

Research Article

Molecular Investigation of the Type VI Secretion System via the *vgrG* Gene among Multidrug-Resistant Ur pathogenic *Escherichia coli*

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Abstract

Bacteria possessing T6SS are highly pathogenic, as the system delivers effector proteins and toxins into host cells. This study aimed to determine whether UPEC strains possess this system by detecting the *vgrG* gene. This study involved the collection of 378 urine samples between November 2025 and February 2026. The samples were collected in the college laboratory. Antibiotic susceptibility testing was performed, and the *vgrG* gene was detected. *E. coli* isolates were identified using the Enterobacteriaceae T-Kit, yielding 82 isolates. Antibiotic susceptibility test results are Ampicillin (95.1%), Cefuroxime (85.37%), Cefotaxime (82.93%), Nalidixic acid (78.05%), Trimethoprim-sulfamethoxazole (76.83%), Trimethoprim (75.61%), Amoxicillin-clavulanate (74.4%), Ampicillin-sulbactam (71.95%), Cefoxitin (71.95%), Levofloxacin (68.29%), Cefazolin (67.07%), Ceftazidime (67.07%), Doxycycline (65.85%), Ciprofloxacin (63.41%), Cefepime (59.76%), Nitrofurantoin (29.27%), Aztreonam (52.44%), Gentamicin (45.12%), Minocycline (43.90%), Mecillinam (37.8%), Tobramycin (36.59%), Kanamycin (32.93%), Piperacillin-tazobactam (30.49%), Fosfomycin (25.61%), Amikacin (28.05%), Polymyxin B (23.2%), Ertapenem (20.73%), Imipenem (19.51%), and Meropenem (17.07%). The results also showed that, among the 44 isolates selected for detection of the *vgrG* gene, 37 isolates (84.1%) carried this gene. This study concludes that the UPEC bacteria in the sample under investigation pose a growing clinical threat characterized by a dual complexity: the widespread prevalence of the T6SS coincides with an alarming rise in resistance rates to conventional antibiotics.



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1. INTRODUCTION

Urinary tract infections (UTIs) are some of the most prevalent bacterial infections globally [1]. Particularly prevalent among older adults and women. Ur pathogenic *E. coli* is the most common bacterium responsible for most acquired urinary tract infections. Infection with this pathogen can manifest as fever, frequent urination, and discomfort and may lead to serious clinical complications, such as urosepsis and kidney failure. UPEC is characterized by remarkable adaptability, posing significant treatment challenges due to a complex array of factors that enable it to evade the host immune defenses and persist within the patient urinary tract [2]. The majority of patients with UTIs experience a recurrence within six months. A significant proportion of these cases is attributed to UPEC strains that are identical to the original strain, the bacteria employ various strategies to adhere, proliferate, resist antibiotic treatment, and persist within the bladder environment [3].

Humans have coexisted with microbes since ancient times. While some bacteria form symbiotic relationships with humans, pathogenic bacteria invade human tissues, causing diseases and bodily damage that can sometimes lead to death. The human quest for treatments against these harmful bacteria led to the discovery and use of antibiotics in the 1940s. Although antibiotics combat disease-causing bacteria, they are also known to adversely affect beneficial microbes; consequently, their use depends on the anticipated benefit, making a precise characterization and thorough understanding of their mechanisms of action indispensable [4]. There are several classifications for antibiotics. Most classification systems include two main categories: one based on the mechanism of action and the other on chemical structure. Based on their mechanism of action, antibiotics are classified into bactericidal and bacteriostatic agents. Bactericidal agents kill bacteria, whereas bacteriostatic agents inhibit growth, making it easier for the immune system to kill the bacteria and eliminate them [5]. Bacterial pathogens employ various methods to invade the host, damage tissues, and suppress the immune response. Bacterial secretion systems play crucial and important roles in enhancing bacterial virulence [6]. It plays a key role in host-pathogen interactions and the survival of the pathogen in various environments [7].



