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IDENTIFICATION OF *CITROBACTER FREUNDII* FROM SKIN WOUNDS IN GOLDFISH (*CARASSIUS AURATUS*) AND DETERMINATION OF ANTIBACTERIAL SUSCEPTIBILITY

Volkan Özavci[#], Hafize Tuğba Yüksel Dolgun[*], Şükrü Kirkan[*]

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Abstract

Citrobacter freundii is a zoonotic pathogen that causes skin injuries and high mortality rates in fish. This study aims to determine the presence of *C. freundii* and antibiotic susceptibility in 50 goldfish (*Carassius auratus*) skin wounds with local bleeding between the ages of 8–18 months in three regions (A, B, and C) located in the West of Turkey. *C. freundii* (22/50; 44%) strains were isolated from wound swab samples by phenotypic and genotypic methods. *C. freundii* isolates were positive for H_2S, mannitol, and indole, and were negative for the gas formation and urea. All *C. freundii* isolates showed 96% similarity to *C. freundii* strain BAO2749 according to the Sanger sequence method. Isolates were also resistant to ampicillin at 57.09% and susceptible to cefoperazone and sulfamethoxazole-trimethoprim by 100%. Statistical analyses were evaluated at a confidence interval of 95%. A p value < 0.05 was considered statistically significant. In conclusion, *C. freundii* isolates can play a significant role in the pathophysiology of infections in goldfish (*Carassius auratus*). It can also cause commercial losses in businesses. For this reason, it will be beneficial to control *C. freundii* pathogenicity in aquarium fish with prophylactic applications to improve product quality and treat diseases in ornamental fish farming.

Key words: goldfish, *Citrobacter freundii*, identification, sequence, antibiogram

[#]Corresponding author.
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Introduction. Goldfish (*Carassius auratus*) are freshwater ornamental fish [1]. *Citrobacter* (*C.*) *freundii* is a gram-negative, motile, facultative anaerobic bacillus, biochemically catalase, citrate, ONPG, and lysine decarboxylase positive, systemic, and opportunistic pathogen that is associated with gill lesions, skin hemorrhages, tail necrosis, absence of mucous cells and mucous glands in the skin, reddening of the body, and high mortality in freshwater fish [2].

Essentially, skin diseases in fish are usually associated with conditions such as poor water quality, an organic load of the aquatic environment, shipping stress, significant temperature changes, and hypoxia. However, temperature, nutrient residues, pH, Cl_2, NO_2, and NO_3 can affect the bacterial flora of the water. Gram-negative rods are responsible for fish diseases and the optimum conditions for the growth of most bacteria are pH 6–8 at 20–42 °C temperatures [3]. Bacterial adhesion to host cells is a crucial step for infection and lesion formation in fish cells. Also, it can cause pathological changes in various organs [4]. Fish skin is a barrier against infection, osmotic pressure, and mechanical injury and is the initial site of most diseases. Therewithal, the primary infection portal for pathogens in fish usually becomes the skin [5]. Excessive use of antibiotics in aquaculture in the treatment of bacterial infections in fish may cause bacterial resistance. Thus, the destruction of the indirectly beneficial microbial flora may also occur [6]. In Turkey, due to the increase in goldfish production and high demand, it has become important to investigate disease and disease factors in fish.

This study aimed to detect the presence of *C. freundii* and to determine the antibiotic susceptibility of the isolates in goldfish (*C. auratus*) with local bleeding skin lesions grown in aquariums in three regions (A: Izmir, B: Aydin, and C: Mugla) in western Turkey.

Materials and methods. ***Sampling.*** Swab samples were taken from 50 *Carassius auratus* fish skin wounds aged 8–18 months reared in the aquarium and having symptoms such as depigmentation, darkening of the skin layer, and cutaneous ulcers in different areas (A region: 5/15, B region: 4/15, and C region: 13/20). The water temperature was 25.0 ± 1 °C. Samples were transported to Aydin Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology under a cold chain in a Stuart transport medium.

Phenotypic identification of Citrobacter freundii. Swab samples were aseptically streaked on MacConkey and blood agar and incubated at 37 °C for 24–48 h. After incubation, Gram staining was performed on the bacteria grown in the culture. Gram (−), catalase (+), oxidase (−), indole (−), H_2S (+), gas formation (+), fermentation of mannitol (+), glucose (+), and lactose (+) isolates were verified as *C. freundii* by the biochemical tests [7].

