RESEARCH ARTICLE

IJVAR International Journal of Veterinary and Animal Research
E-ISSN: 2651-3609 3(2): 25-28, 2020

An Investigation of Food-Borne Bacteria Detected in Fresh Water Fish and Their Antimicrobial Resistance Genes in Turkey

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Received: April 04, 2020
Accepted: May 30, 2020

Abstract
The intense and unconscious use of antibacterial drugs or vitamins to protect fisheries production, fight against fish diseases and promote growth in fish can lead to the emergence of microorganisms resistant to antibiotics and serious public health problems through consumption. Little is known about the food-borne pathogens detected by molecular methods in fish which are grown in fish farms in Turkey and about the antimicrobial resistance genes of food-borne pathogens. In this study, DNA isolations were first made from collected fish muscle samples. Extended-spectrum beta lactamase (blaCTX-M, blaSHV and blaTEM) and carbapanemase (blaKPC, blaOXA-48, blaVIM, blaIMP) genes were investigated in fish tissue and DNA was found to be positive with qPCR. As a result of isolation in 50 fish samples, 70 bacteria were detected by qPCR. Of the 11 samples with *E.coli*, 3 were identified as *E.coli* O157:H7. In the fish samples, *Aeromonas* spp. was detected at the highest percentage (46%), followed by *P.shigelloides*. when the distribution of the extended-spectrum beta-lactamase (ESBL) genes were examined with qPCR in the samples with positive results, blaCTX-M was detected in 22 samples, teblaSHV in 19 samples and blaTEM genes were detected in 10 specimens. In 17 samples of *P. shigelloides* positive, no antimicrobial resistance gene was detected. When the distributions of the carbapenemase genes were found in the positive samples, the blaKPC gene was found to be positive in 6 samples and *Aeromonas spp*. was detected in 5 of these samples. The aim of this study was to analyze the status of food-borne pathogens detected in trout species in the Euphrates River and to analyze and demonstrate the antimicrobial resistance genes of these bacteria using the qPCR method. Food-borne pathogens and antimicrobial resistance genes such as ESBL and carbapanemases, were determined by molecular methods. To be able to reduce the currently increasing rates of antimicrobial resistance, the unconscious use of drugs should be reduced in the treatment of aquatic diseases and the data of the resistance genes in the pathogens detected in fish should be followed molecularly and epidemiologically.

Keywords: Antibacterial Resistance, Euphrates River, Fish, Real-Time PCR

INTRODUCTION

Conservation in aquaculture production, the intense and unconscious use of chemotherapeutic drugs or vitamins to support fish growth, results in the emergence of antibiotic-resistant microorganisms (antibiotic resistance), residual formation in residual food (residues), target organ / tissue damage and and a risk of toxicity in the host such as the deterioration of the fauna in the aquatic environment, all of which are harmful to the environment and human health over time (Yalınkılınç and Kum 2016; Kum et al., 2008). The use of antibiotics in veterinary medicine has improved animal health by reducing the incidence of mortality and infectious diseases (Hou et al., 2015).

In the fish farming sector, the widespread use of antibacterial agents in the treatment of bacterial diseases has been associated with the development of antibacterial resistance in many fish pathogens (Depaola, 1995). In addition, bacteria and antibacterial waste (such as in rivers), caused by humans and animals, have bacteria with antibacterial resistance genes, which are transferred to other bacteria in the flora of this water resource (Daş and Atmaca, 2015). After the consumption of contaminated fish with antibacterial resistant pathogens, these resistant bacteria can be transferred by horizontal gene transfer through interaction with bacteria in the host (Barber and Swygert 2000). In addition, the efficacy of antibacterial drugs can be reduced by the re-use of water with these antibacterial resistance genes by animals or humans (Aravena et al., 2012).Therefore, the World Health Organization has recommended the reduction and limitation of the use of important antibiotics used in human medicine for prophylaxis and growth promotion in veterinary medicine, especially in farm animals and aquaculture, in order to minimize antibacterial resistance (WHO CIA; WHO, 2017). However, resistance to extended-spectrum beta-lactamase in enteric bacteria has increased rapidly both in Turkey and throughout the world (Gür et al., 2009). It is very important to know the antibiotic resistance profiles of the strains for the successful treatment of possible infections in humans (Collignon et al., 2016). Due to the possible transfer of antibiotic resistance genes in fish consumed as food in terms of public health, identification of these genes and identification of bacterial phenotypes and investigation of genetic resistance mechanisms is a necessity (Aslan et al., 2009). In the light of all this information, the aim of this study was to determine the frequency of some pathogenic bacteria and the antibacterial resistance genes of the trout species, which have high economic value in the Euphrates River, using the real-time PCR method.

