Journal of Limnology and Freshwater Fisheries Research
limnofish
http://www.limnofish.org

Isolation of *Citrobacter freundii* from Rainbow Trout (*Oncorhynchus mykiss*) in Freshwater Cage

Mustafa TÜRE* **İlyas KUTLU**

Central Fisheries Research Institute, 61250 Yomra-Trabzon-Turkey

ABSTRACT

In this study, the bacteriological examination was done in case of disease suspect of rainbow trout (Oncorhynchus mykiss) which kept in cages in the Black Sea Region of Turkey. The bacterial agent was identified as Citrobacter braakii by rapid test kit (API 20E, Profile: 1704553), further identification was performed by 16S rRNA gene sequencing. Bacteria were identified as Citrobacter freundii by further molecular analysis. The antibacterial susceptibility of bacteria was also determined for 6 different antibiotics. The bacteria was sensitive to florfenicol, enrofloxacin, oxytetracycline and trimethoprim+sulfamethoxazole and resistant to erythromycin and amoxicillin-clavulanate. The most effective antibiotic was florfenicol. The disease was treated with florfenicol.

Keywords: Fish, cage, citrobacteriosis, antibiotic

ARTICLE INFO

RESEARCH ARTICLE

Geliş : 19.02.2018
Düzeltme : 28.05.2018
Kabul : 27.06.2018
Yayım : 17.08.2018

DOI:10.17216/LimnoFish.396496

**** CORRESPONDING AUTHOR***

mustafa.ture@tarim.gov.tr
Tel : +90 462 341 10 53

Tatlısu Üzerindeki Kafeslerde Üretilen Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss*) *Citrobacter freundii* İzolasyonu

Öz: Bu çalışmada, Türkiye'nin Karadeniz Bölgesi'nde kafeslerde yetiştirilen gökkuşağı alabalıkları (*Oncorhynchus mykiss*) hastalık şüphesi sonrası bakteriyolojik olarak araştırılmıştır. Balıklardan izole edilen bakteriyel etken, hızlı teşhis kiti ile *Citrobacter braakii* olarak isimlendirilmiştir (API 20E, Profil: 1704553). Bakterinin kesin tanımlanması ise 16S rRNA gen bölgesinin sekansı ile gerçekleştirilmiştir ve bakteri *Citrobacter freundii* olarak tanımlanmıştır. Ayrıca bakterinin altı farklı antimikrobiyale karşı antibakteriyel hassasiyeti belirlenmiştir. Bakteri florfenikol, enrofloksasin, oksitetrasiklin ve trimethoprim-sulfamethaksazol antibiyotiklerine karşı duyarlı, eritromisin ve amoksisilin antibiyotiklerine karşı ise dirençli olarak bulunmuştur. En etklili antibiyotik florfenikoldür. Hastalık florfenikol ile tedavi edilmiştir.

Anahtar kelimeler: Balık, kafes, citrobakteriosiz, antibiyotik

Alıntılama
Türe M, Kutlu İ, 2018. Isolation of *Citrobacter freundii* from Rainbow Trout (*Oncorhynchus mykiss*) in Freshwater Cage. LimnoFish. 4(2): 85-89. doi: 10.17216/LimnoFish.396496

Introduction

In recent years, the aquaculture industry has developed in Turkey, especially in the net cages at the sea and lake areas. The many dam lake were used as a suitable area for aquaculture activities in Turkey. According to the recent data, Turkey produces approximately 107013 tons of rainbow trout (*Oncorhynchus mykiss*) in a year in freshwater surround. Approximately 80% of this production is performed in freshwater cage areas (TUIK 2016).

Diseases is a primary problem in aquaculture and can severely impact economic development in many countries. The development of a fish disease is the result of the interaction of pathogen, host and the environment. Success in aquaculture depends on effective fish health management and research (Toranzo et al. 2005). Many fish diseases that affect cultured fish populations are also a threat to wild fish populations. In recent years, it takes attention that regarding the transmission of the diseases between the cultured and wild fish stocks. Some scientific research proves that many diseases affect wild fish populations before the aquaculture industry existed (Olivier 2012; Ture et al. 2018a).