Genotypic identification of Citrobacter freundii. The Sanger sequencing method was applied for the genotypic identification of isolates identified as *C. freundii* with phenotypic tests. PCR was performed using 357F and 1492R universal primers defined in the 16S rRNA gene region [8], and PCR products

were run on agarose gels to compare their sizes. Sequencing was carried out on the isolates that showed images at the target of 1100 base pairs. PCR products were purified using ExoSAP PCR Purification Kit (Thermo Fisher Scientific, USA). The purified PCR products were sequenced with the Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter). Sequence nucleotides were run on the Beckman Coulter CEQ8000 instrument. The nucleotide sequences of the PCR products were analyzed in Standard Nucleotide BLAST® NCBI Genomic Reference Sequences.

Antimicrobial susceptibility test. The antibiotic susceptibility pattern of the *C. freundii* isolates was investigated by the Kirby Bauer disc diffusion method according to the Clinical and Laboratory Standards Institute guidelines [9]. Antimicrobial susceptibility tests were performed on Muller Hinton agar incubated at 37 °C overnight by using commercial discs that contained enrofloxacin (5 µg), florfenicol (30 µg), ampicillin (2 µg), cefoperazone (75 µg), streptomycin (10 µg), sulfamethoxazole-trimethoprim (25 µg), amoxicillin-clavulanic acid (30 µg), and tetracycline (30 µg) (Oxoid, UK).

Water quality test. Aquarium water quality parameters (chlorine, acidity (pH), nitrite, nitrate, carbonate, and overall hardness) were determined using commercially available rapid test strips (JBL® Proaquates) by dipping and holding for 2–3 s in tanks. Then, measured values were compared to the standard values chart explained in the test box.

Statistical analyses. Statistical analyses were performed by the commercial statistical software SPSS version 21.0 (SPSS Inc., USA). The Chi-square test and Fisher's exact test were used to compare the relationship between antibiotic susceptibility and aquarium water quality parameters in *Citrobacter freundii* isolates. The results were evaluated at a confidence interval of 95%. A p value < 0.05 was considered statistically significant.

Results and discussions. The first deaths of goldfish were recorded at a water temperature of 23–25 °C in January-March. Fifty samples were studied in this study and 44% (22/50) of the samples were positive for *Citrobacter freundii* (including H_2S, catalase-positive, and indole, oxidase-negative). The culture was performed on swab samples taken from hemorrhagic or ulcerative skin lesions. Pink colonies (lactose fermenters) where growth was observed in 22 of 50 samples (A:5; B:4; C:13) in MacConkey medium after a 24-hour incubation period, and *C. freundii* was identified in 22 samples by phenotypic diagnostic methods. To fulfill Koch's first and second postulate (the pathogen is present in all cases of the disease and can be isolated from the diseased host) [10] we performed 16S rRNA PCR using 357F and 1492R universal primers. Then, the nucleotide sequence was performed, and the isolates were confirmed as *C. freundii* (96% similarity to *C. freundii* strain BAO2749). The antibiotic susceptibility test was performed using the standard disc diffusion method. *C. freundii* was found resistant to commonly used antibiotics such as ampicillin (59.09%),

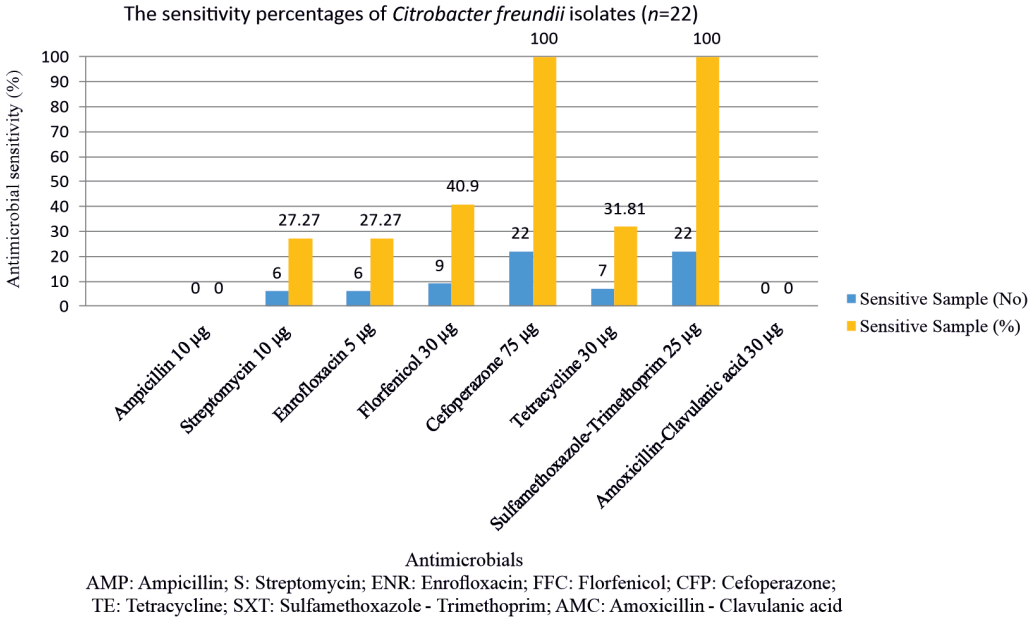


Fig. 1. The antimicrobial sensitivity percentages for *Citrobacter freundii* isolates evaluated as positive in swab samples collected from skin wounds of Goldfish ($n = 22$)

