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Distribution of antibiotic resistance and the presence of vancomycin-resistance genes (*vanA* and *vanB*) in *Enterobacteriaceae* isolated from the Sea of Marmara, the Canakkale Strait and the Istanbul Strait, Turkey

by

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Abstract

We investigated the frequency of antibiotic resistance of *Enterobacteriaceae* and the presence of vancomycin-resistance genes in samples taken from the Sea of Marmara, and the Istanbul and Canakkale Straits, Turkey. Different colony-forming bacteria were isolated and identified with the VITEK 2 Compact 30 system. The antibiotic resistance of the isolates was determined by the disc diffusion method. The isolates were tested against amoxicillin, ampicillin, aztreonam, ceftazidime, cefotaxime, cefuroxime, ofloxacin, vancomycin, tetracycline, kanamycin and gentamycin. The presence of vancomycin-resistance genes (*vanA* and *vanB*) was also investigated. The level of *Enterobacteriaceae* species was higher in the Sea of Marmara than in the Istanbul Strait and the Canakkale Strait. Isolates showing resistance to the greatest number of antibiotics were identified from *E. coli* isolates. The resistance of the selected bacterial isolates were as follows: kanamycin (82%), vancomycin (78%) and ampicillin (60%). Some intermediately vancomycin-resistant *Enterobacteriaceae* isolates had the *vanA* gene. This study provides evidence of widespread bacterial resistance to clinically relevant antibiotics in marine environments. It also contributes to the knowledge on the distribution of antibiotic resistance among *Enterobacteriaceae* and indicates the importance of control measures in domestic water treatment.

Key words: Canakkale Strait, Istanbul Strait, Sea of Marmara, Beta-lactam Antibiotics, PCR, *vanA*, *vanB*

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Introduction

Antibiotics are commonly used in human and veterinary medicine to control bacterial infections (Sarmah et al. 2006). However, due to their poor absorbance in human and animal intestines, the majority of antibiotics are excreted unchanged in feces and urine and eventually find their way into the environment (Schlusener 2006). Through a range of biochemical and physiological mechanisms, antibiotic residues cause development of resistance in bacteria and the antimicrobial resistance has recently been recognized as a worldwide ecological problem (Nordmann et al. 2005; Kaszanyitzky et al. 2007; Kemper 2008). High antibiotic loads rapidly accumulate and persist in aquatic environments (Hirsch et al. 1999) and as a result, a dramatic and global increase has been observed in the number of antibiotic-resistant bacteria, along with the multiple antibiotic resistance and pathogenic bacteria resistance. Antibiotic resistance can originate from gene mutations or horizontal transfer between phylogenetically diverse bacteria. In addition, newly acquired resistance genes may be maintained in new populations in the absence of antibiotic selection pressure. The presence of antibiotic-resistant genes, such as *tet* genes, *van* genes and *sul* genes, has been reported in wastewater, surface water and sediments (Pei et al. 2006; Ram et al. 2007).

The widespread use of vancomycin in treating gram positive bacterial infections throughout the world has resulted in a rapid increase in vancomycin-resistant enterococci (Riberio et al. 2006). The rapid spread of vancomycin resistance among bacteria is due to the location of *van* genes in movable genetic structures, such as plasmids, transposons and integrons. Therefore, vancomycin resistance has been used as a key determinant in natural environments, such as rivers, lakes and seawater (Guardabassi et al. 2000). Different vancomycin-resistance determinants have been demonstrated and classified in categories A, B, C, D, E, G. The most frequently detected vancomycin-resistance genes among the *Enterobacteriaceae* family are *vanA* and *vanB*, and these are the most globally widespread and prevalent phenotypes.

Although antibiotic-resistance of bacterial isolates has been studied in clinical isolates, there are limited studies on the presence of antibiotic-resistant bacteria in Turkish waters. The purpose of this study was to investigate the distribution of multiple antibiotic resistance in isolates of the *Enterobacteriaceae* family from the Sea of Marmara, the Canakkale Strait and the Istanbul Strait. The study was limited to gram negative bacteria, *Enterobacteriaceae*, due to our preliminary results that indicated only <2% of isolates belonged to Enterococcaceae and none of Enterococci isolates were resistant to vancomycin. The presence of vancomycin-resistance genes (*vanA* and *vanB*) in isolates of *Enterobacteriaceae* were also investigated due to the observed resistance to vancomycin.

