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Identification, Characterization, and Pathogenic Investigation of *Aeromonas veronii*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and *Citrobacter freundii* from a Case of Natural Mixed Infection in Common Carp (*Cyprinus carpio*)

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Identification, Characterization, and Pathogenic Investigation of *Aeromonas veronii*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and *Citrobacter freundii* from a Case of Natural Mixed Infection in Common Carp (*Cyprinus carpio*)

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Simple Summary: *Aeromonas veronii*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and *Citrobacter freundii* were opportunistic pathogen and caused fish diseases in aquaculture. The four kinds of bacteria were together isolated from diseased common carp farmed in the aquaculture base of Henan Normal University of China. Artificial infection indicated the four isolates were all highly pathogenic to fish. Similar mixed bacterial infection case in farmed common carp had seldom been reported before, and our study in bacterial drug resistance provided the reference for the treatment of those bacterial fish disease.

Abstract: *Aeromonas veronii*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and *Citrobacter freundii* were opportunistic pathogen widely distributed in water environment and fish population, causing fish diseases under stressful conditions. In this study, bacteria were isolated from diseased common carp *Cyprinus carpio* with symptoms of hemorrhage on body surface, abdominal distention, and flatulence in the intestine. According to the results of biochemical characteristics, *16S rRNA* sequencing analyses, the isolates were identified as *A. veronii*, *A. hydrophila*, *P. shigelloides*, and *C. freundii*. The experimentally infected fish showed identical symptoms as observed in the naturally infected common carp. The LD_{50} of *C. freundii*, *P. shigelloides*, *A. veronii*, *A. hydrophila*, and mixture group were 1.95×10^4, 4.74×10^4, 5.12×10^4, 1.53×10^5, and 5.41×10^4 respectively. Antibiotic resistance results showed that *P. shigelloides*, *A. veronii*, and *A. hydrophila* were sensitive to streptomycin, enrofloxacin, florfenicol, gentamicin, kanamycin, neomycin, norfloxacin, co-trimoxazole, ceftizoxime, and resistant to ampicillin. *C. freundii* was sensitive to streptomycin, gentamicin, and ceftizoxime, moderately susceptible to kanamycin, neomycin, norfloxacin, and ampicillin, and resistant to enrofloxacin, florfenicol, tetracycline, and co-trimoxazole.

Keywords: aquaculture; resident bacteria; challenge experiment; opportunistic infection; antibiotic resistance

1. Introduction

Common carp (*Cyprinus carpio*) is a world-widely cultured fish, which is also one of the most important economic fish species in China [1]. The total production of farmed *C. carpio* was 2.87 million tons in 2023 according to the China Fishery Statistical Yearbook. However, improper management and high-density culture creates ideal conditions for explosive epidemics of diseases [2] . It has been reported that *C. carpio* breeding industry was threatened by a variety of bacterial organisms, such as

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Aeromonas hydrophila, *Pseudomonas fluorescens*, *Pseudomonas alcaligenes*, and *Shewanella putrefaciens* [3–6]. The current key issues that need to be solved urgently are identifying the most commonly disease-causing bacteria in common carp and finding out effective medicine for treatment.

Aeromonas veronii is Gram-negative bacterium that widely distributed in water environment and can cause ulcerative diseases in many kinds of fish [7–14]. The main clinical symptoms of *A. veronii* were hemorrhagic septicemia, fin rot, exophthalmia, and abdominal distention [14]. *A. hydrophila* is another number of *Aeromonas* species that commonly infect aquatic animals. *A. hydrophila* caused hemorrhagic septicemia and lead to massive mortalities in fishes in previous reports [15–19]. *Plesiomonas shigelloides* and *Citrobacter freundii* were both classified into the Enterobacteriaceae family. *P. shigelloides* was reported to be pathogenic and the diseased fish showed protruding eyeballs, swollen anus, and ascites in the abdominal cavity, swelling and hemorrhage [20–24]. *C. freundii* infection was associated with enteritis, necrosis, body reddening, hemorrhage, and septicemia [25–30]. The four kinds of bacteria are all opportunistic pathogen and can be commonly found in normal aquaculture water and healthy fish. They are more likely to cause diseases when fish under stresses, such as temperature change, hypoxia, and parasite infection [31,32].

