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Antimicrobial Resistance Profiles and Tetracycline Resistance Genes of *Escherichia coli* in Mediterranean Mussel and Sea Snails Collected from the Eastern Black Sea (Turkey)

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Abstract: Aquatic environments are often affected by and exposed to anthropogenic pollutants including antimicrobials used as disease prevention and feed additives. Antimicrobial resistance is a major problem both in animal and in human health worldwide. In this study, Mediterranean mussel (*Mytilus galloprovincialis*) and Sea snail (*Rapana venosa*) samples were collected seasonally from the coastline of Black Sea. A total of 54 *Escherichia coli* were isolated from Mediterranean mussel and Sea snail collected from the coast of Artvin, Rize, Trabzon and Giresun, Turkey. Antimicrobial resistance and the presence of tetracycline (*tet*) resistance genes (*tetA*, *tetB*, *tetC*, *tetD*, and *tetE*) in *E. coli* isolates were investigated. Antimicrobial susceptibility test determined that 83.3 % of the isolates exhibited resistance to sulfamethoxazole. Resistance to ampicillin and aztreonam was as 66.7 % and 37.0 % among the tested antimicrobials, respectively. The lowest resistant antimicrobial was florfenicol (1.9 %). *tetC* resistance gene was detected in more than 50% of the isolates. Among the *tet* resistance genes, *tetC* was found in the most common gene followed by *tetB*, *tetA*, *tetE*, and *tetD*. At least one *tet* gene was detected in 88% of the isolates, and 46% of the isolates had two or more *tet* genes. The presence of *tet* resistance genes in *E. coli* in aquatic environments indicates that these isolates may be a reservoir of *tet* resistance genes. They may also exhibit an important role in the spread of genes among the pathogenic and non-pathogenic bacteria.

Keywords: Antimicrobial, *Mytilus galloprovincialis*, *Rapana venosa*, tetracycline resistance gene

Doğu Karadeniz (Türkiye)'den Toplanan Kara Midye ve Deniz Salyangozlarındaki *Escherichia coli*'lerin Antimikrobiyal Direnç Profilleri ve Tetrasiklin Direnç Genleri

Öz: Sucul ortamlar sık sık hastalıklardan korunma ve besin katkı maddesi olarak kullanılan antimikrobiyalleri de içeren karasal kökenli kirleticilere maruz kalmakta ve etkilenmektedir. Antimikrobiyal direnç dünya çapında hem insan sağlığında hem de hayvan sağlığı açısından büyük bir problemdir. Bu çalışmada, Karadeniz kıyılarından mevsimlik olarak kara midye ve deniz salyangozu örnekleri toplanmıştır. Artvin, Rize, Trabzon ve Giresun kıyılarından toplanan kara midye ve deniz salyangozlarında toplam 54 adet *Escherichia coli* suşu izole edilmiştir. *E. coli* izolatlarında antimikrobiyal direnç ve tetrasiklin direnç genlerinin (*tetA, tetB, tetC, tetD,* ve *tetE*) varlığı araştırılmıştır. Antimikrobiyal hassasiyet testi izolatların %83,3'ünün sulfametaksazola karşı dirençli olduğunu göstermiştir. Ampisilin ve aztreonama karşı direnç de sırasıyla %66,7 ve %37,0 olarak hesaplanmıştır. En düşük direncin florfenikole karşı olduğu tespit edilmiştir. İzolatların % 50'sinden fazlasında *tetC* geni bulunmuştur. *tet* genleri arasında, *tetC* en çok bulunan gen olurken bunu *tetB, tetA, tetE* ve *tetD* takip etmiştir. İzolatların %88'inde en az bir tane test edilen direnç geni olduğu tespit edilmiş ve %46'sı ise iki veya daha fazla tet genine sahip olduğu belirlenmiştir. Sucul ortamlardaki *E. coli*'lerde *tet* direnç genlerinin varlığı bu izolatların *tet* direnç genleri bakımından bir rezervuar olabileceğini göstermektedir. Bu izolatlar ayrıca patojenik ve patojenik olmayan bakteriler arasında da bu genlerin yayılmasında önemli bir rol oynayabilir.

Anahtar kelimeler: Antimikrobiyal, *Mytilus galloprovincialis, Rapana venosa,* tetrasiklin direnç geni

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1. INTRODUCTION

Antibiotics or antimicrobials (AMs) are used to treat diseases caused by bacteria in human and animals. The AMs used in animal husbandry and hospitals arrive in aquatic environments and they affect the living organisms in their environments. The occurrence of multiple resistant bacteria to AMs and their corresponding resistance genes has recently raised greater concerns all over the world (Capkin et al., 2015).

