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The Relationship between Copper Tolerance and Antibiotic Susceptibility of *Vagococcus salmoninarum* Isolated from Kılavuzlu Dam Lake

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ABSTRACT

The contamination of aquatic ecosystems by heavy metals has been identified as a significant environmental hazard for aquatic animal species, including fish. The objective of this study was to investigate the tolerance of *Vagococcus salmoninarum* bacteria to copper and the impact of copper exposure on antibiotic susceptibility. The bacterial isolates, comprising six strains, were obtained from rainbow trout reared in cage culture at Kılavuzlu Dam Lake. The initial assessment of antibiotic susceptibility was conducted using the Kirby-Bauer disc diffusion method, which was subsequently validated through the determination of minimum inhibitory concentration (MIC). The broth microdilution method was employed, with the bacterial isolates exposed to concentrations of copper sulphate ranging from 0.1 to 1 mM. The resistance to multiple antibiotics was altered to be significantly altered following exposure to copper sulphate. The minimum inhibitory concentration (MIC) value of copper was observed to be 0.4 mM against *V. salmoninarum*. It was noted that *V. salmoninarum*, which has been exposed to copper in its natural environment, has developed the capacity to withstand stress and has undergone a change in its physiological status.

Keywords: Bacteria, *Vagococcus salmoninarum*, antibiotic resistance, antibiotic susceptibility, copper

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Kılavuzlu Baraj Gölü'ndeki Alabalık İşletmelerinden İzole Edilen *Vagococcus salmoninarum*'da Bakır Toleransı ve Antibiyotik Duyarlılığı Arasındaki İlişkinin İncelenmesi

Öz: Sucul ekosistemlerin ağır metallerle kirlenmesi, balıklar da dahil olmak üzere sucul hayvan türleri için önemli bir çevresel tehlike olarak tanımlanmıştır. Bu çalışmanın amacı *Vagococcus salmoninarum* bakterisinin bakıra toleransını ve bakıra maruz kalmanın antibiyotik duyarlılığı üzerindeki etkisini araştırmaktır. Altı suştan oluşan bakteri izolatları Kılavuzlu Baraj Gölü'nde kafes kültüründe yetiştirilen gökkuşağı alabalıklarından elde edilmiştir. Antibiyotik duyarlılığının ilk değerlendirmesi Kirby-Bauer disk difüzyon yöntemi kullanılarak yapılmış ve daha sonra minimum inhibitör konsantrasyon (MİK) değeri belirlenerek doğrulanmıştır. Bakteri izolatları 0,1 ila 1 mM arasında değişen bakır sülfat konsantrasyonlarına maruz bırakılarak et suyu mikrodilüsyon yöntemi kullanılmıştır. Bakır sülfata maruz kalmanın ardından çoklu antibiyotik direncinin önemli ölçüde değiştiği bulunmuştur. Bakırın minimum inhibitör konsantrasyon (MİK) değerinin *V. salmoninarum*'a karşı 0,4 mM olduğu gözlenmiştir. Doğal ortamında bakıra maruz kalan *V. salmoninarum*'un strese dayanma kapasitesi geliştirdiği ve fizyolojik durumunda bir değişiklik olduğu kaydedilmiştir.

Anahtar kelimeler: *Vagococcus salmoninarum*, antibiyotik duyarlılık, antibiyotik direnç, bakır

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Introduction

Vagococcus salmoninarum is a Gram-positive coccobacillus that was initially described as a *Lactobacillus* strain in 1968 (Austin and Austin 2016). This strain was subsequently defined as *V. salmoninarum* (Wallbanks et al. 1990; Daly 1999).

V. salmoninarum is the causative agent of coldwater streptococcosis. An infectious disease that has been shown to result in high mortality rates and

significant economic losses on fish farms, particularly in rainbow trout and Atlantic salmon (Ghittino et al. 2004; Schmidtke and Carson 1994; Özcan and Barış 2019). However, over the last 30 years, the disease has become established in fish farmed in numerous countries across Europe and the globe (Schmidtke and Carson 1994; Michel et al. 1997; Ghittino et al. 2004; Ruiz-Zarzuela et al. 2005; Salogni et al. 2007; Didinen et al. 2011; Özcan et al. 2014). The mortality rates observed ranged from 20% to 50% per year (Nougayrède et al. 1995; Michel et al. 1997; Ruiz-Zarzuela et al. 2005; Özcan et al. 2016).