According to our knowledge, there is limited disease report of fish which kept in cages of the sea or fresh water in Turkey. In a previous study, *Lactococcus garvieae* isolated from cultured rainbow

trout in freshwater and saltwater cages in the Black Sea Region of Turkey (Ture 2018b). In another study, the rainbow trout marine cages in the Black Sea Region of Turkey were examined for bacterial pathogens and diseases in 2006-2008. Many bacterial isolates were phenotypically identified in fish. One of the isolated bacteria was *Citrobacter freundii* (Kayis et al. 2009). Therefore, fish farms in freshwater and marine cage should be routinely surveyed for fish pathogens.

In this study, rainbow trout reared freshwater cages were investigated in case of the suspected disease in the Black Sea Region of Turkey. *C. freundii* was isolated from rainbow trout with moderate mortality.

Materials and Methods

Sampling and Microbiological Analysis

In the summer of 2017, about 30 moribund rainbow trout (50-100g) were sampled for bacterial examination after a suspected disease on a cage farm in the Gümüşhane province of Turkey. The water temperature was 16°C in cages. For bacterial examination, fish samples were transported to the laboratory (Central Fisheries Research Institute, Turkey, Laboratory of Fish Diseases). Liver and head-kidney samples of fish were aseptically streaked on Tryptic Soy Agar (TSA, Merck) and incubated at 25-30°C for 2 days. Following incubation, typical colonies were selected from the plate and streaked onto same media to check the purity of bacteria. The purified bacteria were biochemically characterized by following biochemical tests: Gram staining, cytochrome oxidase, catalase, and motility. Analytical Profile Index (API 20 E test) was done to identify for bacteria species biochemically (Austin and Austin, 2007; Capkin et al. 2015).

PCR Amplification and Sequencing of Bacteria

The etiologic agent was further confirmed by sequencing of the 16S rRNA genes. For this purpose, extraction of genomic DNA from bacteria was performed as a template for the PCR assay using a boiling technique described by Capkin *et al*. (2015). The optical density and concentrations of DNA were measured by RNA/DNA calculator (ND 8000, Thermo Fisher Scientific). Average, DNA concentrations were adjusted to 40 ng/µl.

The suspected bacteria was identified by a partial DNA sequencing of its 16S rRNA genes. The universal primers, fD1 (AGAGTTTGATCCTGGCTCAG) and rP2 (ACGGCTACCTTGTTACGACTT) were used for PCR amplification (Weisburg et al. 1991). DNA amplification was done with AmpliTag Gold 360 Master Mix (Thermo Fisher Scientific) in a thermocycler (Applied Biosystems) according to the manufacturer's recommendations. Analysis of PCR product was performed using electrophoresis in 1.4% (w/v) agarose gel with 1×TBE (Tris-Borate-EDTA) buffer containing SYBR Green. DNA fragment length was observed with the migration of 100-bp DNA ladder (Bio Basic) and viewed by UV transillumination.

Sequencing reaction was done using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions (In CFRI Laboratory, Turkey). ABI PRISM 3500 Genetic analyzer and POP-7 polymer were used as the separation machine and matrices. The sequence data were analyzed by ABI Prism DNA Sequencing Analysis Software v5.1. The derived nucleotide sequences were described and aligned by NCBI (www.ncbi.nlm.nih.gov/genome/microbes). The obtained consensus sequences were compared with previously published data in GenBank. Phylogenetic relationships of the isolates were estimated using the neighbor-joining (NJ) method in Mega 5.0. The phylogenetic tree was done by using the Mega program (Tamura et al. 2011).

Antibiotic Susceptibility Testing

Following identification, the antibacterial susceptibility of bacteria was also determined for 6 different antibiotics. Antibiotic susceptibility test was performed by the disk diffusion method using commercial disks (Oxoid) on Mueller Hinton Agar (MHA, Merck) plates. The test was done and described according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2014). The commercial antibiotic disks used in this study including florfenicol (FFC, 30 µg), enrofloxacin (ENR, 5 µg), erythromycin (E, 15 µg), oxytetracycline (OT; 30 µg), trimethoprim-sulfamethoxazole (SXT; 25 µg) and amoxicillin-clavulanate (AMC; 30 µg). The duplicate plates were incubated in 30°C at 24h. The isolate was characterized as susceptible or resistant to the antibiotics.