Escherichia coli are used as a fecal indicator organism to monitor bacteriological pollution of aquatic environments and seafood. The occurrence of this species in aquatic environments indicates the area or organisms polluted with feces with animal or human origin. The presence of *E. coli* in aquatic environment became very serious problem all over the world. Pathogenic strains of *E. coli* cause waterborne and foodborne diseases (Avşar and Berber, 2014).

The Black Sea has been threatened by industrial wastewaters, runoff from land human and animal feces and the sewage wastes (Bat and Öztekin, 2016). Many organisms in the aquatic environments can accumulate the contaminants in water, such as heavy metals (Alkan et al., 2012; Bat and Öztekin, 2016) and bacteria (Kacar, 2011).

Mussels are filter-feeding organisms and they actively filter, retain and accumulate the particles from their aquatic environments. Additionally, sea snails or the veined rapa whelk (*Rapana venosa*) mainly feed on mussels, and other bivalves (Bat and Öztekin, 2016). Mediterranean mussel (*Mytilus galloprovincialis*) and sea snail were selected to determine *E. coli* contamination in their tissues because they are important tools of biomonitoring of environmental pollution in aquatic environments (Altuğ and Güler, 2002; Akkan and Mutlu, 2016).

It should be noted that the Black Sea is an important marine environment for fishing and other seafood. The occurrence of AM resistant bacteria in coastal marine organisms may be hazardous for human health and aquaculture facilities because of transferable AMR genes (Grevskott et al., 2017). Therefore, this research aimed to determine the levels of AMR and presence of tetracycline resistance genes of *E. coli* isolated from Mediterranean mussel and Sea snail in Black Sea, Turkey.

2. MATERIAL AND METHODS

Study Area and Sample Collection

Mediterranean mussel and sea snail samples are distributed along the coastline of Black Sea in Turkey. The coastline of Black Sea is exposed to various pollutants by domestic discharge and food industries. Also, lots of streams such as Çoruh River, Fırtına, Değirmendere, and Batlama Streams flow into the Black Sea and it is possible to transfer pollutants to Black Sea via these streams. At least 8-10 Mediterranean mussel and sea snail in every station in a sampling period were collected by diving from twelve stations in the coastline of Artvin, Rize, Trabzon and Giresun, Turkey in four seasons in 2014. The sampling stations are shown in Figure 1.

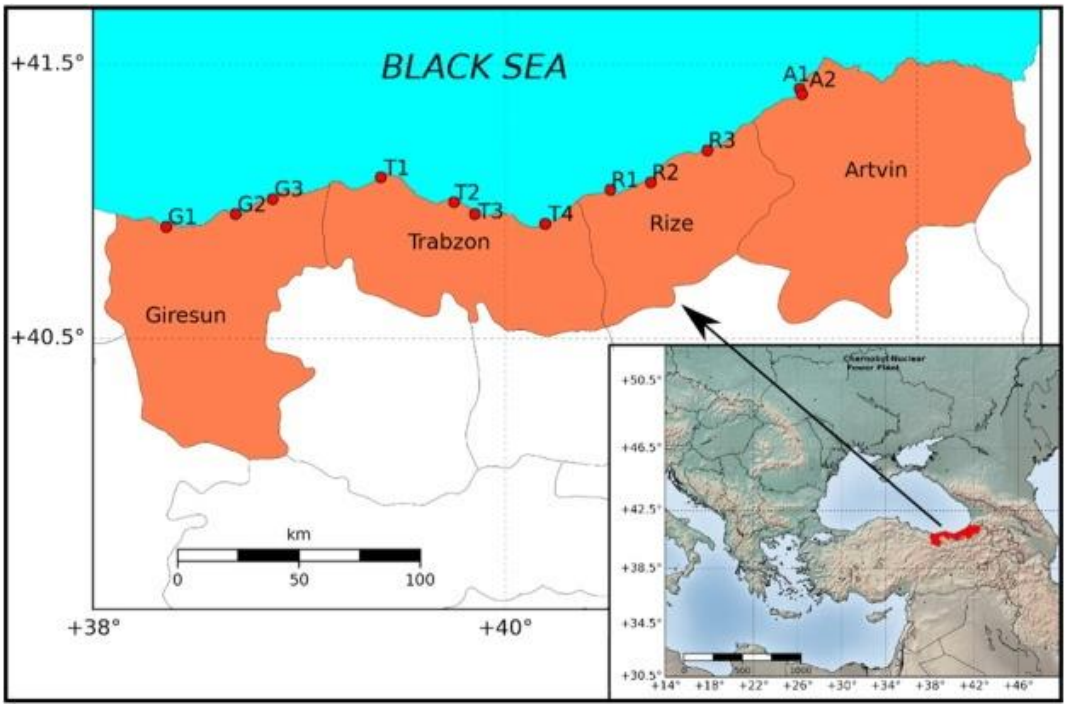

Figure 1. Sampling stations of mediterranean mussels and sea snail along the Eastern Black Sea coast of Turkey (Baltas et al., 2016),