Here, we report a case of bacterial disease naturally occurred in common carp that rearing in the aquaculture base of Henan Normal University in China. The infected fish showed obvious hemorrhage on the body surface, extended abdomen, and flatulence in the intestine. Bacteria were isolated from the ascites and were identified as *A. veronii*, *A. hydrophila*, *P. shigelloides*, and *C. freundii* by 16S rRNA sequencing and biochemical tests. The drug resistance and pathogenicity of four types of bacteria were also analyzed to provide reference for the prevention and treatment measures of similar cases.

2. Materials and Methods

2.1. Fish

The diseased common carp (18.3±2.6 g) was from the aquaculture base of Henan Normal University in Xinxiang city, Henan province, China. The fish were kept in a concrete pond with water depth of 1.5 m. Moribund fish were transported immediately to the laboratory for diagnosis and pathogen isolation. Healthy common carp (16±2.1 g) with no history of disease were provided by a local fish farm in Xinxiang city. Healthy fish were acclimated in aquaria for two weeks prior to infection assay and fed with commercial fed once daily. The water was replaced daily, and water temperature was maintained at 28 ± 1℃. Experiments involving live fish were conducted in accordance with the US National Research Council's "Guide for the Care and Use of Laboratory Animals".

2.2. Isolation, Characterization, and Identification of Bacteria

The ascites of moribund fish were collected and streaked on Luria-Bertani (LB) agar plates with inoculating loop. The plates were incubated at 28℃ for 24 h. The dominant colonies from plates were re-streaked on the LB agar plates three times to obtain pure culture. Total genomic DNA of the isolate was extracted by bacteria DNA extraction kits (Sangon biotech, China). Primers 16S rRNA-F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S rRNA-R (5′-GGTTACCTTGTTACGACTT-3′) were used for PCR amplification of 16S rRNA. The PCR amplification conditions of 16S rRNA were initial denaturation at 94℃ for 5 min, followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 57℃ for 30 s, extension at 72℃ for 1.5 min and a final extension at 72℃ for 10 min. The genes obtained by PCR amplification were cloned to pMD-19 T vector (TakaRa, Japan) and sequenced. The biochemical characteristics were examined using commercial reagent (Hangzhou Microorganism Reagent Co., Ltd., China), and following incubated at 28℃ for 24 h or 48 h. All biochemical tests were repeated for three times, which were performed including oxidase, growth condition at 1% of NaCl, serine dihydrolase, lysine decarboxylase, Voges–Proskauer, indole production, sucrose, lactose, citrate, cellobiose, mannitol, salicin, etc.

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2.3. Antibiotic Susceptibility Testing

The antibiotic susceptibility of aeromonad was determined by the Kirby-Bauer disk diffusion method[33]. Bacterial strains were streaked on Muller Hilton agar plates, and the various antibiotic disks (Difco Laboratories, USA) were applied on the streaked cultures. Disks of gentamycin (10 μg), kanamycin (30 μg), neomycin (30 μg), ceftizoxime (30 μg), florfenicol (30 μg), ampicillin (10 μg), trimethoprim-sulfamethoxazole (23.75/1.25 μg), tetracycline (30 μg), streptomycin (10 μg), norfloxacin (10 μg), and enrofloxacin (10 μg) were used. After 18 h of incubation at 28°C, the sizes of the zone of bacterial growth inhibition were measured. The isolates were classified as sensitive (S), moderately sensitive (M) or resistant (R) according to the National Committee for Clinical Laboratory Standards.