florfenicol (40.90%), amoxicillin-clavulanic acid (31.81%), and streptomycin, enrofloxacin, and tetracycline (27.27%), respectively. In contrast, they were sensitive to cefoperazone and sulfamethoxazole-trimethoprim (100%). The percentages of antimicrobial sensitivity for the *C. freundii* isolates are given in Fig. 1.

In addition, the values obtained for the other regions (A and B) were similar among themselves (Table 1).

It has been reported to be isolated from ulcers and bleeding foci in *C. auratus* species [3]. In this study, *C. freundii* (22/50; 44%) strains were isolated by phenotypic and genotypic methods from swab samples taken from bleeding skin wounds in goldfish grown in aquariums with poor water conditions. This bacterium has a wide distribution area. In addition to goldfish, *C. freundii* infections have been reported in Chinese sturgeon *Acipenser sinensis* [11], *Pseudoplatystoma reticulatum* (Brazilian catfish) [12], angelfish *Pterophyllum scalare* [13], *Labeo rohita* [14], Red snapper [15]. Cao et al. [16] isolated *C. freundii* from freshwater *Anguilla japonica* suffering from tail rot disease as the first report in China.

Antimicrobial studies have been reported worldwide on *C. freundii* in *C. auratus* [4,17]. In this study, the highest resistance rate detected against ampicillin at 59.09%, and the lowest resistance rate was against enrofloxacin and tetracyclin at 27.27%. The highest sensitivity was observed against cefoperazone and sulfamethoxazole-trimethoprim at 100%. It has been reported that *Citrobacter* spp. have been isolated from tetracycline-resistant [Tet(r)] fish [14]. However,

Table 1

Aquarium water quality parameters

NO_3 mg/l	0	10	25	50	100	250	500
A						•	
B					•		
C							•
NO_2 mg/l	0	0.5	2	5	10		
A				•			
B			•				
C					•		
GH	$< 3°$d	$> 4°$d	$> 7°$d	$> 14°$d	$> 21°$d		
A				•			
B			•				
C					•		
CH	0°d	3°d	6°d	10°d	15°d	20°d	
A			•				
B			•				
C					•		
pH	6.4	6.8	7.2	7.6	8.0	8.4	9.0
A				•			
B				•			
C						•	
Cl_2 mg/l	0	0.8	1.5	3.0			
A		•					
B		•					
C			•				

*Cl (chlorine), acidity (pH), general hardness (GH), nitrite (NO_2), nitrate (NO_3), carbonate hardness (CH), °d; degree, A: Izmir, B: Aydin, C: Mugla

tetracycline susceptibility to *C. freundii* has also been reported in some studies [12–14]. In contrast, *Citrobacter* spp. has been reported to be resistant to tetracycline [15]. In this study, *C. freundii* were found less resistant to tetracycline at a low rate. *C. freundii* is resistant to florfenicol because it is widely used in aquaculture [15], and our findings were consistent with this evaluation. It has been reported that *C. freundii* is resistant to trimethoprim and sulfonamides, and sensitive to ampicillin, amoxicillin, and streptomycin [14]. In this study, 100% sensitivity to sulfamethoxazole-trimethoprim and resistance to ampicillin, amoxicillin, and streptomycin were detected. These differences may be seen according to local antibiotic use and frequency. Cao et al. [16] and DE PÁDUA et al. [12] reported that *C. freundii* is resistant to penicillin, ampicillin, and amoxicillin-clavulanic. Our findings corroborate these conclusions.

Water quality is an important key element in the immunological control of fish diseases. Most aquarium fish live in water with a pH ranging from 5.5 to 8.5. The acceptable pH range of goldfish has been reported as 6.0 to 8.0. Also, ammonia is toxic in warmer water, at higher pH, and lower salinity, and can cause acute mor-

tality or chronic sublethal stress. Therefore, ammonia toxicity gets worse in aquariums with higher pH [15]. The highest nitrate (NO_3 mg/l), nitrite (NO_2 mg/l), chlorine, general and carbonate hardness (Cl_2 mg/l, pH, $CaCO_3$ mg/l, etc.), and pH values were reached in aquariums in zone C. The fact that bacteria isolation is the highest in this region confirms the bad conditions. Because of the measured values (Table 1), we could evaluate that the imbalance of parameters and the excess of chemical residues in the water cause injuries to the skin of goldfish and facilitate the growth of that pathogen. The results show that *C. freundii* infections can be effectively identified in aquarium habitats.

