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FULL PAPER

Investigation of the Relationship between Colistin Resistance and Capsule Serotypes in Carbapenem Resistant *Klebsiella pneumoniae* Strains

Basak Baykara[1], Mehmet Cimentepe[2], Tulay Kandemir[3], Fatih Koksal[3]

[1]*Department of Nutrition and Dietetics, Faculty of Health Sciences, Hakkari University, Hakkari, Turkey;*
[2]*Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Harran University, Sanliurfa, Turkey;*
[3]*Department of Medical Microbiology, Faculty of Medicine, Cukurova University, Adana, Turkey*

SUMMARY

Carbapenem-resistant *Klebsiella pneumoniae* is associated with high morbidity and mortality, and capsule serotypes make treatment difficult. The aim of this study is to investigate the relationship between colistin resistance and capsule types in carbapenem-resistant *K. pneumoniae* isolates. In 2018-2020, we conducted our study with 115 carbapenem-resistant *K. pneumoniae* strains diagnosed by matrix-mediated laser desorption ionization time-of-flight mass spectrometry method (MALDI-TOF MS; Bruker Daltonics, Germany). Colistin sensitivities were determined by using DxM MicroScan WalkAway System (Beckman Coulter, ABD) automated system and were then verified by liquid microdilution (MIC). Capsule serotypes were investigated by conventional polymerase chain reaction (PCR) method. Among the carbapenem resistant *K. pneumoniae* isolates, 42% (48) were resistant to colistin and 58% (67) were susceptible to colistin. In the *K. pneumoniae* isolates with colistin resistance 33% (16) K5, 13% (6) K2, 8% (4) K20 4% (2) K1 and 2% (1) K54 and K57 capsule serotypes were found, while in the *K. pneumoniae* isolates with colistin susceptible 12% (8) K5, 4% (3) K2, 3% (2) K20, 1.5% (1) K1 and K54 capsule serotypes were found. Serotype K5 was very frequent in isolates collected from patients with urinary tract diseases. The resistance profile data obtained from the present study can serve as an information base to understand the infection pattern prevailing in the hospital.

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INTRODUCTION

K. pneumoniae, a member of the Enterobacteriaceae family, is an opportunistic pathogen that causes community-acquired and nosocomial infections such as bacteremia, pneumonia, and urinary tract infections in healthy individuals, and especially in immunosuppressive individuals (Munoz-Price *et al.,* 2013). *K. pneumoniae* infections cause prolongation of patients' hospital stay, the need to use more effective and broad-spectrum reserve drugs, increased treatment costs, and rapid resistance of active strains to broad spectrum agents. Recently, infections caused by multidrug-resistant or carbapenem-resistant *K. pneumoniae* isolates have become an important problem worldwide with high mortality and morbidity rates. Because carbapenems have broad-spectrum action, they are used as the first choice for enteric bacteria that produce broad-spectrum ß-lactamase (Munoz-Price *et al.,* 2013, Candevir *et al.,* 2015). According to reports reported by the World Health Organization (WHO), resistance to carbapenemase was found in over 50% of *K. pneumoniae* isolated in 2014 (World Health Organization, 2018). Colistin, an old antibiotic in the polymyxin group, is not preferred due to its side effects, such as nephrotoxicity and neurotoxicity (Cannatelli *et al.,* 2014). However, today it is used together with tigecycline as the last option. Colistin resistance, which is the only treatment option in infections caused by carbapenem-resistant *K. pneumoniae* strains, is one of the most important clinical problems today. The emergence of these colistin-resistant species has been reported to be a result of the intensive and increasingly irrational use carbapenem antibiotics (Falagas *et al.,* 2005).

K. pneumoniae strains have capsule polysaccharide and many other virulence factors. In studies conducted, capsule types K1, K2, K5, K20, and K57 were commonly observed in *K. pneumoniae* strains isolated from patients with urinary tract infection, pneumonia and bacteremia. While K1-K6 serotypes cause significant respiratory infections and septi-

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Corresponding author:
Başak Baykara
E-mail: basakbedir89@gmail.com

cemia, K1-K2 serotypes have been found to be associated with highly invasive diseases (Abbot *et al.,* 2011). It has been stated in studies that geographical differences in which different capsular types are predominant may play a role in the evaluation of the relationship between each *Klebsiella* serotype and clinical symptoms (Cheng *et al.,* 2015). In epidemiological studies, it is thought that there may be a relationship between resistance to antimicrobials, especially carbapenem group antibiotics, and capsule serotypes (Hennequin *et al.,* 2016). The aim of this study is to investigate the relationship between colistin resistance and capsule serotypes in carbapenem-resistant *K. pneumoniae* strains isolated from various clinical samples.