There is evidence to suggest that there is a correlation between the tolerance of bacteria to heavy metals and their resistance to antibiotics. This is a global problem that is currently threatening the treatment of infections in plants, animals and humans (Spain and Alm 2003). It is noteworthy that resistance to these metals is mediated by the same plasmid that determines resistance to drugs. The majority of these metals have recently been identified as either established or potential causes of environmental contamination (Nakahara et al. 1977). Furthermore, the presence of heavy metals in the environment has the potential to induce significant alterations in the structure and functionality of microbial communities. It has been demonstrated that both Gram-negative and Gram-positive bacteria may develop resistance to heavy metals. This resistance may serve as an indicator of the extent of contamination and the degree of exposure to bacteria and heavy metals (Kimiran-Erdem et al. 2015). Previous studies have demonstrated the development of antibiotic resistance in bacterial strains due to heavy metal contamination (Calomiris et al. 1984; McArthur and Tuckfield 2000; Stepanauskas et al. 2006; Garhwal et al. 2014).

The objective of this study was to ascertain the antibiotic susceptibility of *V. salmoninarum* isolates following exposure to copper.

Materials and Methods

Bacteria

The experiments were conducted using *V. salmoninarum* bacteria isolated from six rainbow trout farms with disease outbreaks in Kılavuzlu Dam Lake in Kahramanmaraş province of Turkey (Figure 1). The strains were stored in trypticase-soy broth (TSB) supplemented with 15% (v/v) sterile glycerol at a temperature of -80ºC. The isolate was identified by means of the polymerase chain reaction (PCR) method described by Ruiz-Zarzuela et al. (2005). For each experiment, the bacteria were inoculated on trypticase-soy agar (TSA), and one to three colonies were transferred into TSB and incubated for 48 hours at 22ºC with shaking. The cells were harvested and washed twice with sterile phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 2500 g for 10 minutes. Following the final centrifugation, the cells were re-suspended in sterile PBS and immediately employed in the experiments. The number of bacteria (expressed as colony forming units, CFU) in the suspension was determined through the implementation of 10-fold serial dilutions and the plate count technique on TSA.