Secretion systems are a key factor in the success of bacterial pathogens. Through the proteins they secrete, these systems enable bacteria to adhere to cells, penetrate them, and persist [8]. Bacteria can transport proteins via these systems either directly from the cytoplasm to the extracellular space in a single step or through a two-stage process, depending on the type of system [9]. Bacteria possess diverse strategies for survival and persistence, particularly those inhabiting challenging, competitive environments. The T6SS is one of the most prominent mechanisms used for this purpose. Bacteria use this system to deliver effector molecules that eliminate competitors, thereby ensuring their survival in harsh environments. The system functions by penetrating host cells [10]. Bacteria possessing this system are highly pathogenic, as the system confers distinct advantages: it delivers toxins to neighboring pathogens to enhance competitive survival, and translocates protein effectors into host cells, disrupting lipid membranes, cell walls, and cellular structures while also participating in various cellular functions, such as promoting colonization, enhancing survival, facilitating adhesion, and evading the immune system [11]. Multiple proteins are involved in the formation of T6SS in *E. coli*, encoded by specific genes. Among these is the *vgrG* gene, which encodes the valine-glycine repeat protein G (VgrG), a needle-like structural protein located at the tip of the system that punctures the target cell, mimicking the contractile tail of bacteriophages [12]. Given the pivotal role of T6SS in enhancing the virulence and pathogenicity of UPEC strains, this study aimed to molecularly investigate the presence of this system by detecting the *vgrG* gene, a key genetic marker for the system in UPEC, while also determining the strains' antimicrobial susceptibility profiles.

2. METHOD

2.1. Sample Collection

This study involved the collection of 378 mid-stream urine samples from male and female patients suffering from urinary tract infections, comprising 182 samples from males and 196 from females, between November 2025 and February 2026. Sample selection was based on the clinical judgment of physicians at the participating healthcare facilities. A random sampling strategy was adopted to ensure fair representation of the samples taken from patients. Samples were collected from hospitals in Diyala Governorate, Iraq (Baqubah Teaching Hospital, Al-Batool Teaching Hospital, and Al-Khalis General Hospital) as well as from several private medical laboratories. The samples were stored in a freezer at -20°C until use.

Ethical approval (No. CEPEC/07, dated January 3, 2025) required to conduct the study and collect samples was obtained from the relevant authorities.

2.2. Phenotyping Identification

The samples obtained from the patients were cultured in the college laboratory on blood agar, Eosin Methylene Blue agar, and MacConkey agar (manufactured by HiMedia, India). Samples were considered positive for urinary tract infection (UTI) if they exhibited bacterial growth at a concentration of at least 10^5 colony-forming units per milliliter (CFU/mL). Confirmatory diagnosis of *E. coli* was performed using biochemical tests with the Render-Enterobacteriaceae T-Kit (manufactured by Shenzhen Render Bio-tech Co., Ltd.).

2.3. Antimicrobial Susceptibility Test

Thirty antibiotics were used in this study, and the antibiotic susceptibility of the *E. coli* isolates was tested using the methods described below:

2.3.1 Antibiotic susceptibility testing using disks

Antibiotic sensitivity testing was performed using the following antibiotic discs: Mecillinam, kanamycin, doxycycline, nalidixic acid, norfloxacin, fosfomycin, and trimethoprim. Using the Kirby-Bauer disc diffusion test [13].

2.3.2 Antibiotic susceptibility testing using Render-Kit

Antibiotic susceptibility testing was performed using a test kit (Enterobacteriaceae T-Kit, Shenzhen Render Bio-tech Co., Ltd., China) following the manufacturer's instructions. The kit tested for the following antibiotics: ampicillin, ampicillin/sulbactam, piperacillin/tazobactam, amoxicillin /clavulanic acid, cefazolin, cefuroxime, cefotaxime, ceftazidime, ertapenem, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, minocycline, trimethoprim-sulfamethoxazole, and nitrofurantoin.

2.3.3 Bacterial susceptibility test for polymyxin B

Bacterial susceptibility to polymyxin B was tested by determining the minimum inhibitory concentration [14].

2.4 DNA Extraction

To extract DNA, a procedure performed in the college laboratory, *E. coli* isolates were first cultured in nutrient broth and incubated for 24 hours at 37°C. DNA extraction was performed using the PureLink™ DNA Mini Kit (manufactured by Thermo Fisher Scientific, USA) following the manufacturer's protocol. The DNA was eluted in 100 µL of buffer and subsequently stored at -20°C.