MATERIALS AND METHODS

Identification of isolates and antibiotic susceptibility tests

In 2018-2020, 115 carbapenem-resistant *K. pneumoniae* isolated from clinical specimens were included in the study. MALDI-TOF MS (Bruker Daltonics, Germany) was used to identify *K. pneumoniae* strains isolated from various clinical samples sent to the microbiology laboratory. Growing strains were also identified outside of automated systems using catalase, oxidase and other biochemical tests that were cultivated on 5% sheep blood agar, Endo agar, MacConkey agar, and EMB media, and incubated at 37°C for 24 hours. Antibiotic susceptibilities of *K. pneumoniae* isolates were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria using the automated system of the DxM MicroScan WalkAway System (Beckman Coulter, USA)[10]. The susceptibility of isolates determined carbapenem-resistant with automated systems to ertapenem (10 µg), imipenem (10 µg) and meropenem (10 µg) group antibiotics were investigated by disk diffusion test. Colistin (Sigma-Aldrich, St. Louis, MO) susceptibility tests were performed by using the liquid microdilution method. MIC was determined by using the liquid microdilution method in the concentration range of 0.125-128 mg/L. This study was conducted with the approval of the Cukurova University Faculty of Medicine Ethics Committee (Date: 02.10.2018 and Decision number: 31).

Investigation of capsule serotypes and genotypic gene region

The isolates included in the study were genotypically confirmed by looking at the 16S-23S ITS region in *K. pneumoniae*. Strains were classified according to capsule serotypes using the conventional PCR method. DNA extraction was in Luria Broth using the boiling method. The cells were heated to 95°C for 15 minutes in a dry heat block and the supernatant obtained after centrifugation was stored at -20°C for PCR analysis. For the PCR mix, 12.5 µl of 10X PCR buffer (Fermentas, USA) was prepared by adding 0.25 µl of each primer, 7 µl of RNase-free water and 5 µl of DNA. PCR analyses were performed on the thermal cycle device (MJ Mini Personal Thermal Cycler, BioRad). The primers shown in *Table 1* were used for the genes of capsule serotypes and 16S-23S ITS gene, which are common in our country (Liu *et al.,* 2008, Fang *et al.,* 2004, Turton *et al.,* 2008, Fang *et al.,* 2007). Amplification products were examined for the presence of bands in the gel imaging system (Gel logic 1500 imaging system, Kodak Company, NY, USA) using 2% agarose gel electrophoresis with ethidium bromide.

Statistical analysis

The logistic regression model was performed for the multivariate analysis to identify risk factors for mortality. We used the X^2 test and the Fisher exact test (if necessary) to find the relationship between serotypes and other variables. P-value <0.05 was considered statistically significant. The data were analyzed with SPSS statistics (Version 20) program (IBM Corporation).

Table 1 - *Primary sequences of 16S-23S ITS gene region and capsule types found in K. pneumoniae.*

	Primer	*Annealing Isısı*	*bp*	*Reference*
6S-23S ITS	*K. pneumoniae* F ATTTGAAGAGGTTGCAAACGAT *K. pneumoniae* R TTCACTCTGAAGTTTTCTTGTGTTC	57 °C	130 bp	Liu *et al.* (2008)
K1	MagAF GGTGCTCTTTACATCATTGC MagAR GCAATGGCCATTTGCGTTAG	57 °C	1283 bp	Fang *et al.* (2004)
K2	K2wzyF GACCCGATATTCATACTTGACAGAG K2wztR CCTGAAGTAAAATCGTAAATAGATGGC	57 °C	641bp	Turton *et al.* (2008)
K5	K5wzxF360 TGGTAGTGATGCTCGCGA K5wzxR639 CCTGAACCCACCCCAATC	55 °C	280 bp	Turton *et al.* (2008)
K20	wzyK20-F CGGTGCTACAGTGCATCATT wzyK20-R GTTATACGATGCTCAGTCGC	55 °C	741 bp	Fang *et al.* (2007)
K54	wzxK54F CATTAGCTCAGTGGTTGGCT wzxK54R GCTTGACAAACACCATAGCAG	55 °C	881 bp	Fang *et al.* (2007)
K57	wzyK57F CTCAGGGCTAGAAGTGTCAT wzyK57R CACTAACCCAGAAAGTCGAG	55 °C	1037 bp	Fang *et al.* (2007)