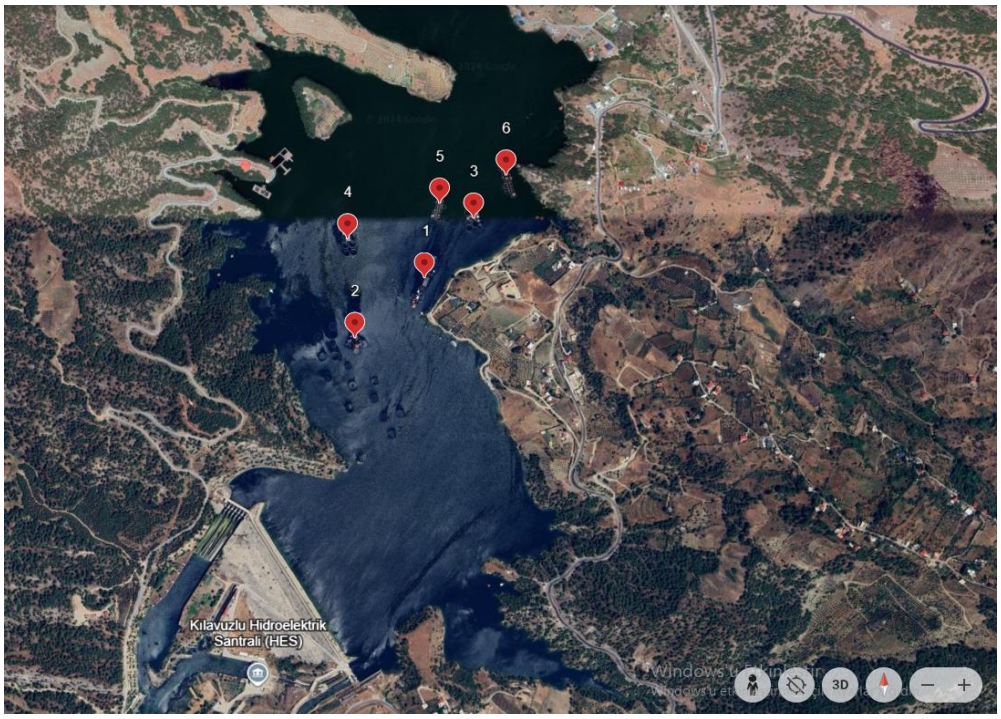

Figure 1. Map of the sampling station

Exposure to copper and minimum inhibitory concentration

To each of the triplicate tubes containing TSB, copper sulphate was added to obtain final concentrations of 0 and 0.1 to 1 mM. A suspension of *V. salmoninarum* (50 µl of $5x10^6$ CFU ml^{-1}) was added to each tube, mixed, and incubated for 48 hours at 22ºC. Following a 48-hour incubation period, 50 µl of sample from each tube was cultured in sterile agar plates containing copper in order to test bacterial recovery. The samples were incubated for 48 hours at 22 degrees Celsius, after which the number of bacterial colonies was counted.

Antibiotic susceptibility testing of the isolates

The antimicrobial susceptibility patterns were determined using Mueller-Hinton medium and commercial antimicrobial disks. The antimicrobial susceptibility patterns were determined using the following antimicrobial disks: kanamycin (30 µg), ampicillin (10 µg), enrofloxacin (10 µg), eritromycin (15 µg), gentamicin (10 µg), neomycin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), penicillin G (10 IU) and trimetoprim/sulfametaxazol (25 µg). Antimicrobial susceptibility testing was conducted in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines using the Kirby-Bauer disc diffusion method (CLSI 2011). The results for the antibiotic susceptibility patterns before and after copper exposure were recorded and subjected to analysis to ascertain any changes in the patterns.

Result

All isolates isolated from rainbow trout in six cage culture farms in Kılavuzlu Dam Lake showed high resistance to copper and the minimum inhibitory concentration (MIC) for copper was 0.4 mM. Following exposure to copper, no change was observed in the organisms' phenotypical properties. However, all isolates exhibited altered antibiotic susceptibility characteristics. Figure 2 depicts the varying inhibition levels of copper against *V. salmoninarum*. After exposure to copper, all antibiotics in this study demonstrated increased resistance in *V. salmoninarum* isolates.

The copper strains demonstrated resistance to furazolidone, gentamicin, and trimethoprim-sulfadiazine. All strains demonstrated sensitivity to kanamycin, ampicillin, neomycin, chloramphenicol, ciprofloxacin, enrofloxacin, erythromycin and penicillin.

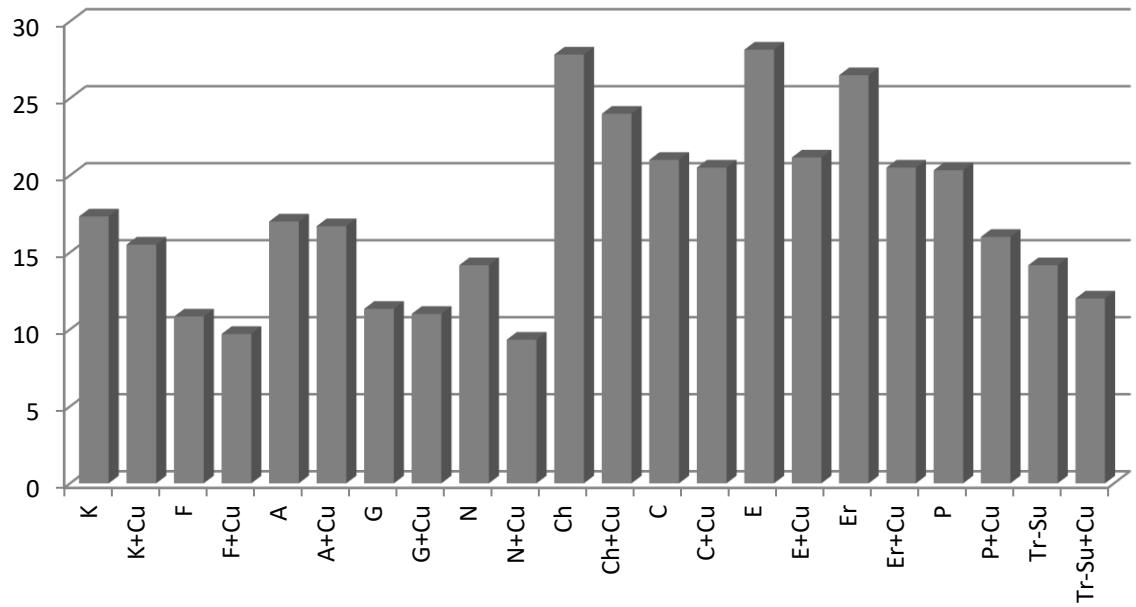

Figure 2. Antibiotic susceptibility of *V. salmoninarum* not exposure and exposure to 0.4 mM copper. K: *Kanamycin*, F: Furazolidon, A: Ampicillin, G: *Gentamicin*, N: *Neomycin*, Ch: Chloramphenikol, C: Ciprofloxacin, E: Enrofloxacin, Er: Eritromisin, P: Penisillin, Tr-Su: Trimethoprim-Sulphadiazine.