2.4. Experimental Infections

The infection assay was carried out in healthy common carp. The bacterial concentration determined by plating 10 -fold serial dilutions onto LB agar plates. The median lethal dosage (LD_{50}) was calculated based on the total cumulative mortality (%) as described by Reed and Muench (1938) [34]. For LD_{50} determination, 240 carp individuals were randomly divided into six groups, including five infected groups and one control group. Infected groups (40 fish per group) were intraperitoneally injected with *A. veronii*, *A. hydrophila*, *P. shigelloides*, *C. freundii* and a mixture of four kinds of bacteria (equal numbers of each kind) at the concentration of 1.0×10^4, 1.0×10^5, 1.0×10^6 and 1.0×10^7 colony-forming unit (CFU)/fish (10 fish injected for each concentration), respectively. The control group was injected with the same dose of sterile physiological saline. The experiment was repeated once. The mortalities and clinical signs of all the groups were recorded every day for 15 d post-infection. Morbid fish were subjected to routine bacteriological examination for re-isolation and re-identification of the organism.

3. Results

3.1. Clinical Signs and Isolation of Bacteria from Diseased Common Carp

The clinical signs of diseased fish showed hemorrhage on gill cover and lower jaw, extended abdomen, swollen anus, ascites, and intestinal flatulence (Figure 1). The bacteria isolated from the diseased common carp were Gram negative. Colonies were buff, translucent, circular, convex and intact edge on LB. Ten colonies were selected and *16S rRNA* were obtained and sequenced, the results showed that five strains of the isolates were *A. veronii*, two strains were *P. shigelloides*, two strains were *C. freundii*, and one strain was *A. hydrophila.*

3.2. Biochemical Characteristics

The isolates of *A. veronii* and *A. hydrophila* had positive biochemical reactions for indole, Voges–Proskauer, arginine dihydrolase, lysine, glucose, sucrose, and mannose, but negative for inositol. *A. hydrophila* could utilize arabinose, but could not *A. veronii*. Both *A. veronii* and *A. hydrophila* were able to grow in peptone water under 8% NaCI. *C. freundii* made utilization of citrate, glucose, raffinose, sorbitol, xylose, and urea, while *P. shigelloides* was incapable of metabolizing those molecules. *P. shigelloides* produced indole, lysine decarboxylase, and ornithine decarboxylase. Both *C. freundii* and *P. shigelloides* possessed motility (Table 1).

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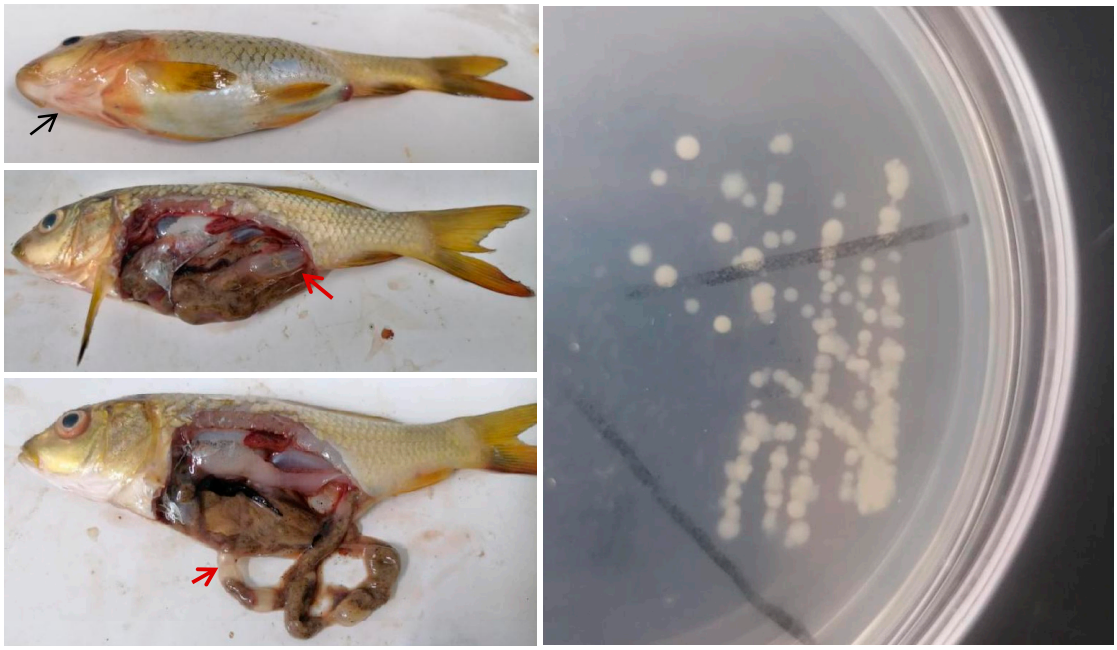