RESULTS

In the distribution of carbapenem-resistant *K. pneumoniae* isolates according to type of clinic, 63 (55%) were general intensive care, 37 (35%) were service clinics and 15 (10%) were various polyclinics. Of the isolates, urine was 44% (51) followed by blood (n=28, 24%), tracheal aspirate (n=14, 12%), wound (n=10, 9%), sputum (n=7, 6%) and from other samples (n=5, 5%).

In our study, among 115 carbapenem-resistant *K. pneumoniae* isolates, 42% (48) were resistant to colistin and 58% (67) were susceptible to colistin. Of the 48 isolates with colistin resistance, 27 (56%) were isolated from general intensive care, 20 (42%) were from service clinics and 1 (2%) was from a urology outpatient clinic (p<0.05). Urine was the most common (50.8%) clinical specimen from which colistin-resistant *K. pneumoniae* was isolated, followed by blood (n=13, 27%), tracheal aspirate (n=8, 17%), wound (n=5, 11%), sputum (n=4, 8%) and bile (n=1, 2%). Of the 67 isolates susceptible to colistin, 36 (54%) were isolated from general intensive care, 17 (25%) were from service clinics and 14 (21%) were from various polyclinics (p<0.05). In isolates susceptible to colistin 51% (34) were in urine, 22% (15) in blood, 9% (6) in tracheal aspirate, 7.5% (5) in wound, 4.5% (3) in sputum and peritoneal fluid, and 1.5% (1) in cerebrospinal fluid (CSF) (*Table 2*). Colistin resistance and susceptibility isodes were seen more in urinary system and blood circulation system infections than in the source of infection (p<0.05, *Figure 1*).

Carbapenem-resistant *K. pneumoniae* isolates were found to be resistant to imipenem in 56% (64), meropenem in 85% (98) and ertapenem in 99% (114) of cases (*Figure 2*). The MIC of colistin obtained from broth microdilution is shown in *Figure 3*. The MIC of carbapenem-resistant *K. pneumoniae* isolates against colistin ranged from 0.03125 to 64 mg/L.

In our study, a 130 bp band image of the *K. pneumoniae* 16S-23S ITS gene region was observed in all 115 carbapenem-resistant *K. pneumoniae* isolates (*Figure 4*).

The capsule is the significant virulence factor associated with both antibiotic resistance and severity of infection. Six capsular serotypes, such as K1, K2, K5, K20, K54 and K57 were examined from 48 colistin-resistant *K. pneumoniae* isolates. K5 was the most prevalent serotype (n=16, 33%), followed by K2 (n=6,

Table 2 - *Distribution of isolates according to clinical samples.*

Material	*Carbapenem-resistant K. pneumoniae isolate (n) (%)*	*Colistin-resistant K. pneumoniae isolate (n) (%)*	*Colistin-sensitive K. pneumoniae isolate (n) (%)*
Urine	51 (%44)	17 (%35)	34 (%51)
Blood	28 (%24)	13 (%27)	15 (%22)
Tracheal Aspirate	14 (%12)	8 (%17)	6 (%9)
Wound	10 (%9)	5 (%11)	5 (%7.5)
Sputum	7 (%6)	4 (%8)	3 (%4.5)
Peritoneum	3 (%3)	0	3 (%4.5)
CSF	1 (%1)	0	1 (%1.5)
Bile	1 (%1)	1 (%2)	0
Total	*115 (%100)*	*48 (%100)*	*67 (%100)*