MATERIALS AND METHODS

Evaluation was made of 50 samples of fish from the Euphrates River (C. carpio, C. luteus, C. umbla, C. macrostomum, C.gibelio, C. trutta, C. auratus, C. kais, L. abu, L. regium, A. grypus, A. marmid, A.mossulensis). The fish samples were collected from fishermen at different points of the Atatürk Dam on the Euphrates River and were

taken to the Laboratory of Pharmacology and Toxicology in the Faculty of Veterinary Medicine. Approximately 4-5 g of epoxy muscle of the fish was washed with distilled water and dried on filter paper. These samples were then weighed, packed into polyethylene bags and stored at -20 ° C until analysis.

DNA isolations were performed from the collected 50 fish samples using the High Pure PCR template preparation kit (Roche Diagnostics GmBH, Mannheim, Germany) according to the manufacturer's instructions. The DNA samples were stored at -80˚C until qPCR processing. DNA was studied in real-time PCR using the primers in Table 1. The LightCycler 480 Sybr Green Master I kit was used with the primers in accordance with the manufacturer's instructions. The primer was added to each reaction with a final volume of 0.5 µM. The total reaction volume was 20 µL and the template DNA was used as 5 µL.

Extended spectrum beta-lactamase (blaCTX-M, blaSHV and blaTEM) and carbapanemase (blaKPC, blaOXA-48, blaVIM, blaIMP) genes were investigated in fish tissue DNA with positive qPCR. Carbapenemase genes in these DNA samples were identified using the LightCycler 480 II system (Roche Diagnostics GmbH, Mannheim, Germany) multiplex real-time PCR method using the Lightmix modular kit (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions; blaKPC, blaOXA-48, blaVIM, blaIMP genes were studied. To determine the extended spectrum beta-lactamase genes BlacTX-M, blaSHV and blaTEM, blaCTX-M, blaSHV and blaTEMqPCR test kits (Techne, Germany) were used respectively, in the LightCycler 480 system separately.

Table 1. QPCR primers used to detect pathogens in fish samples

Pathogen	Primary	Oligonucleotide Sequence	Region	Reference
E. coli	*E.coli-F*	GACCTCGGTTTAGTTCACAGA	malBpromoter	Wang et. al, 1997
	E.coli-R	CACACGCTGACGCTGACCA		
E. coli O157:H7	O157-F	GTAGGGAAGCGAACAGAG	hlyA	
	O157-R	AAGCTCCGTGTGCCTGAA		
Shigella spp.	Shi-F	CTTGACCGCCTTTCCGATAC	ipaH	
	Shi-R	CAGCCACCCTCTGAGAGTA		
Vibrio vulnificus	VV-F	CTCACTGGGGCAGTGGCT	Cytolysin	
	VV-R	CCAGCCGTTAACCGAACCA		
Vibrio parahemolyticus	VP-F	GAATTCGATAGGGTGTTAACC	genomic DNA	
	VP-R	ATCCTTGAACATACGCAGC		
Salmonella spp.	Sal-F	CGTTTCCTGCGGTACTGTTAATT	invA	Lee et. al, 2008
	Sal-R	AGACGGCTGGTACTGATCGATAA		
Plesiomonas shigelloides	PS-F	GCGAGCGGGAAGGGAAGAACC	hugA	Herrera et. al, 2006
	PS-R	GTCGCCCCAAACGCTAACTCATCA		
Aeromonas spp.	A16SF	GGGAGTGCCTTCGGGAATCAGA	16S rRNA	Robertson et. al, 2014
	A16SR	TCACCGCAACATTCTGATTTG		

RESULTS

As a result of isolation in 50 fish samples, 70 bacteria were detected with qPCR. Of 11 samples with *E. coli*, 3 were determined as O157:H7. *Aeromonas spp.* was found to be high in fish samples (46%), followed by *P. shigelloides* (Table 2).