Figure 1. Symptoms of naturally infected fish (left) and colonies of isolated bacteria on LB plate (right). Black arrow showed the hemorrhage on body surface, red arrow showed the flatulence in intestine.

Table 1. Biochemical Characteristics of isolated strains.

Test	*P. shigelloides*	*C. freundii*	Test	*A. veronii*	*A.hydropha*
H_2S production	-	+	Glucose (gas production)	+	+
Phenylalanine deaminase	-	-	Sucrose	+	+
Gluconate	-	-	Mannose	+	+
Indole reaction	+	-	Indole reaction	+	+
Voges–Proskauer	-	-	Voges–Proskauer	+	+
Citrate	-	+	Arabinose	-	+
Motility	+	+	Arginine dihydrolase	+	+
Glucose (gas production)	-	+	Inositol	-	-
Lysine decarboxylase	+	-	Lysine	+	+
Ornithine decarboxylase	+	-	Unsalted peptone water	+	+
Raffinose	-	+	3% NaCI peptone water	+	+
Sorbitol	-	+	6% NaCI peptone water	+	+
Adonitol	-	-	8% NaCI peptone water	-	-
Xylose	-	+	10% NaCI peptone water	-	-
Urease	-	+			

Note: "+" is masculine and "-" is feminine.

3.3. Experimental Infections

To confirm the pathogenicity of the four types of bacteria, the challenge assay was carried out in healthy common carp. Fish were intraperitoneally injected with different dose of each bacteria or a mixture of four bacteria. The experimentally infected fish showed identical symptoms as observed in the naturally infected common carp, which showed hemorrhage on body surface, abdominal distention, ascites, and flatulence in the intestine (Figure 2). The LD_{50} of *C. freundii*, *P. shigelloides*, *A. veronii*, and *A. hydrophila* were 1.95×10^4, 4.74×10^4, 5.12×10^4, and 1.53×10^5 respectively. The LD_{50} of

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mixture group was 5.41×10^4. There were no clinical symptoms or death in the control group (Table 2). Furthermore, four kinds of bacteria were re-isolated from the experimental infected fish, as confirmed by colonial morphology observation, physiological and biochemical characteristics analyses, and *16S rRNA* sequencing analyses.

3.4. Determination of Antimicrobial Resistance

The antibiotic resistance patterns of four bacteria were valued by the size of the inhibition zones around each disc, showed that *P. shigelloides*, *A. veronii*, and *A. hydrophila* were sensitive to streptomycin, enrofloxacin, florfenicol, gentamicin, kanamycin, neomycin, norfloxacin, co-trimoxazole, ceftizoxime, and resistant to ampicillin. *C. freundii* was sensitive to streptomycin, gentamicin, and ceftizoxime, moderately susceptible to kanamycin, neomycin, norfloxacin, and ampicillin, and resistant to enrofloxacin, florfenicol, tetracycline, and co-trimoxazole (Table 3).

Table 2. Cumulative mortality of experimentally infected fish by isolates.

