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Determination of antimicrobial resistance gene variations using Tet and Str genes in freshwater fish species

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ABSTRACT

With the rapid development of aquaculture, antibiotics are widely used for prophylactic and therapeutic purposes to reduce economic losses caused by disease outbreaks. Considering that most antibiotics applied to humans and animals are partially metabolized and not eliminated, it is evident that these antibiotic residues can have negative effects on natural aquatic organisms after reaching the receiving environment, such as rivers and reservoirs. Therefore, it is believed that this indiscriminate use of antibiotics is now beginning to affect aquatic organisms in natural environments, outside of closed environments. In this study, tissue samples were taken from seven fish species in the Fırat River. Specific primer sets were designed for Tet and Str genes, which are known to play a role in antibiotic resistance mechanisms. The changes in gene expression levels were then examined. The results showed that the expression levels of Tet and Str genes associated with antibiotic resistance were more than two-fold higher in *Cyprinus carpio* and *Chondrostoma regium* species compared to a control group that did not use antibiotics. A moderate expression level was observed in *Capoeta trutta*, *Acanthobrama marmid*, *Capoeta umbla*, and *Barbus grypus* species. In addition, in *Luciobarbus mystaceus* species, the Tet gene was expressed at a meaningless level, while the Str gene was downregulated. Therefore, it is believed that this species may not have encountered or has previously encountered antibiotics at low levels, leading to the control levels of the resistance mechanism.

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Introduction

The aquaculture industry has shown five times more growth in recent years to meet the demand for high-quality protein (1). However, infectious, and non-infectious diseases in fish pose serious problems for their production and development (2-4). The fish's resistance decreases as the balance between the environment and host is disrupted, making them more susceptible to diseases. As a result, disease progression increases, leading to higher mortality rates (5). While factors such as poor water quality (pollution, mixing with sewage water, temperature, pH, oxygen levels in the water, etc.), unbalanced nutrition, and stress may be predisposing factors, deaths occur more frequently because of infectious diseases caused by bacteria, viruses, parasites, and fungi (6).

As a result of the widespread use of antimicrobial agents, multiple drug-resistant bacterial pathogens have been found to be present in both terrestrial and aquatic ecosystems (7). In addition, it has been reported that there are high levels of antibiotic-resistance genes in and around aquaculture areas, even in the absence of selective pressure from antibiotics (8-10). Even if antibiotic use is discontinued, the fish recirculating water system is a determinant indicator of the spread of antimicrobial resistance genes, in conjunction with water quality parameters such as pH, COD, TP, TAN, Mg, Ca, Fe, and Zn, coupled with heavy metals and microbial community shifts (10-11). Various studies have shown that aquatic culture environments are significant reservoirs of antibiotic-resistance genes (1, 12). Fecal contamination of surface water, river water, wetlands, and even drinking water has led to the spread of such resistance (13-16).

Antibiotic-resistant bacteria (17) and antibiotics (18) being released into the environment in varying amounts has raised concerns about the emergence of antibiotic-resistance genes (ARGs) in recent years (19). While antibiotic resistance is a long-standing and naturally occurring phenomenon in bacterial communities (20), the intensification of anthropogenic activities has increased the prevalence of antibiotic-resistant bacteria and their ARGs (21).

ARGs can be transferred and disseminated between different bacterial genera via horizontal gene transfer (HGT) through mobile genetic elements (MGEs), which can enhance antibiotic resistance in bacterial communities (1, 22). Currently, ARGs are considered emerging pollutants that can reduce the therapeutic effectiveness of antibiotics and pose a risk to the environment and human health. Therefore, antibiotic-resistance genes have become a major public safety concern (1).

The study focused on fish from the Cyrinidae family, which has a wide range of species and is important for the Fırat River system. *C. carpio*, one of the most widely cultivated species in the world, was used as a reference species because it had not been exposed to any diseases or drugs. Studies have shown the negative effects of antimicrobial

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resistance genes on natural fish in waters where aquaculture facilities are densely located.