Results

The diseased fish displayed anorexia, lethargy, hemorrhage and darkened skin color externally. Also, ascites and hemorrhage in the internal organs were observed. The cumulative mortality was approximately 10% (This information belonging to the farmer). Bacteria were isolated from a total of 20 fish samples (20/30). Gram staining and oxidase negative, motility and catalase positive short rod bacteria were evaluated as *Citrobacter braakii*

(Figure 1) by API 20E test (Profil: 1704553, % ID: 99,8).

The further identification of bacteria was also performed by DNA sequencing of its 16S rRNA gene. The bacteria has the expected 1500-bp PCR amplification product was shown in Figure 2. The bacteria were identified to species level as a *C. freundii*. The sequencing results obtained from the 16S rRNA gene region were compared with different isolates that registered in the database. The 16S rRNA gene sequence of *C. freundii* strain was demonstrated to have ≥ 98% similarity with reference strains including previously published (*C. freundii* (MF428814.1), *C. werkmanii* (CP019986.1), *C. braakii* (KT764982.1) and *C. murliniae* (KU161313.1) from GenBank. The bacteria has been deposited in GenBank databases (GenBank Acc. Number: MG797671). The phylogenetic tree was shown in Figure 3.

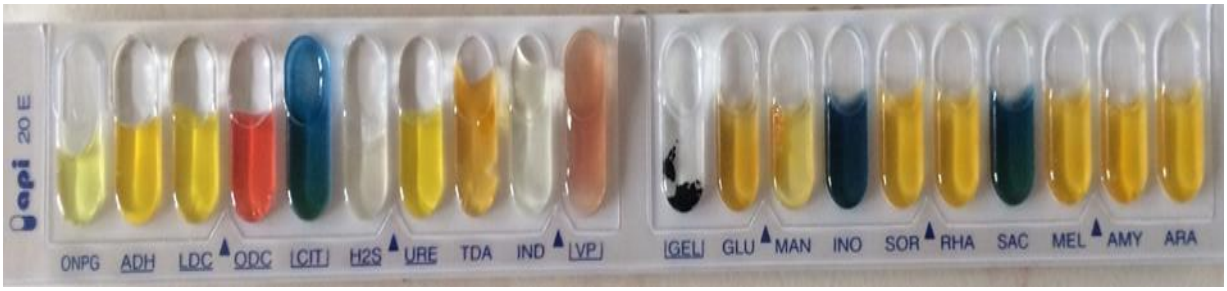

Figure 1. *Citrobacter braakii* from API 20E, (Profil: 1704553).

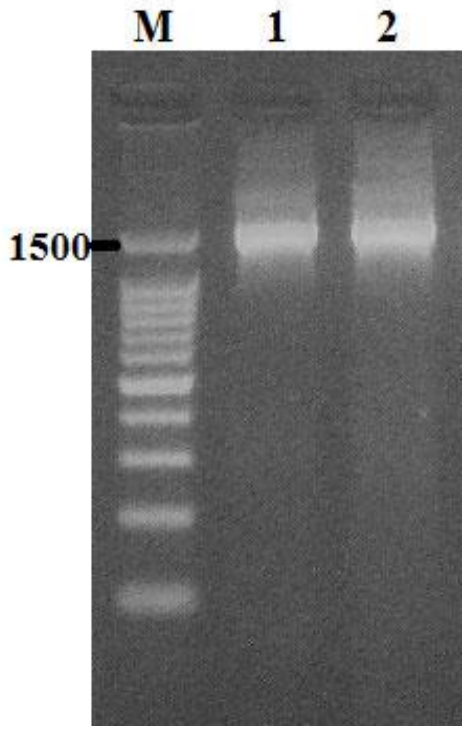

Figure 2. Gel electrophoresis image of PCR product belonging to 2 *C. freundii* strains. M: 100 bp DNA marker, 1 and 2: *C. freundii* strains.

Antimicrobial susceptibility test indicated that bacteria were sensitive to florfenicol, enrofloxacin, oxytetracycline and trimethoprim+sulfamethoxazole and resistant to erythromycin and amoxicillin-clavulanate. The most effective antibiotic was florfenicol.