2.5 Amplification of the *vgrG* Gene

From the 82 *E. coli* isolates, a representative sample of 44 isolates was selected for *vgrG* gene detection. The selection was based on the antibiotic resistance profiles of the isolates. UPEC isolates exhibiting varying resistance patterns were chosen to ensure diversity among them. The study aimed to detect T6SS in clinically significant strains from the region where the samples were collected. The *vgrG* gene was detected in the bacterial isolates under study using PCR with a primer specifically designed for this study. The PCR cycling program consisted of the following stages: an initial denaturation step (94°C) for 3 minutes, 34 cycles comprising denaturation (94°C) for 45 seconds, annealing (57°C) for 45 seconds, and extension at 72°C for 60 seconds, and a final extension step (72°C) for 3 minutes. The size of the product upon amplification of the *vgrG* gene is 508 bp. Primers specific to the *vgrG* gene were designed using the NCBI Primer-BLAST tool. The reference gene sequence was retrieved from the NCBI GenBank database. We verified the efficiency and specificity of the primers by performing BLASTn searches against the nucleotide database to ensure no off-target binding with other genes or organisms occurred. Additionally, the primers were checked for the formation of secondary structures such as self-dimers and hairpins to ensure high amplification efficiency during the PCR. The sequence of the primer used for amplification is shown in (Table 1).

Table 1: The primer sequence used to detect the *vgrG* gene in this study.

Gene	Sequence	Product size (bp)	References
<i>vgrG</i>	F: ATACCCGCCTTGCGTACAAA	508	Present Study
	R: CTACAAGCGCAACGTGCTAC		

2.6 Statistical Analysis

The R software (version 4.4.0, R Foundation for Statistical Computing) was used to conduct all statistical analyses for the study. Categorical variables were summarized using descriptive statistics, while means with standard deviations and medians with interquartile ranges were used for other variables, depending on the data distribution. The chi-square test of independence was used to assess the statistical significance of the relationships between the variables and the categories of etiologies. Furthermore, Cramér's V coefficient was calculated to evaluate the strength of these associations, and a statistical significance level of $p < 0.05$ was adopted.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of *E. coli* isolates

The current study involved the collection of 378 urine samples from male and female patients with urinary tract infections. The samples were distributed as 182 from males and 196 from females. A total of 241 samples (63.76%) showed positive growth on the culture media (Blood Agar, MacConkey Agar, and Eosin Methylene Blue [EMB] Agar). Of these, 141 were from females (58.42%) and 100 were from males (41.49%). Conversely, 137 samples (36.24%) showed no growth (negative results) on the culture media. Additionally, 55 samples showed positive growth exclusively on Blood Agar, representing 22.80% of the total number of samples that exhibited positive growth on the aforementioned media. The number of samples showing positive growth on the aforementioned media reached 186 (77.20%), comprising 159 lactose-fermenting isolates (85.48%) and 27 non-lactose-fermenting isolates (14.52%) (Table 2).

Table 2: Baseline demographic, clinical, and microbiological characteristics of the study samples (n = 378).

Variable	Category	n (%)	p-value
Sex	Male	182 (48.7)	0.007
	Female	196 (51.3)	
Age group	<5 years	60 (15.9)	<0.001
	5–18 years	129 (34.1)	
	19–40 years	116 (30.7)	
	>40 years	73 (19.3)	
Residence	Baqubah	229 (60.6)	—
	Khalis	90 (23.8)	
	Jadidat Alshat	46 (12.2)	
	Buhriz	9 (2.4)	
	Almuqdadaya	3 (0.8)	
	Balad Ruz	1 (0.3)	
Collection site	Al-Batool Hospital	122 (32.3)	—

	Baqubah Hospital-Consulting	98 (25.9)	
	Khalis Hospital	86 (22.8)	
	Alqanat Laboratory	25 (6.6)	
	Jadidat Alshat Laboratory	20 (5.3)	
	Buhriz Laboratory	19 (5.0)	
	Umm Alqura Laboratory	5 (1.3)	
	Almuqdadiya Laboratory	3 (0.8)	
Underlying disease status	None	341 (90.2)	<0.001
	Diabetes	4 (1.1)	
	Hypertension	5 (1.3)	
	Renal disease	9 (2.4)	
	Cardiovascular disease	2 (0.5)	
	Other comorbidity	2 (0.5)	
	Multiple comorbidities	15 (4.0)	
Recurring infection	No	257 (68.0)	<0.001
	Yes	121 (32.0)	
Culture result	No growth	137 (36.2)	
	Lactose fermenter	71 (18.8)	
	Gram-positive	53 (14.0)	
	<i>E. coli</i> pure	49 (13.0)	
	Non-lactose fermenter	26 (6.9)	
	<i>E. coli</i> + other(Mixed growth)	33 (8.7)	

