPEC [7,47]; this suggests that the iron receptors are particularly important for biofilm growth. Similar to others [48,49], we found that the presence of iron scavenger receptors is associated with antibiotic resistance. It is possible that simultaneous presence of virulence factors and resistance genes on resistance transferable elements such as integrons and conjugative plasmids increases the possibility of spreading
both virulence traits and antibiotic resistance via horizontal gene transfer [49]. Conversely, we found that susceptibility to antibiotics such as SXT was significantly associated with the presence of the chuA iron gene. We infer that UPEC isolates are subject to the “biological fitness cost” phenomenon, in which bacteria shut down expression of some non-essential genes in order to preserve energy [50].

In conclusion, we identified associations between different virulence factors (including the iron uptake receptors, biofilm and hemolysin production) and antibiotic resistance among the UPEC isolated from Iranian patients. These findings further our understanding of the role that these virulence factors play in the pathogenicity of UPEC isolates, which in turn could help in the development of treatment or preventive strategies against UTIs.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This work was financially supported by the Pasteur Institute of Iran.

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