Research on antibiotic resistance, a global issue, highlights the scientific community's interest in this topic. Therefore, it is important to evaluate the emergence and prevalence of antibiotic resistance genes. In this study, the expression levels and differences of the Tet and Str genes, which are commonly used and play a role in antimicrobial resistance mechanisms (23), were investigated in seven different fish species sampled from two different regions of the Fırat River. The relationships between environmental factors such as water pollution, the presence of nearby facilities, excessive use of antibiotics, contaminated water entering the river, and global changes in water were also examined in relation to the presence of antimicrobial resistance in fish.

Materials and Methods

Sampling A total of 70 fish belonging to 7 different fish species (*Barbus grypus*, *Chondrostoma regium*, *Capoeta trutta*, *Acanthobrama marmid*, *Capoeta umbla*, *Cyprinus carpio*, *Luciobarbus mystaceus*) were obtained from two regions (Figure 1) through fishermen: one region with intense fish farming activities (9th Region of the Fırat River (1)), and the other region with almost no fishing activities (Kömürhan Region (2)). Five fish of each species were collected. Disease-free and antibiotic-free *C. carpio* fish were also obtained from the Elazığ Agriculture Directorate and used as a control group. Muscle tissue (50 mg) was taken from the area below the dorsal fin of each fish and placed into centrifuge tubes. Then, 300 µL RNA later was added to each tube, and the tubes were stored at -20°C.

Water parameters in the areas where fish were obtained from the Fırat river were measured as follows: In region 1, the water temperature was measured as 9.6°C, pH as 7.2, and oxygen level as 8.3 mg/L. In region 2, the water temperature was measured as 10°C, pH as 6.8, and oxygen level as 7.8 mg/L.

RNA isolation

Total RNAs were isolated from 25 mg of muscle tissue from each of the different fish species (7 species, n=5x2) using the RNeasy Mini Kit (Qiagen) and the Qiacube instrument, following the manufacturer's instructions.

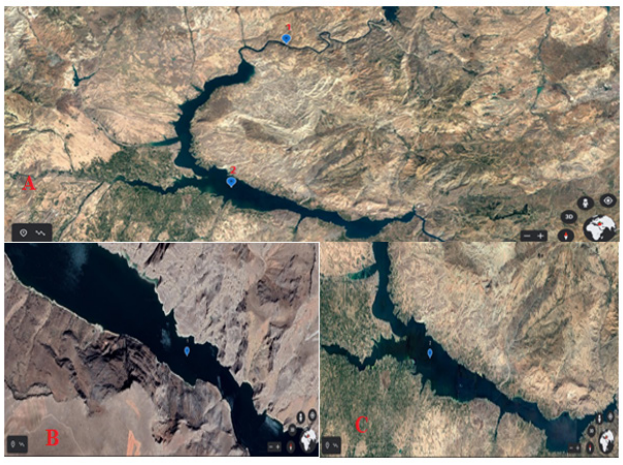

Figure 1. Sampling locations of fish specimens in the study. A: Regions 1 and 2, B: Region 1, C: Region 2 (24).

cDNA synthesis

Isolated RNAs were diluted to a concentration of 1 ng/µl for each sample using Real-Time PCR, and then cDNAs were synthesized using the RT2 First Strand Kit (Qiagen). The cDNA synthesis protocol was as follows: 5 µl of RNA was mixed with 2 µl of GE buffer and incubated at 42 °C for 5 minutes. The reaction was then completed by adding 4 µl of 5X Reaction Buffer, 1 µl of Primer (Primer Array System, UK), and 2 µl of Reverse Transcriptase Mix to bring the total volume to 20 µl. The final concentration was achieved by incubating at 42 °C for 15 minutes and then at 95 °C for 5 minutes in PCR (25).