Escherichia coli Isolation and Identification

Internal organs of Mediterranean mussel and sea snail samples were separated. Then, a total of 25 g samples of both mussel and sea snail in each station were separately homogenized with sterile saline water (0.85 %). 1000 µl homogenate was inoculated to Luria Bertani (LB) Broth (Merck), and incubated at 35 °C for 2 days. Then, 200 µl suspension was streaked on the surface of Eosin Methylene Blue (EMB) Agar (Merck). After two-day incubation at 35 °C, dark colonies with a metallic green on the medium were subcultured to obtain pure isolate. For biochemical characterizations of the bacteria, gram staining, catalase, cytochrome-oxidase, indole production, methyl red, voges proskauer, citrate, and API 20E (Biomerieux, Marcy l'Etoile, France) tests were performed. Isolates were identified and stored in a Tryptic Soy broth with 15% glycerol at -70 °C (Brenner and Farmer, 2015).

Determination of AMR

AMR of the isolates were determined by Disk Diffusion Test using commercial disks (Bioanalyse) according to the guidelines of Clinical and Laboratory Standards Institute (CLSI 2014). Commercial disks included tetracycline (TE; 30 µg), oxytetracycline (T; 30 µg), ampicillin (AM; 10 µg), aztreonam (ATM; 30 µg), chloramphenicol (C; 30 µg), florfenicol (FFC; 30 µg), sulfamethoxazole/trimetophrim (SXT; 25 µg), kanamycin (K; 30 µg), sulfamethoxazole (SMZ; 100 µg), and gentamycin (CN; 10 µg). The isolates were characterized as resistant or susceptible to the AMs according to the inhibition zone diameters measured. Also, *E. coli* ATCC 25922 was used as control organism (CLSI, 2014).

PCR Assays for Detection of Tetracycline Resistance Genes

All *E. coli* isolates were tested for the presence of tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetD*, and *tetE*). The genomic DNA was extracted from the *E. coli* isolates with a boiling technique. Briefly, a few pure colonies were chosen and suspended in 250 µl sterile water and then heated at 95°C for 10 min. Moreover, it was centrifuged at 17000 × g for 10 min and the supernatant was used for PCR experiments. Selected primers were used to amplify tetracycline resistance genes by PCR (Table 1).

Thermal cycling was performed with a T100 Thermal Cycler PCR system (Bio-Rad). Each reaction mixture for PCR contained 12.5 µl of *Taq* 2× Master Mix (NEB, New England BioLabs), 80-100 ng of the template DNA, 100 pmol of each primer (Macrogen) in a 25 µl. The PCR amplification conditions consisted of initial denaturation at 95°C for 30 s; denaturation at 95°C for 30 s, annealing at 54-60°C (see Table 1) for 45 s, and extension at 68°C for 45 s. PCR reaction was performed for 35 cycles and a final cycle was performed at 68°C for 1.5 min. Controls consisted of the PCR mixture containing (1) no DNA template (reagent control) and (2) DNA from bacteria known to contain the different resistance genes tested for (positive control). 15 µl aliquots of PCR was run at 100 V for 45 min in 1% agarose gel prepared in 0.5× TAE buffer. Ethidium bromide was used to stain the gels and the gels were viewed by UV transillumination.

Table 1. Tetracycline resistance gene primers

Primer Name	Sequencing (5' - 3')	Target Gene	PCR Product (bp)	Annealing Temperature (°C)
tetA F	GCTACATCCTGCTTGCCTTC	*tet A**	210	54
tetA R	CATAGATCGCCGTGAAGAGG			
tetB F	TTGGTTAGGGGCAAGTTTTG	*tet B**	659	55
tetB R	GTAATGGGCCAATAACACCG			
tetC F	CTTGAGAGCCTTCAACCCAG	*tet C**	418	54
tetC R	ATGGTCGTCATCTACCTGCC			
tetD F	AAACCATTACGGCATTCTGC	*tet D**	787	55
tetD R	GACCGGATACACCATCCATC			
tetE F	CGGCGTGGGCTACCTGAACG	*tet E***	1180	60
tetE R	GCCGATCGCGTGAAGTTCCG			

** Ng et al., 2001; ** Guardabassi et al., 2000*

3. RESULTS AND DISCUSSION

All Mediterranean mussel and sea snail samples were tested for the occurrence of *E. coli*. According to biochemical properties, it was determined that a total of 54 *E. coli* strains were isolated and identified. Of these, a total of 35 strains were isolated from Mediterranean mussels and 19 from the veined rapa whelk. According to the seasonal presence of the isolates, the highest number isolation was carried out in the summer while the lowest isolation was in winter (Figure 2). There were different number of isolation according to the sampling stations. Eighteen and nineteen strains were isolated from Mediterranean mussel (n:11+12) and sea snail (n:7+7) in Trabzon and Giresun, respectively (Figure 3).