Samples in the current study demonstrated a statistically significant association between gender and pathogen category ($\chi^2 = 17.88$, $p = 0.0065$). Age group also showed greater statistical significance regarding pathogen classification ($\chi^2 = 157.46$, $p < 0.001$), with isolate types varying according to patients' age groups. Pathogen category was strongly associated with recurrent infections ($\chi^2 = 113.60$, $p < 0.001$), and the presence of comorbidities in patients also showed strong statistical significance ($\chi^2 = 77.05$, $p < 0.001$). Overall, these results indicate that clinical and demographic variables significantly influence the microbiological data of the samples, as shown in (Table 3).

Table 3: Association between baseline characteristics and pathogen/culture-result category using chi-square test. (n = 378).

Variable	Test	χ^2	Df	p-value	Cramér's V
Sex vs. pathogen category	Chi-square	17.88	6	0.0065	0.217
Age group vs. pathogen category	Chi-square	157.46	18	<0.001	0.373
Recurring infection vs. pathogen category	Chi-square	113.60	6	<0.001	0.548
Any comorbidity vs. pathogen category	Chi-square	77.05	6	<0.001	0.451

Initially, *E. coli* isolates were characterized based on their morphological properties following culture on the aforementioned media. The results of the current study showed that 82 isolates were lactose-fermented, exhibiting bright pink colonies with defined edges. Furthermore, on EMB agar, the isolates displayed colonies with a distinctive metallic green sheen, a highly characteristic trait of *E. coli*. Subsequently, biochemical identification was performed using the Render-Enterobacteriaceae T-Kit, and the results of the current study confirmed that all 82 bacterial isolates obtained were identified as *E. coli*. The results of the biochemical tests for the isolates are presented in (Table 4).

Table 4: Results of biochemical tests for the identification of *E. coli*.

Test Name	Symbol	Result
Lysine Decarboxylase	LDC	Positive
Citrate Simmons	C	Negative
Glucose Fermentation	GLU	Positive
Esculin Hydrolysis	ESC	Negative
Tryptophan Deaminase	TDA	Negative
Voges-Proskauer	VP	Negative

Fermentation-Sorbitol	SOR	Positive
Melibiose-Fermentation	MEL	Positive
Fermentation-Rhamnose	RHA	Positive
Arabinose-Fermentation	ARA	Positive
Cellobiose-Fermentation	CEL	Positive
Beta-galactosidase	ONPG	Positive
Arginine Dihydrolase	ADH	Negative
Ornithine Decarboxylase	ODC	Positive
Urease	URE	Negative
Hydrogen Sulfide	H ₂ S	Negative
Gelatin Liquefaction	GEL	Negative
Indole	IND	Positive
Mannitol-Fermentation	MAN	Positive
Inositol-Fermentation	INO	Negative
Fermentation-Saccharose	SAC	Positive
Fermentation-Amygdalin	AMY	Positive
Lactose-Fermentation	LAC	Positive
Citrate	CIT	Negative

Antibiotic sensitivity test of *E. coli* isolates

Susceptibility testing was performed on 82 UPEC isolates against 30 antibiotics. The bacterial isolates demonstrated resistance to each of the antibiotics, with the corresponding resistance percentages shown in (Table 5).

Table 5: Results of testing for resistance to the antibiotics used in the study.