Materials and methods

Sampling areas

Seawater samples were collected from three different marine environments: the Sea of Marmara (2011-14), the Istanbul Strait (2006-07) and the Canakkale Strait (2011-12), Turkey (Fig. 1). The sampling areas in the Sea of Marmara were eutrophic, whereas those in the Istanbul and Çanakkale Straits were oligotrophic based on the analyses of nutrients, chlorophyll-α and indicator bacteria (Çardak & Altuğ 2010). The samples were collected from a total of 46 stations (6 stations in the Istanbul Strait; 14 stations in the Sea of Marmara and 26 stations in the Çanakkale Strait; Fig. 1) and all samples were transported daily to the laboratory.

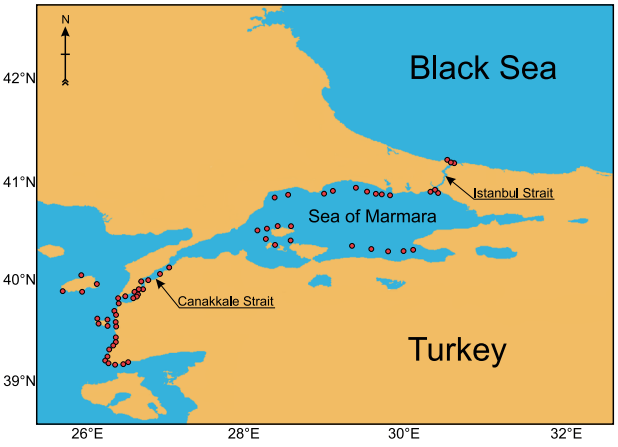

Figure 1

Study area: the Sea of Marmara, Istanbul Strait and Canakkale Strait

Bacteriological Analyses

The water samples were transferred into 250 ml sterile, brown glass bottles under aseptic conditions and processed on board of the research vessel Yunus-S. The water samples were filtered through a 0.45 µm membrane filter with a metal vacuum

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filtering set (Millipore, Germany). The membrane filter was then placed on m-Endo, m-FC (Sartorius AG, Germany) and inoculated (0.2 ml) in duplicate on Endo agar (Merck Darmstadt, Germany). The plates were incubated for 48 h at 37±0.1°C (at 44.5±0.1°C for *Escherichia coli*), and the colonies on the plates were evaluated (APHA 1999). At the end of the incubation period, different colonies were picked and re-streaked several times to obtain pure cultures. The pure isolates were gram-stained and then identified using gram-negative (GN) (gram [-] fermenting and non-fermenting bacilli), gram-positive (GP) (gram [+] cocci and non-spore-forming bacilli) and BCL (gram [+] spore-forming bacilli) cards in an automated micro-identification system, VITEK 2 Compact 30 (Biomereux, France). The identification cards are based on the established biochemical methods and newly developed substrates. Biochemical tests (46 tests for BCL, 43 tests for GP and 47 tests for GN) were performed to measure the use of carbon sources, enzymatic activities, inhibition and resistance. Calculations were performed on raw data and compared with thresholds to determine reactions for each test. On VITEK 2 Compact, the results of the test reactions appear as '(–)' or '(+)'. The reactions in the parentheses were considered an indicator of weak reactions, too close to the test threshold (Abele-Horn et al. 2006).