Table 2. Distribution of pathogens in fish samples detected positive with qPCR

Pathogen	N	%
Escherichia coli w/o O157:H7	8	16.00%
Escherichia coli O157:H7	3	6.00%
Salmonella spp.	9	18.00%
Shigella spp.	0	0.00%
Plesiomonas shigelloides	17	34.00%
Aeromonas spp.	23	46.00%
Vibrio vulnificus	2	4.00%
Vibrio parahaemolyticus	8	16.00%

The distribution of extended spectrum beta-lactamase genes was examined and the results are shown in Table 3.

Table 3. Distribution of qPCR extended spectrum beta-lactamase (ESBL) genes in positively detected samples

Pathogen	N	blaCTX-M	blaSHV	blaTEM
Escherichia coli w/o O157:H7	8	6(75%)	4(50%)	3(37.5%)
Escherichia coli O157:H7	3	2(66.7%)	1 (33.3%)	1(33.3%)
Salmonella spp.	9	1(11%)	5(55.5%)	2(22%)
Shigella spp.	0	0	0	0
Plesiomonas shigelloides	17	0	0	0
Aeromonas spp.	23	13(55.6%)	9 (39%)	0
Vibrio vulnificus	2	0	0	1
Vibrio parahaemolyticus	8	0	0	3
Total	70	22	19	10

The distribution of carbapenemase genes was examined and the results are shown in Table 4.

Table 4. Distribution of qPCR-polysapapenemase genes in positive samples

Pathogen	N	blaKPC	blaOXA-48	blaVIM	blaIMP
Escherichia coli w/o O157:H7	8	1 (12.5%)	1 (12.5%)	0	0
Escherichia coli O157:H7	3	0	0	0	0
Salmonella spp.	9	0	0	0	0
Shigella spp.	0	0	0	0	0
Plesiomonas shigelloides	17	0	0	0	0
Aeromonas spp.	23	5 (21.8 %)	0	0	0
Vibrio vulnificus	2	0	0	0	0
Vibrio parahaemolyticus	8	0	0	0	0
Total	70	6	1	0	0

DISCUSSION AND CONCLUSION

Low-dose and long-term antibiotic use in food-bearing animals cause resistant bacteria. The transfer of such antibiotic resistance from animals to humans occurs through animal food containing antibiotic-resistant MOs consumed by humans without sufficient heat treatment. It can also be caused by the use of animal fertilizers or animal faeces which contain resistant MO, in the cultivation of plants and the consumption of these products by humans. When the antibacterial resistance genes detected in the study were examined, it was seen that in Gram negative bacteria, which are important in terms of public health and transmitted to humans through food, ESBL resistance genes were more abundant (Ecll w / o 0157: H7 at 75% blaCTX-M, 50% blaSHV, 37.5% blaTEM; blaCTX-M, 55.5% blaSHV, 22% blaTEM; *Aeromonas spp.* 56,52% blaCTX-M, 39% blasSHV; *Vibrio vulnificus*, blaTEM 50%: *Vibrio parahaemolyticus* 8 positive strains, 37.5%). When the distribution of carbapenemase genes was examined, only *Aeromonas spp.* (21.8%) blaKPC level resistance gene was detected. In many previous studies, resistance to beta lactamase enzymes has been determined, similar to the findings of the present study (Pfeifer et al., 2010). With the exception of *Aeromonas spp.*, it can be said that the Carbapenems may be effective as the causative agent.