Primer designs specific to antibiotic-resistance genes

Specific primer designs for antibiotic-resistance genes Gen ID: 109080013 sequences were used for the Tet gene. The obtained primer sequence was synthesized as follows: 5'-TGGTATGGGGTTGCATGAGT-3' (20 bp length, 59°C Tm, 50% GC content, 0.00 any, 0.00 3'th, 0.00 hairpin, 0.00 product size: 249 bp) for the forward primer and 5'-AAAGATCTGGACTGCAGGCT-3' (20 bp length, 59.01°C Tm, 50% GC content, 0.00 3'th, 0.00 hairpin, product size: 249 bp) for the reverse primer. Gene ID: 109048221 was used for the Str gene. The obtained primer sequence was designed as follows: 5'-CTCTCTA-TAGGTGCCCGGTG-3' (20 bp length, 59.04°C Tm, 60% GC content, 0.00 any, 0.00 3'th, 0.00 hairpin, 0.00 product size: 229 bp) for the forward primer and 5'-CGATGAG-TGAGAAAGCTGCC-3' (20 bp length, 58.99°C Tm, 55% GC content, 0.00 any, 0.00 3'th, 0.00 hairpin, 0.00 product size: 229 bp) for the reverse primer (Figure 2).

Real-Time PCR analysis

For the determination of antibiotic resistance gene expression differences, the primer design was performed for the Tet gene using Gene ID: 109080013 sequence data and for the Str gene using Gene ID: 109048221 sequence data. Real-Time PCR analysis protocol was created using expression primers for Tet and Str genes. A total of 20 µl volume was prepared by adding 12.5 µl SybrGreen qPCR Master Mix, 1 µl Forward and Reverse Assay Primer, 6.5 µl H2O, and 5 µl cDNA. The PCR protocol included denaturation at 95 °C for 10 min, annealing at 94 °C for 15 s, and 60 °C for 30 s for 40 cycles (25).

Statistical analysis

Using the ct values obtained after the PCR analysis, gene expression levels were determined by the $2^{\Delta\Delta ct}$ formula and binomial data were obtained. The expression levels of resistance-associated genes were determined using the ANOVA test with $p<0.05$ values and graphical results obtained (25).

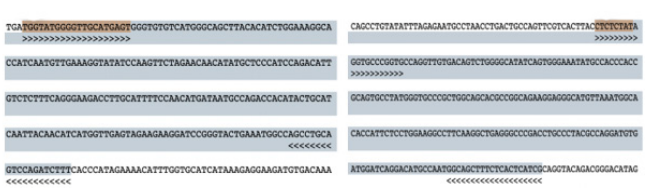

Figure 2. Primer and PCR amplicon size region of expression primers (Left: Tet Gene, Right: Str Gene).

Results

According to the Tet gene expression results, compared to the control groups, gene expression levels were upregulated in the first region samples of *C. carpio* species by 2.6-fold, in *C. regium* species by 2.2-fold, in *C. trutta* species by 1.6-fold, in *A. marmid* species by 1.9-fold, in *C. umbla* species by 1.6-fold, and in *B. grypus* species by 1.5-fold. In *L. mystaceus* species, the gene expression level was the same as the control group with a 1.1-fold expression level, and no significant change in expression was observed. The Tet gene expression level graphs for fish species collected from the first region are given below (Figure 3A).

In the second region samples, gene expression levels were upregulated in *C. carpio* species by 2.4-fold, in *C. regium* species by 2.1-fold, in *C. trutta* species by 1.4-fold, in *A. marmid* species by 1.6-fold, in *C. umbla* species by 1.4-fold, and in *B. grypus* species by 1.4-fold. In *L. mystaceus* species, the gene expression level was the same as in the control group, and no significant change in expression was observed. The Tet gene expression level graphs for fish species collected from the second region are given below (Figure 3B).

According to the expression results of the Str gene; in the samples collected from the first region, the gene expression level was overexpressed by 3.2-fold in *C. carpio* species, upregulated by 2.4-fold in *C. regium* species, upregulated by 1.7-fold in *C. trutta* species, upregulated by 2.2-fold in *A. marmid* species, upregulated by 1.7-fold in *C. umbla* species, upregulated by 1.65-fold in *B. grypus* species, and downregulated by 0.9-fold in *L. mystaceus* species. The Str gene expression graph from the samples collected from the first region is given below (Figure 4A).