Antibiotic Name	Number of Resistant Isolates	Percentage %
Ampicillin	78	95.1
Mecillinam	31	37.8
Amoxicillin–clavulanate	61	74.4
Ampicillin–sulbactam	59	71.95
Piperacillin–tazobactam	25	30.49
Cefazolin	55	67.07
Cefuroxime	70	85.37
Cefoxitin	59	71.95
Cefotaxime	68	82.93
Ceftazidime	55	67.07
Cefepime	49	59.76
Imipenem	16	19.51
Meropenem	14	17.07
Ertapenem	17	20.73
Aztreonam	43	52.44
Amikacin	23	28.05

Gentamicin	37	45.12
Tobramycin	30	36.59
Kanamycin	27	32.93
Doxycycline	54	65.85
Minocycline	36	43.90
Nalidixic acid	64	78.05
Ciprofloxacin	52	63.41
Levofloxacin	56	68.29
Trimethoprim	62	75.61
Trimethoprim–sulfamethoxazole	63	76.83
Fosfomycin	21	25.61
Nitrofurantoin	24	29.27
Norfloxacin	46	56.10
Polymyxin B	19	23.2

The results of antibiotic susceptibility testing in the current study revealed high resistance among UPEC bacteria to numerous antibiotics. These findings are particularly concerning regarding commonly used antibiotics. The highest resistance was observed against ampicillin (95.1%) , cefuroxime (85.37%), Cefotaxime (82.93%), Nalidixic acid (78.05%), Trimethoprim (76.83%), Trimethoprim-sulfamethoxazole (75.61%), and Amoxicillin-clavulanate (74.4%), the resistance rates were 71.95% for both ampicillin-sulbactam and ceftazidime, 68.29% for levofloxacin., Cefazolin and Ceftazidime (67.07% each), Doxycycline (65.85%), Ciprofloxacin (63.41%), Nitrofurantoin (29.27%), Aztreonam (52.44%), Gentamicin (45.12%), Minocycline (43.90%), Mecillinam (37.8%), Tobramycin (36.59%), Kanamycin (32.93%), Piperacillin-tazobactam (30.49%), Fosfomycin (25.61%), Amikacin (28.05%), Polymyxin B(23.2%), Ertapenem(20.73%), and Imipenem(19.51%), as for the drug Meropenem, which showed the lowest resistance rate at 17.07%, this necessitates reducing reliance on antibiotics with high resistance rates and turning to therapeutic alternatives that are more effective [15].

The findings of the current study align with global research indicating a significant rise in bacterial resistance to conventional antibiotics, and they support recommendations for the optimal use of antibiotics avoiding indiscriminate application to curb the spread of antibiotic-resistant strains [16].

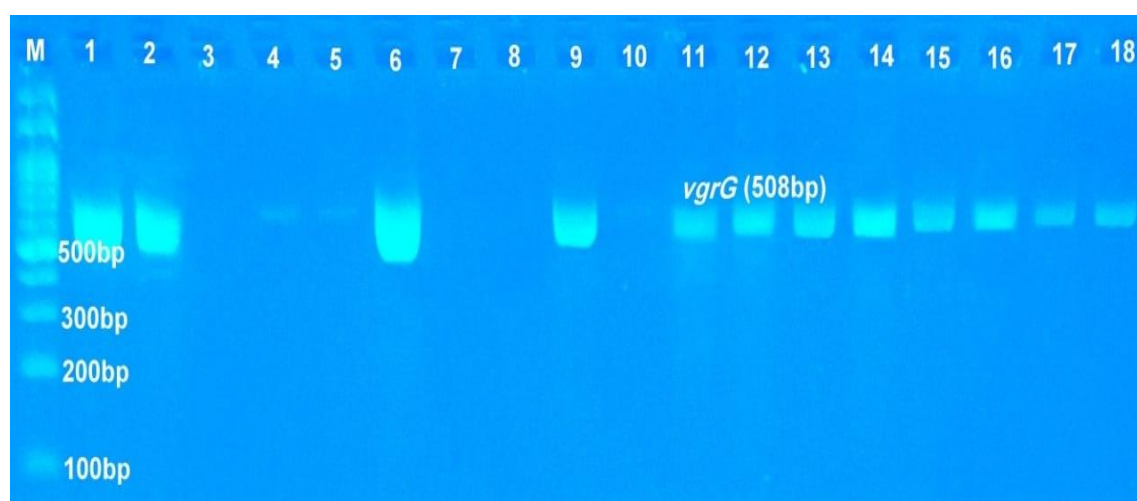


Figure 1: PCR products for the molecular detection of the *vgrG* gene in UPEC via gel electrophoresis using a 1.5% agarose gel (100 V, 60 minutes). The size of the product upon amplification of the *vgrG* gene is 508 bp. Lane M: DNA ladder (100-3000 bp).