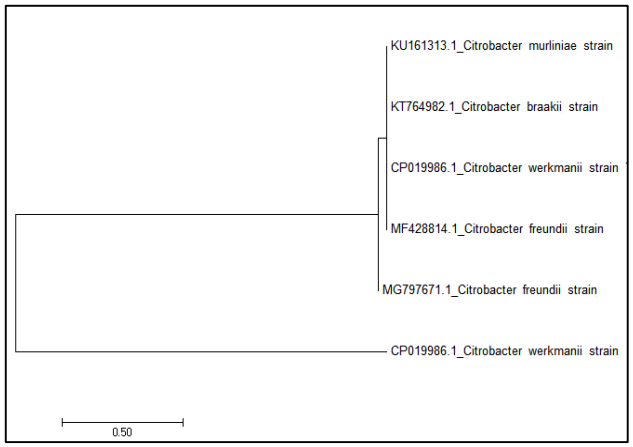

Figure 3. Phylogenetic tree based on 16S rRNA gene sequence comparison, obtained with the NJ method showing the *C. freundii* strain with related taxa.

Discussion

In the current study, rainbow trout reared freshwater cage systems were investigated in terms of bacterial fish pathogens in the Black Sea Region of Turkey. *C. freundii* isolated from rainbow trout with moderate mortality. The fish were treated with antibiotics (florfenicol).

In the previous study, typical and atypical *Citrobacter* species were defined by molecular methods in Turkey. However, the *Citrobacter* species were identified as *Citrobacter* sp. based on 16S rRNA gene sequence comparison. In contrast to the results detailed in our study, it was detected that some of the *Citrobacter* species with biochemical characteristics were atypical and showed oxidase-positive reactions (Duman et al. 2017).

C. freundii can be pathogenic to fish. Gram-negative motile bacterium *C. freundii* is an opportunistic pathogen. Hence, stress and environmental pollution play a key role in the occurrence of infection (Sanz 1991). There is limited research on the pathogenicity of *Citrobacter* genus in cultured fish. *C. freundii* was first reported as an emerging bacterial fish pathogen from aquarium sunfish (*Mola mola*) in Japan (Sato et al. 1982). This species was subsequently isolated from trout in USA and Spain with a mixed infection, and from carp in India (Sanz 1991; Karunasagar et al. 1992). In our country, *C. freundii* was isolated from rainbow trout in Trabzon but heavy mortalities were not observed (Kayış et al. 2009). In addition to, *C. braakii* belonging to *Citrobacter* genus was first reported as a fish pathogenic bacteria in Turkey (Altun et al. 2013).

Antibiotics are usually used worldwide for treating bacterial diseases in humans and animals, including fish (Zhang et al. 2009). The results of antimicrobial susceptibility test indicated that *C. freundii* strain was sensitive to florfenicol, enrofloxacin, oxytetracycline and trimethoprim+sulfamethoxazole and resistant to erythromycin and amoxicillin-clavulanate. In contrast to the results detailed in this study, Duman et al. (2017) reported that *Citrobacter* sp. isolates were resistant to florfenicol, sulfonamides, and tetracycline antimicrobials according to the broth micro dilution method. In another study, it was reported that *C. braakii* isolate was sensitive to gentamicin but resistant to enrofloxacin, florfenicol, amoxicillin, oxytetracycline and sulfamethoxazole-trimethoprim according to the disc diffusion method (Altun et al. (2013). The antibiotic susceptibility may vary according to the many factors including bacterial species and isolation area. Florfenicol is a relatively new antibiotic, and it has successfully been used for the treatment of bacterial fish diseases in Turkey (Kayis et al. 2009; Ture et al. 2016). Therefore, the fish were treated with florfenicol in this case of *C. freundii* infection.

In the current study, the bacterial strain was tried to identified by both API 20E and 16S rRNA gene sequencing methods. However, there is a poor relationship between the phenotypic and genotypic identification methods. According to the API 20E result, bacteria were evaluated as *C. braakii*. However, the bacteria were identified as *C. freundii* by 16S rRNA gene sequencing methods. It is known that molecular methods including DNA fingerprinting were more discriminative than the other methods.