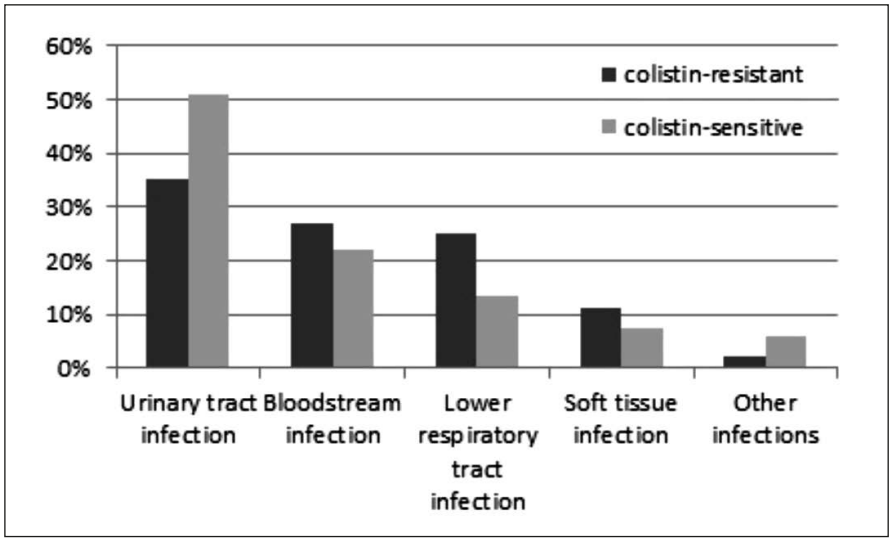

Figure 1. *Distribution by source of infection from which colistin-susceptible and resistant isolates.*

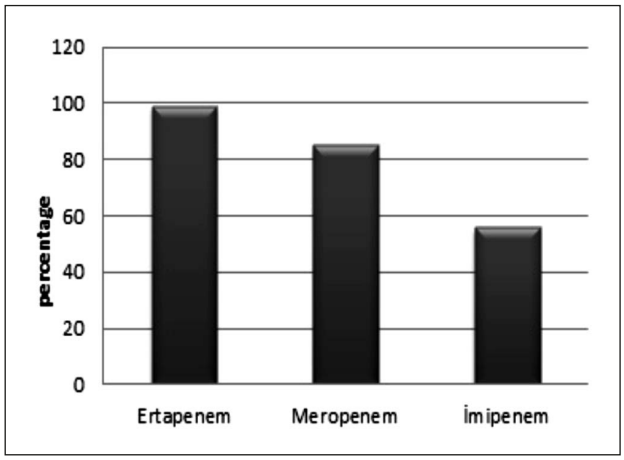

Figure 2. *Distribution of antibiotics studied by disc diffusion of K. pneumoniae isolates.*

13%), K20 (n=4, 8%), and K1 (n=2, 4%), while K54 and K57 were found in only 1 (2%) isolate ($p<0.05$). In the distribution of capsule serotypes according to type of clinic, 5 different capsule types in the general intensive care unit, 4 in the ward and 2 in the outpatient clinic were detected. In colistin susceptible to *K. pneumoniae* isolate, serotype K5 was the most prevalent (n=8, 12%) followed by K2, K20, and K1 and K54, which accounted for 3 (4%), 2 (3%), and 1 (3.5%) isolates, respectively. Colistin-susceptible isolates were seen in 4 different capsule serotypes in the general intensive care unit, 3 in the service clinic and 2 in the outpatient clinic.

In our study, carbapenem-resistant *K. pneumoniae* capsule serotypes with colistin resistance were mostly found in urine (K1, K5, K20, K54 and K57), blood (K1, K2, K5 and K20) and tracheal aspirate