Antibiotic Resistance

Two or three colonies of each isolate were suspended in 5 ml of marine broth 2216 (Oxoid, UK) and diluted with sterile water against the 0.5 McFarland turbidity standard to approximately 106 cells per ml. Two milliliters were then swabbed on marine agar (Oxoid, UK). The β-lactam antibiotics included penicillin, cephalosporins (first, third and fourth generations), monobactams and carbapenems. Antibiotic disks (Oxoid, UK) containing amoxicillin (30 µg), ampicillin (10 µg), aztreonam (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefuroxime (5 µg), ofloxacin (5 µg), vancomycin (30 µg), tetracycline (30 µg), kanamycin (5 µg) and gentamycin (120 µg) were placed on marine agar plates and incubated for 2-3 days at 37°C. The antibiotic resistance of the isolates was examined by the Kirby–Bauer method. The percentage of the selected bacteria in samples that exhibited antibiotic resistance was measured on nutrient agar plates. The base medium selected for this test was Mueller-Hinton agar (Oxoid, UK) because of its international recognition in antimicrobial susceptibility testing. The results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (CLSI, 2006). All isolates that showed 'resistant' or 'intermediate' behavior were classified as 'resistant' or otherwise as 'sensitive'.

The antibiotic resistance of the GN bacteria isolated from the three marine areas was described for the period between 2006 and 2014. The Multiple Antibiotic Resistance (MAR) index of a given sample was calculated by the equation a/(bxc), where "a" represents the aggregate antibiotic resistance score of all isolates from a sample, "b" is the number of isolates, and "c" is the number of isolates from a sample (Krumperman 1983). Bacterial isolates that displayed resistance to three or more antibiotic agents were designated as multiply antibiotic-resistant (ranging from 2 to 10).

Detection of *vanA* and *vanB* genes by PCR

In this study, *vanA* and *vanB* genes were used to verify vancomycin resistance in *Enterobactericae,* as they were found to be associated with 78% of the vancomycin resistance (Hanaki et al. 2004). To isolate DNA for PCR, vancomycin-resistant isolates were inoculated into 3 ml of LB broth (L3022, Sigma-Aldrich, Saint Louis, MO, USA) and incubated for 20 h at 37°C with shaking. The overnight-cultured cells (1.5 ml) were harvested by micro-centrifugation (13,000 g, 10 min), and the supernatant was decanted. The pellet was re-suspended in 500 µl of deionized water. The cells were lysed by boiling for 10 min, followed by removal of the debris by centrifugation (13,000 g, 10 min). Then, 1 µl of the supernatant was used as a template for PCR amplification. The PCR reactions were carried out in a Mastercycler personal thermal-cycler (Eppendorf, USA) in a final volume of 50 µl using a 1 × Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer (Table 1) and 1.0 U of Taq polymerase (Fermentas, Lithuania). Primers were obtained using the Integrated DNA Technologies (USA).

PCR reactions for the *vanA* and *vanB* genes were performed with an initial cycle of denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, elongation at 72°C for 1 min and a final cycle at 72°C for 10 min. An aliquot of each PCR product was checked by electrophoresis on the 1% (w/v) vertical agarose gel at 70 V for 45 min in 1 × TAE buffer (Tris-Acetate-EDTA, pH 8).

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Table 1

Sequence of each primer (forward, f and reverse, r) used to amplify the two genes/intergenic regions searched using PCR and the respective expected amplicon sizes

Gene / IR	Sequence	Amplicon size (bp)	Annealing temperature (°C)	Reference
vanA	f 5'-GGGAAAACGACAATTGC-3'	732	54	Dutka-Malen 1995
	r 5'-GTACAATGCGGCCGTTA-3'			
vanB	f 5'-ATGGGAAGCCGATAGTC-3'	635	54	Ribeiro et al. 2007
	r 5'-GATTTCGTTCCTCGACC-3'			

Results

Identification and Phylogenetic Analysis of the Isolates

A total of 286 randomly selected bacterial isolates (106, 140 and 40 from the Istanbul Strait, the Sea of Marmara and the Canakkale Strait, respectively) were identified by the VITEK 2 Compact 30 system. More than 98% of these isolates were affiliated with the genus level. Most of the identified bacteria (85%) were GN (Table 2).