According to the expression results of the Str gene; in the samples collected from the second region, the gene expression level was expressed by 2.1-fold in *C. carpio* species, upregulated by 1.3-fold in *C. regium* species, upregulated by 1.36-fold in *C. trutta* species, upregulated by 1.6-fold in *A. marmid* species, upregulated by 1.25-fold in *C. umbla* species, upregulated by 1.25-fold in *B. grypus* species, and downregulated by 0.75-fold in *L. mystaceus* species. The Str gene expression graph from the samples collected from the second region is given below (Figure 4B).

According to these results, the control groups yielded normal values in the study. The expression levels of the Tet and Str genes reached their highest levels in the *C. carpio* species. Significant expression was also observed in *C. regium*, *C. trutta*, *A. marmid*, *C. umbla*, and *B. grypus* species, but their expression levels were close to the control group. In *L. mystaceus*, the Tet gene was found to have an insignificant expression level, and in terms of the Str gene, they were down-regulated compared to the control group (Figure 5).

In the study, it was observed that the expression levels of antibiotic-resistance genes associated with Tet and Str genes were more than 2-fold higher in *C. carpio* and *C. regium* species compared to a control group without antibiotic use. In *C. trutta*, *A. marmid*, *C. umbla*, and *B. grypus* species, a lower but significant expression level

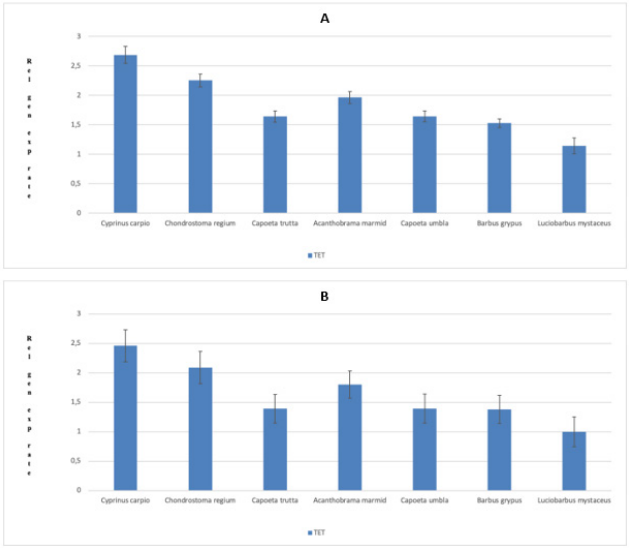

Figure 3. Expression levels of the same fish species collected from the first region (A) and the second region (B) compared to the control group in terms of Tet gene expression.

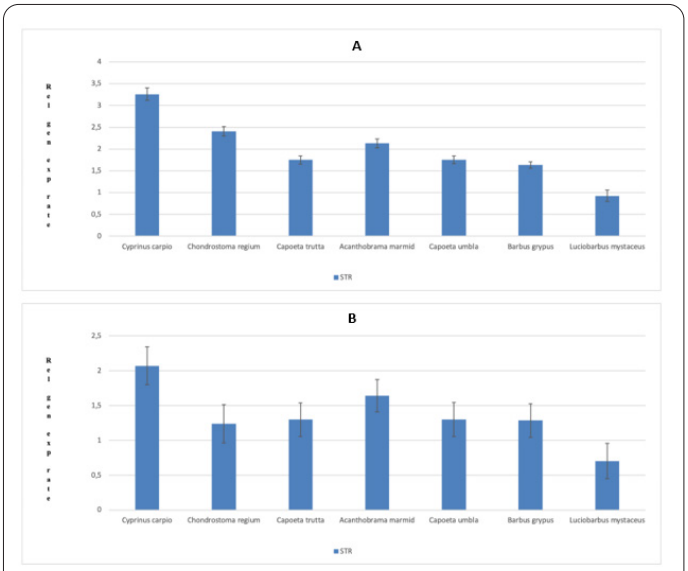

Figure 4. Expression levels of the same fish species were taken from the first (A) and second (B) regions of the Str gene, compared to the control group.