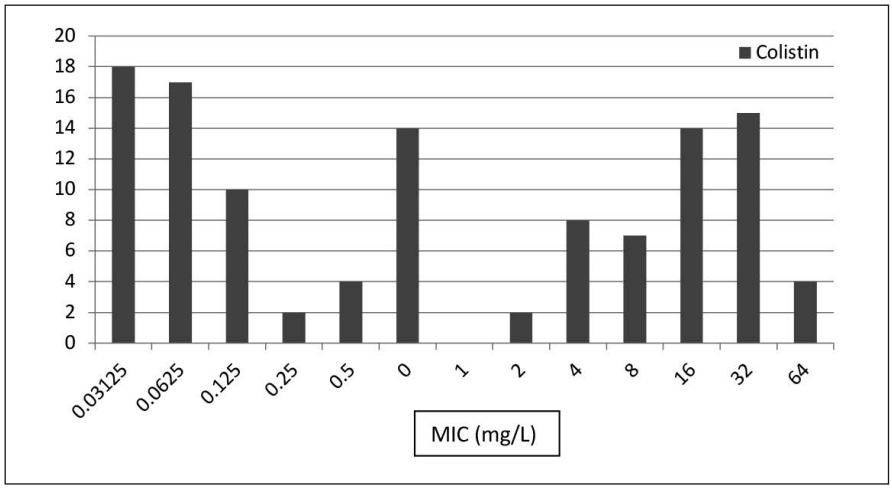

Figure 3. *MIC distributions of colistin studied with liquid microdilution of carbapenem-resistant K. pneumoniae isolates.*

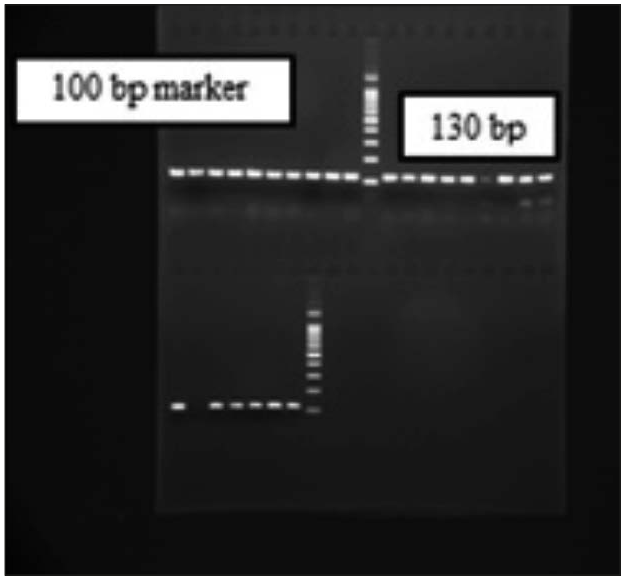

Figure 4. *Band image of K. pneumoniae 16S-23S ITS gene region amplification assay.*

(K2 and K5). One different serotype was seen in the samples of wound (K5), sputum (K2) and CSF (K5); the capsule serotype was not detected in other body fluids. Colistin-susceptible *K. pneumoniae* capsule serotypes were found in urine (8 isolates belongin to serotypes K2, K5, K20 and K54), blood (5 isolates belongin to serotypes K1, K5 and K20), wound (1 isolate belongin to serotype K5), and sputum (1 isolate belonging to serotype K2) (*Table 3*).

DISCUSSION

K. pneumoniae is isolated from common nosocomial infections such as pneumonia, urinary tract infections, surgical site infections and bacteremia. Resistance formed in *K. pneumoniae* strains is rapidly spreading among strains of carbapenem-resistant genes carried by plasmids. Today, the increase in colistin use has caused colistin resistance to reach alarming levels (Kalem *et al.,* 2016). Colistin-resistant *Klebsiella* spp. isolates were first reported in America

Table 3 - *Distribution of capsular serotypes in colistin-resistant and susceptible K. pneumoniae strains by sample type.*

	Colistin-resistant K. pneumoniae						*Colistin-sensitive K. pneumoniae*					
Material	*K1*	*K2*	*K5*	*K20*	*K54*	*K57*	*K1*	*K2*	*K5*	*K20*	*K54*	*K57*
Urine	1	2	9	3	1	1		2	4	1	1	
Blood	1	2	3	1			1		3	1		
Tracheal Aspirate		1	2									
Wound			1						1			
Sputum		1						1				
Peritoneum												
CSF			1									
Bile												
Total	*2*	*6*	*16*	*4*	*1*	*1*	*1*	*3*	*8*	*2*	*1*	