3.2. Detection of T6SS by the *vgrG* Gene

Molecular detection results via PCR amplification of the *vgrG* gene showed its presence in 37 (84.1%) of the 44 UPEC isolates. (Figure 1) illustrates the electrophoresis results of the PCR product. The results of the current study are of significant clinical and statistical importance. The presence of the *vgrG* gene, a key component of the T6SS in UPEC, indicates that the bacterial isolates under study possess competitive and aggressive capabilities. Molecular analysis revealed that 84.1% (37 of 44) of the isolates carried the *vgrG* gene, demonstrating the widespread prevalence of this secretion system among local UPEC strains. From a biological perspective, the T6SS functions as a molecular spear launched by UPEC bacteria to penetrate neighboring cells. The VgrG protein encoded by this gene constitutes the spear's pointed tip. Its role is not merely structural; it often acts as an effector protein that directly injects toxins into target cells [12].

Possession of this gene confers competitive advantages upon UPEC within the patient urinary tract. The system function is not limited to attacking human cells but extends to targeting competing bacteria. Consequently, bacterial strains carrying this gene can eliminate beneficial bacteria and colonize the environment highly effectively, which explains the presence of these isolates in patient clinical samples. Given that 84.1% of the isolates under study carry this gene, it strongly suggests that most UPEC infections in this study were caused by virulent strains. From a clinical perspective, the presence of the *vgrG* gene may serve as a predictor of infection severity and the potential for progression beyond simple cystitis to more serious complications or dissemination to other sites.

Numerous global studies have confirmed the widespread prevalence of T6SS among extra-intestinal pathogenic *E. coli* (ExPEC) isolates—particularly those from patients with urinary tract infections—compared to commensal isolates. This highlights the system's role as a key virulence factor that enhances the bacteria's ability to adhere to and penetrate host tissue barriers, thereby facilitating their survival and colonization within the urinary tract and their dissemination to other sites in the body. This result confirms that the T6SS represented by the *vgrG* gene serves as a lethal weapon for UPEC. Its high prevalence (84.1%) poses an additional challenge for therapeutic protocols [17].

The relationship between the *vgrG* gene—associated with the T6SS and phenotypic antimicrobial resistance—was investigated in 44 *E. coli* isolates. Overall, resistance to most antibiotics was observed regardless of the presence or absence of this gene. Resistance to ampicillin (AM) reached 100% across all groups, indicating that all isolates exhibited resistance. Regarding drug combinations of beta-lactams and beta-lactamase inhibitors (such as AMC and SAM), resistance was observed in both isolates possessing the gene and those lacking it. The same pattern was observed regarding resistance to the cephalosporin group (CZ, CTX, CAZ, and FEP). There were no statistically significant differences between the isolates based on the presence or absence of the *vgrG* gene. Finally, a 100% resistance rate was recorded against the carbapenem group (IPM).

The absence of statistically significant differences in antimicrobial resistance among isolates regarding the presence of this gene (with all P-values reaching 1.000) indicates that the *vgrG* gene played only a minor role in the observed resistance. Furthermore, the high resistance rates recorded across all groups suggest that other resistance mechanisms independent of the genes under study, such as efflux mechanisms, may be the primary systems responsible for this resistance.

4. Conclusion

The current study on UPEC isolates revealed that they pose a clinical threat in the study area, a threat characterized by complexity due to the widespread prevalence of the T6SS alongside significantly high rates of resistance to conventional antibiotics. Molecular detection via PCR revealed that 84.1% of the isolates carried the *vgrG* gene, a key component of the T6SS, confirming the significant capacity of UPEC isolates to compete, colonize, and damage host tissues. This may explain the severity of the infection, as well as the challenges associated with treating it and limiting its spread. Furthermore, susceptibility test results revealed a marked decline in the efficacy of currently used antibiotics. Commonly used antibiotics in the study area, specifically ampicillin, cefotaxime, and ciprofloxacin, exhibited high resistance rates, rendering them largely unsuitable for therapeutic use.

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