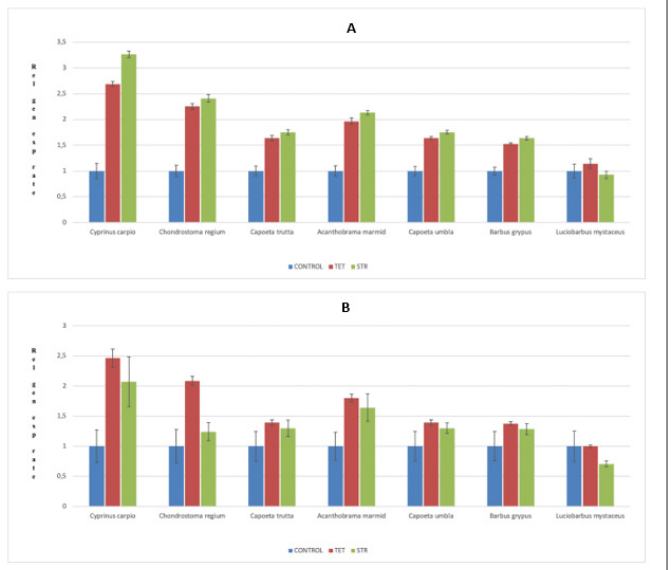

Figure 5. Expression levels of the same fish species were taken from the first (A) and second (B) regions of both genes compared to the control group.

was observed. In addition, in *L. mystaceus* species, the Tet gene was expressed at a nonsignificant level, and the Str gene was down-regulated. Therefore, it is thought that this species either did not encounter or encountered antibiotics in the past at low levels and reached the control levels of the resistance mechanism. The reason for the different change rates in these expression levels is thought to be due to both the dilution of antibiotic residues used in the natural environment by rivers and the potential difference in exposure of the fish species used extensively in the study to antibiotics (Figure 5).

Discussion

In the study where Tetracycline, Mg^{+2}, Ca^{+2}, Na^{+1}, and K^{+1} parameters were evaluated in terms of turbidity for the characterization of water quality in the tributaries of the Fırat-Dicle basin, it was stated that Tetracycline, Mg^{+2}, and Ca^{+2} was observed intensively. They indicated that the reason for the intense appearance of these parameters is due to farming and agricultural factors. They also reported that tetracycline was found intensively in these regions due to intensive animal husbandry activities (26). This study conducted by us is similar to the study where we detected the presence of antimicrobial resistance genes in two separate regions on the Fırat River system and proved the presence of antibiotics. When the same fish species were compared in our study, it was observed that the resistance of genes was 1-2 degrees higher on average in the first region where aquaculture facilities were predominantly located. At the same time, more agricultural activities are carried out in the areas close to the first region. We can conclude that the recent changes in temperatures and drought may also be effective in the emergence of resistance genes. We can also conclude that in waters mixed with the river water without treatment, especially with the decrease in rainfall, more accumulation may have occurred in the waters.

In the study conducted to detect heavy metals in water and *C. trutta* fish in the Karakaya Dam Lake region on the Fırat River, Cu, Zn, and Fe heavy metals were reported to be present. They also reported that heavy metal accumulation in fish is more than in waters (27). We can think that there is an effect of heavy metals formed in various ways because of water pollution in the detection of resistance genes in *C. trutta*, which is one of the fish species we used in common with this study. In the same region, in the study conducted to detect heavy metals in *L. esocinus* fish, they also found Cu, Zn, and Fe heavy metals and reported that they were more in fish (28).

Çapkin et al. (19) reported in their study investigating antibiotic resistance and the presence of resistance genes in trout that TetA was the second most common antibiotic resistance gene. They emphasized that the multiple antibiotic resistance index was above the critical threshold for almost all of the isolated bacteria. They suggested that their findings could play an important role in the development of antibiotic resistance in aquatic environments and the spread of resistance genes among bacteria. Our study found similar results in terms of the widespread prevalence of the Tet gene.

Cizek et al. (29) used Tet (A and E) genes to determine the antimicrobial susceptibility of *Aeromonas* spp isolates obtained from carp in their study. They detected Tet genes in 40% of their samples and stated that TetE was dominant. Our study is similar in terms of the detection and dominance of Tet genes. In a comparative study of the antibiotic resistance of bacteria isolated from different aquatic systems, 7 different fish species were used, and when the multiple antibiotic resistance indexes of the bacteria were analyzed, it was found that the MAR index was above the threshold value of 0.2 in all systems (30).