In conclusion, cultured rainbow trout were investigated for bacterial fish pathogens after a suspected disease in a freshwater cage area. *C. freundii* was isolated from rainbow trout with moderate mortality.

Acknowledgment

We would like to express our appreciation to the Central Fisheries Research Institute, which founded this study.

References

Altun S, Duman M, Büyükekiz AG, Özyiğit MO, Karataş S, Turgay E. 2013. Isolation of *Citrobacter braakii* from Rainbow Trout (*Oncorhynchus mykiss*). ISR J AQUACULT-BAMID. 1(9).

Austin B, Austin DA. 2007. Bacterial fish pathogens, diseases of farmed and wild fish. 4th ed. Godalming, England: Springer-Praxis 552 p.

Capkin E, Terzi E, Altinok I. 2015. Occurrence of antibiotic resistance genes in culturable bacteria isolated from Turkish trout farms and their local aquatic environment. Dis Aquat Org. 114:127-137. doi: 10.3354/dao02852

CLSI 2014. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement, M100-S24.

Duman M, Saticioglu IB, Buyukekiz A, Altun S. 2017. Molecular characterization and antimicrobial resistance profile of atypical *Citrobacter gillenii* and *Citrobacter* sp. isolated from diseased rainbow trout (*Oncorhynchus mykiss*). J GLOB ANTIMICROB RE. 10(9): 136-142. doi: 10.1016/j.jgar.2017.05.014

Karunasagar I, Karunasagar I, Pai R. 1992. Systemic *Citrobacter freundii* infection in Common carp, *Cyprinus carpio* L., fingerlings. J FISH DIS.15(1):95-98. doi: 10.1111/j.1365-2761.1992.tb00642.x

Kayis S, Capkin E, Fikri B, Altinok I. 2009. Bacteria in rainbow trout (*Oncorhynchus mykiss*) in the Southern Black Sea Region of Turkey - a survey. ISR J AQUACULT-BAMID. 61(4):339-344.

Olivier G. 2012. Disease interactions between wild and cultured fish- Perspectives from the American Northeast (Atlantic Provinces). Bull. Eur. Ass. Fish Pathol. 22(2):103-109.

Sanz F. 1991. Rainbow trout mortalities associated with a mixed infection with *Citrobacter freundii* and IPN virus. Bull. Eur. Ass. Fish Pathol. 11:222-4.

Sato N, Yamane N, Kawamura T. 1982. Systemic *Citrobacter freundii* infection among Sunfish mola mola in matsushima aquarium. Bulletin of the Japanese Society of Fisheries. 48(11):1551-1557. doi: 10.2331/suisan.48.1551

Tamura K, Peterson D, Peterson N, Stecher G,Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. MOL BIOL EVOL. 28(10): 2731–2739. doi: 10.1093/molbev/msr121

Toranzo AE, Magarinos B, Romalde JL. 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246(1-4):37-61.doi: 10.1016/j.aquaculture.2005.01.002

TurkStat 2016. Turkey Statistical Institute. Available from http://www.turkstat. gov.tr/Start.do, Access date: 05.02.2018.

Ture M, Alp H. 2016. Identification of Bacterial Pathogens and Determination of Their Antibacterial Resistance Profiles in Some Cultured Fish in Turkey. *J VET RES*. 60(2): 141-146. doi:10.1515/jvetres-2016-0020

Ture M, Altinok I, Alp H. 2018b. Effects of Cage Farming on Antimicrobial and Heavy Metal Resistance of *Escherichia coli*, *Enterococcus faecium*, and *Lactococcus garvieae.* Microbial Drug Resistance doi: 10.1089/mdr.2018.0040

Ture M, Mısır DS, Altuntaş C, Kutlu İ. 2018a. A Survey of Some Bacterial Fish Pathogens on Whiting (*Merlangius merlangus euxinus*) in Eastern Black Sea Coast, Turkey. Turk J Fish & Aquat Sci. 18(12):42-46. doi: 10.4194/1303-2712-v18_11_09

Weisburg WG, Barns SM, Pelletier DA, Lane, DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 173(2):697-703. doi:10.1128/jb.173.2.697-703.1991

Zhang XX, Zhang T, Fang HHP. 2009. Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol. 82(3):397−414. doi: 10.1007/s00253-008-1829-z