Analysis of the bacterial isolates revealed a predominance of γ-proteobacteria (89%), belonging to 12 genera: *Enterobacter* (34.2%), *Klebsiella* (22%) and *Citrobacter* (12%). Among these, there was a high relative abundance of genera belonging to *Enterobacter* (Istanbul Strait) and *Klebsiella* (Sea of Marmara). Fewer bacteria isolates belonged to the genera *Serratia*, *Pantoea* and *Raoultella*. The distribution of *Enterobacteriaceae* bacterial genera differed considerably across the three different areas in the samples collected in 2006 and 2014. The highest similarity in *E. coli* isolates was observed

Table 2

Diversity of Enterobacteriaceae in the sampling areas

Species	Sampling areas		
	1	2	3
Cedecea lapagei Grimont et al. 1981	+	+	-
Citrobacter braakii Brenner et al. 1993	+	+	+
C.freundii (Braak 1928) Werkman and Gillen 1932	+	+	+
Enterobacter aerogenes Hormaeche and Edwards 1960	+	+	+
E. cloacae (Jordan 1890) Hormaeche and Edwards 1960	+	+	+
E. sakazakii (Farmer et al. 1980)	+	+	+
Escherichia coli T. Escherich, 1885	+	+	+
Hafnia alvei Møller, 1954	+	+	-
Klebsiella pneumoniae ssp. pneumoniae Schroeter 1886 Trevisan 1887	+	+	+
K. ornithinolytica Sakazaki et al. 1989	+	+	-
K. oxytoca (Flügge 1886) Lautrop 1956	+	+	+
K. planticola Bagley et al. 1982	+	+	-
Pantoea agglomerans (Ewing and Fife 1972) Gavini et al. 1989	+	+	-
Proteus vulgaris Hauser 1885	+	+	+
P. mirabilis Hauser 1885	+	+	+
Providencia rettgeri (Hadley 1918) Brenner et al. 1978	+	+	-
Raoultella terrigena (Izard et al. 1981) Drancourt et al. 2001	+	+	-
Salmonella enterica subsp. arizonae (Borman 1957) Le Minor and Popoff 1987	+	+	+
S. typhimurium (Loeffler 1892) Castellani and Chalmers 1919	+	+	+
Serratia marcescens Bizio 1823	+	+	+
S. odorifera Grimont et al. 1978	+	+	-
S. plymuthica (Lehmann and Neumann 1896) Breed et al. 1948	+	+	-
S. liquefaciens (Grimes and Hennerty 1931) Bascomb et al. 1971	+	+	-
Yersinia enterocolitica (Schleifstein and Coleman 1939) Frederiksen 1964	+	+	-

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between bacterial communities from the Istanbul Strait and the Sea of Marmara.

Antibiotic resistance profiles

Figure 2 depicts the percentage of isolates resistant to the panel of antibiotics tested. The patterns of resistance in the three locations were dissimilar.

The results showed that the isolates had high resistance to kanamycin (82%), vancomycin (78%) and ampicillin (60%). Some of the *Enterobacteriaceae* isolates isolated from marine water had *vanA* and *vanB* genes and they were intermediately resistant to vancomycin.

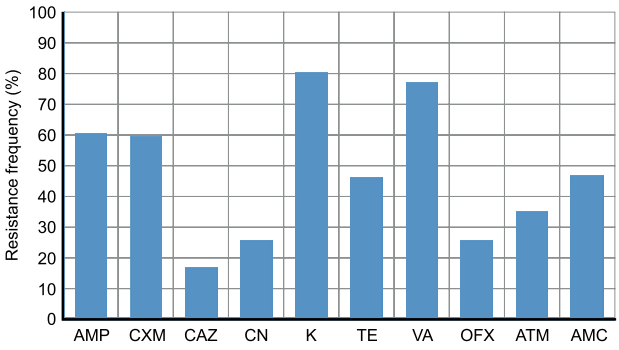

Figure 2

Antibiotic resistance of *Enterobacteriaceae* isolates from the Istanbul Strait, the Çanakkale Strait and the Sea of Marmara (AMP: ampicillin; CXM: cefuroxime; CAZ: ceftazidime; CN: gentamycin; K: kanamycin; TE: tetracycline; VA: vancomycin; OFX: ofloxacin; ATM: aztreonam; AMC: amoxicillin; CTX: cefotaxime)

Many isolates showed resistance to at least two antibiotic derivatives, as well as to penicillin. Only 27% of the isolates were sensitive to antibiotics other than penicillin. Among all the *Enterobacteriaceae* isolates, the highest frequency of anti-bacterial resistance was to kanamycin (82%). The frequency of carbapenem-resistant isolates was 22%.