in 2009, and then outbreaks due to colistin- and carbapenem-resistant *K. pneumoniae* were identified in hospitals in many countries, such as Greece, South Korea and the USA (Munoz-Price *et al.,* 2013). The proportion of colistin-resistant cases among carbapenem-resistant *K. pneumoniae* cases were reported to be 36.1% in Italy, 13% in the United States, 27.6% in India, and 10.7%, 25.6% and 22% in different years in Italy (Capone *et al.,* 2013, Rojas et al., 2017, Bhaskar *et al.,* 2017, Parisi *et al.,* 2015). In our country, most studies have shown that colistin-resistance was found to be 3% in 2014, 7.7% in 2016, 36.4% in 2017 and 39.5% in 2019 among carbapenem-resistant *K. pneumonia*e isolates (Aydemir 2016, Aygar 2020, Özkul Koçak *et al.,* 2019). In our study, among 115 carbapenem-resistant *K. pneumoniae* isolates, 42% were resistant to colistin and 58% were susceptible to colistin. Unless alternative treatment methods are available and attention is paid to rational use of antibiotics in colistin-resistant *K. pneumoniae* infections, it may lead to increased colistin-resistance, prolongation of hospital stay, the need to use more effective and broad-spectrum reserve drugs, increased treatment costs, and rapid resistance of active strains to broad-spectrum antibiotics.

Several virulence factors have been identified in *K. pneumoniae*, including lipopolysaccharide adherence factors, siderophore activity, and capsular serotype. Among these factors, capsular serotypes are the most studied virulence factors in *K. pneumoniae* (Khaertynov *et al.,* 2018; Remya *et al.,* 2018). Today, 77 different serotypes have been defined as antigenic in *Klebsiella* species. *K. pneumoniae* strains are associated with a variety of diseases due to the difference in capsular polysaccharides (Shelenkov *et al.,* 2020). Some strains are more virulent, while others are less virulent than capsule serotypes. The presence of high doses of *K. pneumoniae* capsular polysaccharide may cause immunological paralysis. Various capsule serotypes, including K1, K2, K5, K20, K54, and K57, are often associated with community-acquired invasive septicemia, pyogenic liver abscess syndrome, and pneumonia (Hasani *et al.,* 2020).

In this study, 33%, 13%, and 8% of isolates were identified as K5, K2, and K20 serotypes, respectively, potentially putting the patients at risk of increased pathogenicity. Compared with other investigations, our data revealed a lower prevalence rate of K1, K54, and K57 serotypes (Wasf *et al.,* 2016; Sahoo *et al.,* 2019). A recent study reported from Iran found K54 as the most frequent (68%) capsular serotype, while K1 (8%) was the least frequent (Tavakol M *et al.,* 2017). However, contrary to our findings, a higher frequency of serotype K5 (60%) was reported. Another study from south India highlighted the occurrence of K1 and K2 serotypes in carbapenem-resistant *K. pneumoniae* (Remya *et al.,* 2018). Differences in the frequency of capsular serotypes may be due to differences in the types of samples collected in other studies. Capsular serotypes are most commonly isolated in urine and blood. In terms of the clinical sample in which *K. pneumoniae* with colistin resistance is isolated, it was seen that isolates obtained from urine and blood culture are more common than in other clinical samples; therefore, infections are mostly due to urinary system and bloodstream infections. In our study, it was thought that the higher number of urine samples (compared to other clinical samples) sent to microbiology laboratories may have been a factor in this situation.

CONCLUSION

Evaluating the synergistic effects of colistin with other antibiotics and applying combination therapies could be the most appropriate option to prevent rapidly spreading colistin resistance. Unnecessary use of antibiotics should be avoided in colistin resistance; resistance should be monitored by applying surveillance programs, and infection control measures should be followed to reduce the spread of resistance. Epidemiological studies suggested that there may be a relationship between resistance to antimicrobials, especially carbapenem group antibiotics, and capsule serotypes. The clonal determination of possible relationships between carbapenem-resistant strains

easily acquiring resistance to broad-spectrum antibiotics, as well as other virulence factors affecting prognosis, such as capsule structure, is important. Information on the distribution of capsular serotypes in specific diseases and in medical wards with a special pattern of antibiotic resistance could aid physicians in prescribing appropriate treatments.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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