Discussion

The phenomenon of antimicrobial resistance has recently been identified as a significant global ecological issue (Levy 2001). The occurrence of antibiotic resistance in the environment may be attributed to the presence of heavy metals and other toxic substances (Schwarz et al. 2004; Baker-Austin et al. 2006).

The present study has demonstrated that kanamycin, ampicillin, neomycin, chloramphenicol, ciprofloxacin, enrofloxacin, erythromycin and penicillin are effective in controlling *V. salmoninarum*. It is therefore recommended that farmers employ these antibiotics for prophylactic and therapeutic purposes in the culture of rainbow trout. As reported by Ruiz-Zarzuella et al. (2005), strains of *V. salmoninarum* isolated from farms in the north-eastern region of Spain demonstrated sensitivity to erythromycin and oxytetracycline. In a further study, Tanrıkul et al. (2014) demonstrated that strains of *V. salmoninarum* isolated from diseased rainbow trout exhibited sensitivity to amoxicillin, ampicillin, enrofloxacin, norfloxacin, oxolinic acid and florfenicol.

In this study, the minimum inhibitory concentration (MIC) values of copper against all strains of *V. salmoninarum* were found to be identical (0.4 mM). As reported by Nies (1999) and Roane and Kellogg (1995), the MIC of copper for *Escherichia coli* was found to be 1 mM and 10 mM, respectively. Zhou et al. (2015) demonstrated that exposure to five heavy metals (Pb, Cu, Zn, Cr (VI) and Hg) resulted in antibiotic susceptibility in *Pseudomonas fluorescens*, with a reported MIC value for copper of 100 mg/ml. The MIC of bacteria resistant to copper exhibited variability between bacterial groups.

Copper has the potential to disrupt cell function in multiple ways, since several mechanisms acting simultaneously may reduce the ability of microorganisms to develop resistance against copper (Michels et al. 2005). In the present study, we also demonstrated that antibiotic susceptibility could provide valuable insights into the exposure of copper. The findings indicated that exposure to varying doses of copper resulted in an elevated level of antibiotic resistance in *V. salmoninarum*. A previous experiment demonstrated that elevated antibiotic resistance levels in bacteria exposed to heavy metals were observed (Garhwal et al. 2014). The analysis of multiple antibiotic-resistant bacteria isolated from a mariculture farm in China revealed a high prevalence of chloramphenicol-resistant isolates (Dang et al. 2006). Similarly, the co-selection of antibiotic resistance by heavy metals in freshwater microcosms and industrially polluted streams has been demonstrated (McArthur and Tuckfield 2000; Stepanauskas et al. 2006). Calomiris et al. (1984) reported a positive correlation between Cu, Pb, and Zn contaminants and multiple antibiotic resistance in bacteria in the drinking water. In the study conducted by Garhwal et al. (2014), 30 clinical isolates of Gram-negative (25 strains) and Gram-positive (5 strains) bacteria were included in the analysis. The antibiotic and lead resistance patterns were studied using the disc diffusion method. Furthermore, lead exposure was found to significantly elevate antibiotic resistance activities in clinical isolates (Garhwal et al. 2014).

In conclusion, the presence of heavy metals in the environment has been identified as a contributing factor in the development of antibiotic resistance among bacterial populations. Furthermore, prolonged exposure to these heavy metal residues may result in the emergence of a heavy metal-resistant bacterial strain. To date, there is a paucity of literature on the heavy metal resistance patterns of bacteria isolated from fish farms. It is therefore recommended that the effects of heavy metals in aquatic systems on bacterial populations be monitored.

Ethical Approval

All animal studies were approved by the Animal Ethics Committee of KSÜZİRHADYEK and Research Institute (Protocol number: 2013/5-1).

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