Sekhar et al. (2017), isolated 150 *E. coli* bacteria from the intestine content (catla catla) of 150 freshwater fish in the Andhra Pradesh river, analyzed the E16S gene using PCR, investigated ESBL resistance with the disc diffusion method and determined ESBL resistance in 16 isolated bacteria. There were found to be blaTEM, blaAmpC, blaCTX-M, blaSHV and blaOXA genes in isolates 13,9,6 and 4 as molecules with genetic multiplex PCR. Similarly, in the current study, in *E. coli* isolates 75% blaCTX-M, 50% blaSHV, 37.5% blaTEM resistance genes were obtained. These findings indicate that resistance genes detected in *E. coli* isolates can be seen not only in a local region but in different countries and this problem is an international problem. In a study by Kahraman et al. (2017), 400 fish and other seafood products from retail outlets were isolated with *A. hydrophile* and *P. shigelloides* with the conventionalculture method and their assets were confirmed using PCR. *A. hydrophile* and *P. shigelloides* were isolated in 5.71% of the other seafood products, while only *P. shigelloides* were isolated in the fish samples. In the current study, *P.shigelloides* were isolated from 17 positive samples (34%) and a higher bacterial density was determined than in the study by Kahraman et al. (2017).

Lv et al.(2018) and Xin et al.(2019) examined fish in the southern China Guangzhou region and fish consumed in Tianjin in the Gulf of Bohai, and Roschanski et al. (2017) examined 160 different marine products in Germany, and identified the same bacteria and resistance genes as those in the current study with parallel results.

In a study in Switzerland, Boss et al. (2016), investigated enrichment methods for the detection of specific public health-related MDR bacteria, such as methicillin-resistant *S. aureus* and *E. coli* in fish and seafood, and studied the bacterial strains isolated from the application of extended-spectrum β-lactamases and carbapenemases. The antimicrobial agent panel was tested with the MIC test, obtaining similar results of resistance genes, especially *E.coli,* to those of the current study which were obtained using a different method, the real-time PCR method.

Ryu et al. (2012), studied *E. coli* in 2663 specimens collected from the port of Seoul and studied 70 of 179 resistant isolates with PCR. Among the antimicrobial resistance genes, tetB and tetD, tetracycline resistance genes were found in 29 (41.4%) isolates and 14 (20%) isolates, respectively. The beta-lactamase resistance gene, blaTEM was also found in 15 (21.4%) isolates, and the Aminoglycoside resistance gene, aadA, in 18 (25.7%) isolates.

Due to the distribution of similar pathogens and resistance genes detected in this study, it can be concluded that aquaculture facilitates the spread of multi-resistant bacteria and resistance genes.

The accuracy of the method used in this study was proven by Coppens et al. (2019), Robertson et al. (2014), and Herrera et al (2006), in studies to determine the bacterial resistance alone or with a molecular method. It was stated that MIC and real-time PCR (qPCR) should be used in combination so that faster and more reliable results can be obtained, with the support of the reliability of the qPCR technology.

In this study to determine the resistance level of the extended spectrum beta-lactamase genes of the bacteria isolated in fish, the results demonstrated that the problems of the treatment of bacterial diseases with antibiotics such as penicillin and cephalosporin can lead to future risks in terms of public health (Ishida et al., 2010; Serrano, 2005).

In the South-eastern Anatolia region of Turkey, which is rich in water resources of rivers and dams, fish farming and the numbers of fish farms are increasing. This increase has led to an increase in the unconscious use of antibacterial drugs. The high rate of antibiotic resistance determined in this study indicates that excessive amounts of human or animal waste and antimicrobial agents are released directly or indirectly into the Euphrates river. The level of unconsciousness use of the drugs in fisheries activities will lead to the ineffectiveness of the antibacterial drugs used for treatment in public health as well as pollution of the ecosystem. As a result, greater importance and attention must be given to the currently increasing levels of antimicrobial resistance threatening public health as treatment periods of the diseases are prolonged, entailing greater economic costs and patient losses. Because of the health risks associated with the use of antibiotics in animal

products, awareness about the conscious use of antibiotics should be increased, the number and variety of antibiotics used in edible fish should be limited, and the data on the pathogens and resistance genes determined in fish should be monitored molecularly and epidemiologically.

Conflict of Interest

The authors declared that there is no conflict of interest

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