The relationship between antibiotic susceptibility of *C. freundii* isolates and aquarium water quality parameters are presented in Table 2.

There was no significant relationship between the all antibiotics susceptibility of the *C. freundii* isolates and the aquarium water quality parameters ($p < 0.05$).

All results obtained in this research are given in the heatmap (Fig. 2).

Conclusion. It was concluded that prophylactic practices should be considered for the production of healthy goldfish in aquarium breeding. In addition, the effective use of antibiotics in the treatment of goldfish in *C. freundii* diseases is important in the prevention of resistance.

Table 2

The relationship between antibiotic susceptibility and aquarium water quality parameters

			Aquarium water quality parameters				
			NO_3	150–250	500		
			NO_2	2–5	10		
Antimicrobials	Antibiotic effectiveness	Number of *Citrobacter freundii* isolates ($n = 22$)	GH	$> 7^\circ$d - $> 14^\circ$d	$> 21^\circ$d	X^2	p
			CH	6°d	15°d		
			pH	7.6	8.4		
			Cl_2	0.8	1.5		
AMP	Resistant	13		7	6	2.2	0.203
	Sensitive	9		2	7		
S	Resistant	6		3	3	0.282	0.655
	Sensitive	16		6	10		
ENR	Resistant	6		3	3	0.282	0.655
	Sensitive	16		6	10		
FFC	Resistant	8		2	6	2.2	0.203
	Sensitive	14		7	7		
CFP	Resistant	0		0	0	—	—
	Sensitive	22		9	13		
TE	Resistant	6		2	4	0.196	1
	Sensitive	16		7	9		
SXT	Resistant	0		0	0	—	—
	Sensitive	22		9	13		
AMC	Resistant	7		5	2	3.956	0.074
	Sensitive	15		4	11		

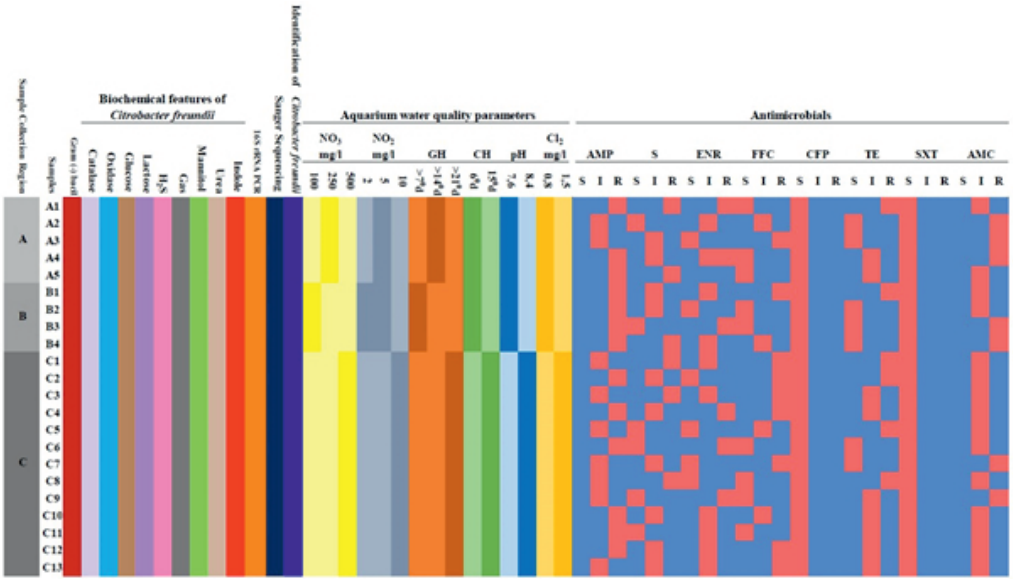


Fig. 2. Heatmap; all results for *Citrobacter freundii* isolated from skin wounds of goldfish ($n = 22$). [In the aquarium water quality parameters, dark colours represent higher values for the relevant parameter of regional samples, and light colours represent the lowest values. The red colour represents positive values and the blue colour represents negative values for antimicrobial activity.]

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Department of Microbiology
Faculty of Veterinary Medicine
Dokuz Eylül University
Kiraz/Izmir, 35890, Türkiye
e-mail: volkan.ozavci@deu.edu.tr

Department of Microbiology
Faculty of Veterinary Medicine
Aydin Adnan Menderes University
Efeler/Aydın, 09010, Türkiye
e-mails: tugba.yuksel@adu.edu.tr
skirkan@adu.edu.tr