Group	Concentration (CFU)	Fish	Dead fish number on day after challenge														Accumulative mortality	LD_{50} value (CFU)
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Control	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0%	
P. shigelloides	1.0×10^7	10	0	0	0	0	4	1	1	3	1	0	0	0	0	0	0	100%	4.74×10^4
	1.0×10^6	10	0	0	0	0	2	1	0	3	0	2	0	0	1	0	0	90%	
	1.0×10^5	10	0	0	0	0	0	0	0	3	1	2	0	1	0	0	0	70%	
	1.0×10^4	10	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	20%	
C. freundii	1.0×10^7	10	0	0	0	0	4	2	3	1	0	0	0	0	0	0	0	100%	1.95×10^4
	1.0×10^6	10	0	0	0	0	1	1	2	3	1	1	1	0	0	0	0	100%	
	1.0×10^5	10	0	0	0	0	0	1	0	1	5	1	1	0	0	0	0	90%	
	1.0×10^4	10	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	30%	
A. veronii	1.0×10^7	10	2	4	3	0	0	0	0	0	0	0	0	0	0	0	0	90%	5.12×10^4
	1.0×10^6	10	1	0	0	0	4	2	0	1	0	0	0	1	0	0	0	90%	
	1.0×10^5	10	1	0	0	0	0	1	0	2	1	0	0	2	0	0	0	70%	
	1.0×10^4	10	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	20%	
A. hydrophila	1.0×10^7	10	1	0	0	0	3	1	2	0	0	1	0	2	0	0	0	100%	1.53×10^5
	1.0×10^6	10	0	0	0	0	1	0	1	1	4	0	1	0	0	0	0	80%	
	1.0×10^5	10	0	0	0	0	0	0	0	0	1	0	1	2	0	0	0	40%	
	1.0×10^4	10	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	10%	
Mixed infection	1.0×10^7	10	0	0	0	0	3	1	1	2	2	1	0	0	0	0	0	100%	5.41×10^4
	1.0×10^6	10	0	0	0	0	1	1	0	1	2	1	2	0	1	0	0	90%	
	1.0×10^5	10	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	50%	
	1.0×10^4	10	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	30%	

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Figure 2. Symptoms on the epithelial surface and inside the body of experimentally infected fish, showing hemorrhage on body surface, abdominal distention, ascites, and flatulence in the intestine. A1, A2, *A. veronii* infection, B1, B2, *A. hydrophila* infection, C1, C2, *P. shigelloides* infection, D1, D2, *C. freundii* infection, E1, E2, mixed infection.

Table 3. Susceptibility of bacterial isolates to antibiotics.

Antibiotic	Drug concentration（μg/ disc）	Inhibition zone diameter (mm)			
		A.veronii	*A.hydrophila*	*C. freundii*	*P.shigelloides*
Streptomycin	10	19^{S}	15^{S}	15^{S}	15^{S}
Enrofloxacin	10	40^{S}	23^{S}	10^{R}	30^{S}
Florfenicol	30	30^{S}	32^{S}	0^{R}	28^{S}
Gentamicin	10	21^{S}	19^{S}	20^{S}	19^{S}
Kanamycin	30	20^{S}	20^{S}	15^{I}	18^{S}
Neomycin	30	17^{S}	18^{S}	17^{I}	18^{S}
Tetracycline	30	11^{R}	25^{S}	0^{R}	21^{S}
Norfloxacin	10	34^{S}	23^{S}	14^{I}	27^{S}

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Co-trimoxazole	23.75/1.25	19^{S}	20^{S}	0^{R}	13^{I}
Ceftizoxime	30	44^{S}	40^{S}	32^{S}	37^{S}
Ampicillin	10	10^{R}	10^{R}	14^{I}	0^{R}

Note: "S" is sensitive, "I" is intermediate, and "R" is resistant.