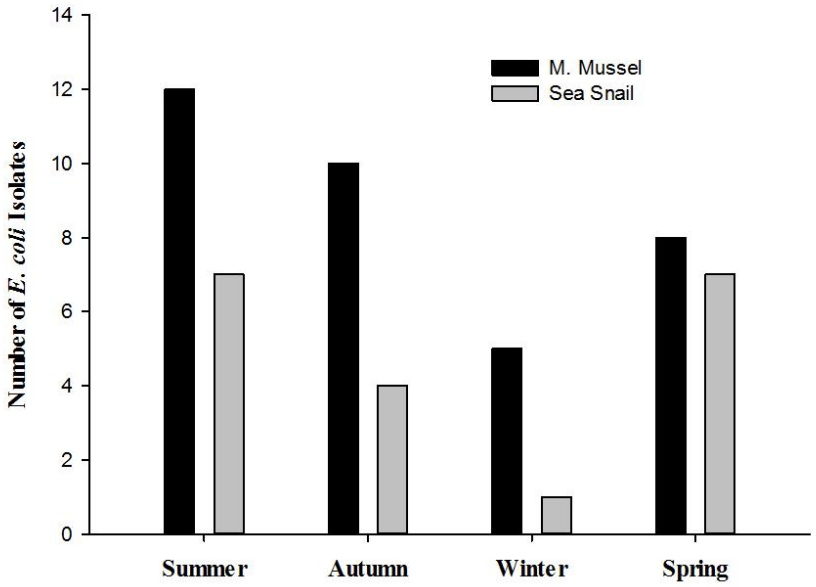

Figure 2. Seasonal isolation number and distribution of the *E. coli* isolates

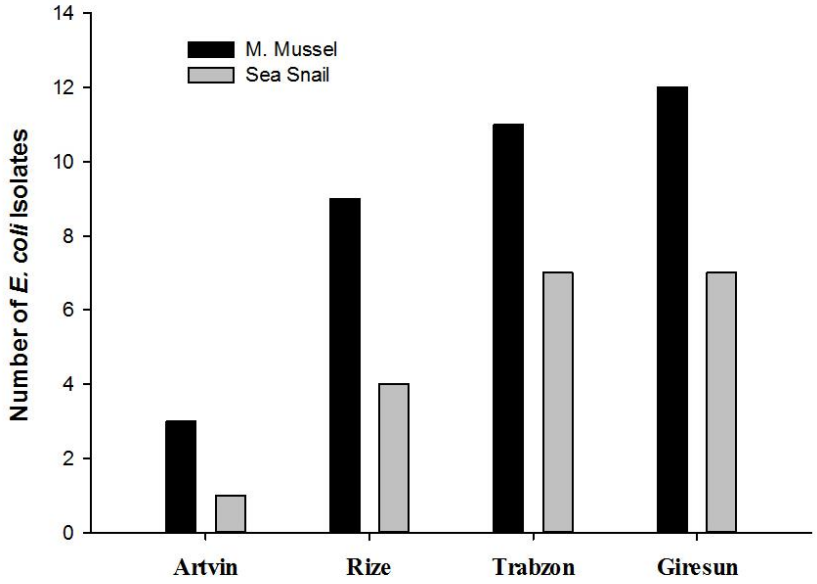

Figure 3. Locational distribution of the *E. coli* isolates

According to AMR tests, it was determined that 83.3 % of the isolates were resistant to sulfamethoxazole. Resistance to ampicillin and aztreonam were as 66.7 % and 37.0 %, respectively. The lowest resistance was determined to Florfenicol (1.9 %) (Table 2). Additionally, in this study, it was also examined that whether the bacteria had the *tet* resistance genes or not. Tetracycline resistance genes of *E. coli* isolates are summarized in Table 3. Of the tested resistance genes (*tetA*, *tetB*, *tetC*, *tetD* and *tetE*), the most common gene was detected to be *tetC* (68.5 %). The *tetC* gene was followed by *tetB* (27.8 %), *tetA* (16.7 %), *tetE* (14.8 %) and *tetD* (11.1 %). It was determined that 88.0 % of *E. coli* isolates carried at least one *tet* gene and 46.0 % carried two or more *tet* genes. According to sampling stations, *E. coli* isolated from Trabzon and Rize had more resistance gene than Artvin and Giresun in terms of at least one resistance gene (Table 3).