In a study on the detection of antibiotic resistance genes in duck and fish farming ponds, 17 types of antimicrobial resistance genes were detected and TetA was noted as a potential indicator for the abundance of tetracycline resistance genes in these classes (12). Our study also shows similarities in terms of the detection and prevalence of the Tet gene. In their study to detect antimicrobial resistance genes in the intestines of pigs and carp, Libisch et al. (31) detected the TetE resistance gene only in carp. Our study also shows parallelism by detecting the presence of the same gene in muscle tissue using carp as the fish species. While our study used muscle tissue, their study examined the abundance of bacterial communities and antimicrobial resistance genes using four antimicrobial resistance genes in intestinal mucus and determined the bacterial community composition associated with it. In contrast to our study, they used blaTEM, ermB, qnrS, and sulI antimicrobial resistance genes and detected only SulI and QnrS genes in carp, while Tem genes were found in brown trout and mustache fish. They explained that their findings indicate that anthropogenic activities not only increase the pollution of aquatic environments but also contribute to the emergence and spread of antibiotic resistance in organisms living in these environments (21).

In their study titled "Environmental antibiotic resistance poses a great threat to human health: An increasing concern," Xiong et al. (32) investigated antibiotic concentrations and antibiotic resistance genes in water, sediment, and freshwater fish in China, highlighting the potential risks to human health posed by the presence of potentially resistant and pathogen-associated taxonomic groups in fish ponds. While the study did not investigate antibiotics in water, the increased antibiotic resistance genes observed between fish from different regions provided insight into the surrounding aquatic environment.

In their study, "Composition and distribution of bacterial communities and antibiotic resistance genes in fish from four aquaculture systems," Zhang et al. (1) emphasized the potentially harmful effects of fish-related antibiotic resistance genes (ARGs) on food safety and human health through the transfer of the food chain. In this study, ARGs and bacterial communities in the fish gut, mucosal skin, and gill filaments were comprehensively evaluated in four different aquaculture systems using hybrid aquaculture products. The research showed that 9 ARGs were detected in the gut and mucosal skin and 6 ARGs in the gill filaments. Our study showed similar characteristics in that the presence of antibiotic-resistance genes was detected, unlike the use of different tissues and different ARGs. Additionally, the presence of Tet and Str ARGs in economically valuable fish species in our study supports the notion that antibiotic resistance could have harmful effects on food safety and human health.

In their study on duck-fish polyculture farming, Zhou et al. (33) emphasized the significant and positive correlation between Cu and Zn, as well as their relationship with antibiotic-resistance genes. Our study aims to detect

antimicrobial resistance genes in fish and is similar to the use of Tet genes. Previous studies conducted in the Fırat River (27-28, 34) have found heavy metal presence in fish, which can be associated with the antimicrobial resistance genes used in our study and guide future research.

In their study on the variation of antibiotic resistance genes between river water and fish, Zhou et al. (35) noted that antibiotic resistance genes are particularly high in summer and autumn. Our study aligns with this finding as our samples were obtained in October when antibiotic-resistance genes were identified. Their data also suggests that bacteria and ARGs in fish and water can be linked, with ARGs using bacteria as a medium for transmission between fish and water.

Our study determined the prevalence of Tet and Str genes in fish, which are commonly used in gene expression studies. It was found that *C. carpio* had the highest resistance among the seven species examined, although it was not at dangerous levels. This study demonstrates the presence of antimicrobial resistance genes in fish due to global changes such as climate change, pollution in water, and the presence of aquaculture facilities. Our research can fill the gaps in this field and provide direction for future studies.

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Interest conflict
Conflict of interest The authors declare that they do not have any conflict of interest in the research work and publish the article.

Author's contribution
Removing samples from fish SD, laboratory procedures SD and ŞÖ, statistics and analyzes ŞÖ, writing of the article and evaluation of results SD and ŞÖ.

Ethical approval
In order to conduct the research, Fırat University Animal Experiments Local Ethics Board numbered 2022/06; "Since there is a study with dead animal texture, there is no need to obtain an ethical committee certificate." certificate.

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