4. Discussion

A. veronii, *A. hydrophila*, *C. freundii*, and *P. shigelloides* were all opportunistic pathogen widely distributed in water environment and fish population, causing fish diseases under stressful conditions, such as overcrowding, low oxygen concentration, and high water temperature [35]. The disease occurred in this case maybe because of the high water temperature and overfeeding, as the fish stop dying after stop feeding. *C. freundii* and *P. shigelloides* had been reported to cause severe hemorrhagic septicemia in internal organs of Chinese sturgeons that cultured in a research Institute [36], and co-infection by *Aeromonas bestiarum*, *Aeromonas sobria*, and *P. shigelloides* was observed in Mexican golden trout, leading to red and inflamed lesions in the abdomen and mouth, and hemorrhage in fins and gills [31]. However, it's the first time that co-infection of *A. veronii*, *A. hydrophila*, *C. freundii*, and *P. shigelloides* in common carp was reported. Experimental infections showed that fish injected with four strains of bacteria all developed the similar clinical symptoms, which were hemorrhage, abdominal distention, ascites, and flatulence in the intestine (Figure 2), the same with the sign in naturally infected fish (Figure 1). So it was difficult to identify which kind of bacteria was the main cause of the disease from symptoms. But since half of the ten isolated strains were *A. veronii*, and the LD_{50} of mixture group was close to that of *A. veronii* group, it was most likely that *A. veronii* were the main pathogen.

Biochemical Characteristics of four bacteria were in accordance with the description of *A. veronii*, *A. hydrophila*, *C. freundii*, and *P. shigelloides* in Bergey's Manual [37]. *A. veronii*, *A. hydrophila*, and *C. freundii* all can utilize glucose to produce gas (Table 1), that explained why the intestine of diseased fish was filled with gas (Figure 2). The flatulence was more obvious in the mixed infection group, indicating more gas was produced in mixed infection than that of single bacterial infection. The LD_{50} determination showed that *C. freundii* was the most virulent bacteria, and the virulence of *A. hydrophila* was the weakest. However, the mixed infection didn't enhance the bacterial virulence, and the virulence of mixed bacteria was equivalent to that of *A. veronii* (Table 2).

Antimicrobial susceptibility testing indicated that the isolates of *P. shigelloides*, *A. veronii*, and *A. hydrophila* were susceptible to streptomycin, enrofloxacin, florfenicol, gentamicin, kanamycin, neomycin, norfloxacin, co-trimoxazole, cefazoxime, and resistant to ampicillin (Table 3). Simillarly, *P. shigelloides* isolated from Chinese sturgeon was sensitive to gentamicin and neomycin, and resistant to norfloxacin and ampicillin [36]. In our case, *C. freundii* was sensitive to streptomycin, gentamicin, and ceftizoxime, moderately susceptible to kanamycin, neomycin, norfloxacin, and ampicillin, and resistant to enrofloxacin, florfenicol, tetracycline, and co-trimoxazole (Table 3). In previous study, *C. freundii* isolated from largemouth bass was sensitive to gentamicin, norfloxacin, tetracycline, and co-trimoxazole, but resistant to kanamycin. *C. freundii* from angelfish was sensitive to tetracycline, resistant to enrofloxacin, florfenicol, and ampicillin. It seems that *C. freundii* strains from different fish showed different drug-resistant characterizations. The susceptibility test is essential for the drug selection in treatment.

5. Conclusions

In this study, we isolated *A. veronii*, *A. hydrophila*, *C. freundii*, and *P. shigelloides* from diseased common carp. The biological characteristics of four types of bacteria were determined. Artificial infection in fish showed four strains of bacteria were all highly pathogenic and could cause the disease with hemorrhage and abdominal distention syndrome, indicating the naturally infected fish maybe died of mixed infection. The drug sensitivity of the bacteria was also tested. The findings in

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this study will provide reference for the diagnosis, treatment, and prevention of the disease caused by mixed bacterial infection.

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Institutional Review Board Statement: This study was approved by the Laboratory Animal Ethics Committee of the College of Fisheries of Henan Normal University (Approved ID: 2023081101).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available by the authors upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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