Table 2. Number and percentage of antimicrobial resistance/susceptibility of *E. coli* isolated from mediterranean mussel and sea snail

	Resistant		Susceptible	
Antimicrobials	**n**	**%**	**n**	**%**
Oxytetracycline T30	10	18.5	44	81.5
Ampicillin AM10	36	66.7	18	33.3
Aztreonam ATM30	20	37.0	34	63.0
Tetracycline TE30	10	18.5	44	81.5
Chloramphenicol C30	3	5.6	51	94.4
Kanamycin K30	13	24.1	41	75.9
Florfenicol FFC30	1	1.9	53	98.1
Trimetphrim/ Sulfamethoxazole STX25	6	11.1	48	88.9
Sulfamethoxazole SMZ100	45	83.3	9	16.7
Gentamicin CN10	7	12.9	47	87.1

Table 3. The percentage of tetracycline resistance genes in *E. coli*

Locations	*Tetracycline Resistance Genes*							
	N	***tetA***	***tetB***	***tetC***	***tetD***	***tetE***	***One gene***	***Two or more genes***
Artvin	4	25.0	50.0	50.0	0.0	0.0	75.0	50.0
Rize	13	7.7	46.2	69.2	15.4	15.4	92.3	61.5
Trabzon	18	5.6	33.3	72.2	11.1	11.1	94.4	27.8
Giresun	19	31.6	5.3	68.4	10.5	21.1	84.2	52.6
General	**54**	**16.7**	**27.8**	**68.5**	**11.1**	**14.8**	**88.0**	**46.0**

Lots of researches have documented the microbial characteristics of mussels (Kacar, 2011; Avşar and Berber, 2014) and sea snails (Altuğ and Güler, 2002). The number of *E. coli* isolates obtained in this study was higher in summer and autumn but lower in winter and spring. The high *E. coli* contamination in the Mediterranean mussel and sea snail may be related to various environmental conditions such as water temperature, food availability and biological cycles of the livings (Kacar, 2011). Additionally, according to stations, a total of 18 and 19 *E. coli* strains (more than Rize and Artvin) were isolated from Trabzon and Giresun coastlines, respectively. The coastline of these points may be exposed to high industrial and domestic discharges. Furthermore, a growing number of researches indicates that *E. coli* from natural environments (Rees et al., 2015) show increasing resistance to various classes of AMs. AM resistant fecal bacteria from seafood or humans may be disseminated among humans or water and food. Contaminated seafood such as mussels and sea snails may cause to reach humans via consumption and handling (Grevskott et al., 2017). Ampicillin, sulfamethoxazole, erythromycin, streptomycin, and neomycin are frequently used AMs in Turkey for bacterial diseases (Capkin et al., 2015). A highly resistance was detected to sulfamethoxazole, ampicillin, and aztreonam in all isolates in this study (Table 2). The results of this study agreed with the previous studies conducted in marine environment. The overuse of these AMs in humans or food-producing animals may have led to resistance in *E. coli* isolates in this study.

In addition to findings of antimicrobial resistance, the presence of *tet* genes in different bacterial species from different aquatic environments has previously been investigated (Balta et al., 2010; Capkin et al., 2015). Tetracycline, chlortetracycline and oxytetracycline are the most frequently used AMs used in human diseases, animal husbandry and in aquaculture worldwide (Capkin et al., 2015). It has been reported that *tetA* and *tetB* were detected in the river origin bacteria of Black Sea (Sandalli et al., 2010). Another study screening tetracycline genes of *E. coli* from fish and seafood (Ryu et al., 2012) has reported the genetic determinant of *tetB* was the predominant followed by *tetD*. In this study, tetracycline resistance gene determinant *tetC* was detected to be high frequency followed by *tetB*, *tetA*, *tetE* and *tetD* in all isolates (Table 3). The difference may be different geographical locations, isolation time and aquatic environments.

4.CONCLUSION

This study suggests that Mediterranean mussel and sea snail may become a reservoir for the AM resistant bacteria and allow the dissemination of tetracycline genes among the pathogenic and nonpathogenic bacteria in the marine environment. The prevalence of multidrug resistant organisms and the corresponding genes in aquatic surroundings may be a problem for human and animal life to treat diseases caused by bacteria.

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