The highest frequency of vancomycin-resistant isolates was obtained from the samples taken from the Istanbul Strait. The isolates isolated from the Sea of Marmara had the second highest frequency (Table 3). The lowest frequency of vancomycin-resistant bacteria was recorded in the samples obtained from the Canakkale Strait. The isolates from the Istanbul Strait were significantly more resistant ($p < 0.05$; 17-19%) than those from the Canakkale Strait and the Sea of Marmara.

Multiple Antibiotic Resistance (MAR) Index

The results indicated that there was a temporal and spatial change in the resistance profiles (Fig. 3). There was a high frequency of multi-resistant bacteria among the isolates from all three areas: the Sea of Marmara (0.27), the Canakkale Strait (0.18) and the Istanbul Strait (0.36). In general, the MAR index did not show any significant difference ($p = 0.543$) and ranged from 0.27 to 0.73 among the areas in all the sampling periods.

Enterobacter and *Klebsiella* isolates represented the majority of bacterial isolates retrieved from the samples. Two percent of these isolates were susceptible to all tested antimicrobials, 6.5% were resistant to a single antimicrobial, and 89.9% were multi-resistant. The most common phenotypes were

Table 3

Frequency of antibiotic resistance (%) of Enterobacteriaceae isolates from the Istanbul Strait, the Canakkale Strait and the Sea of Marmara

Isolates	AMP 10 µg	CXM 30 µg	CAZ 30 µg	CN 120 µg	K 5 µg	TE 30 µg	VA 30 µg	OFX 5 µg	ATM 30 µg	AMC 30 µg	CTX 30 µg
Cedecea spp.	100	50	100	0	50	0	50	50	0	0	0
Citrobacter spp.	100	0	50	0	0	100	0	0	0	0	0
E. coli	66.6	66.6	7.4	7.4	14.9	37.1	18.5	25.6	7.4	29.6	18.5
Enterobacter spp.	57.1	57.1	85.7	14.3	21.4	42.9	0	14.2	7.1	35.7	21.4
Klebsiella spp.	72.7	50	45.5	9.1	18.1	36.3	27.7	31.8	4.54	45.4	18.1
Pantoea spp.	66.6	66.6	66.6	0	16.6	33.3	50	50	0	50	66.6
Proteus spp.	100	100	80	0	20	60	40	40	20	40	0
Providencia spp.	50	50	50	0	0	50	50	0	0	50	50
Raoultella spp.	100	57.1	71.4	0	28.5	42.8	0	42.8	28.5	28.5	0
Salmonella spp.	100	100	90	0	10	66.6	66.6	0	0	0	0
Serratia spp.	62.5	56.2	87.5	6.25	12.5	43.75	43.75	6.25	0	37.5	12.5

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those resistant to aminoglycosides (82%), ampicillin (60%) and amoxicillin-clavulanic acid (36.3%).

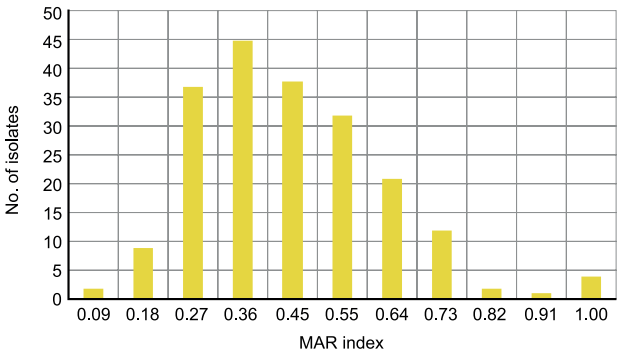

Figure 3

MAR index of the isolates from the Istanbul Strait, the Canakkale Strait and the Sea of Marmara

Detection of *vanA* and *vanB* genes

In this study, *vanA* and *vanB* genes were examined in 100 out of 278 isolates. In antibiotic-resistant members of the *Enterobacteriaceae* family species, only five isolates contained the *vanA* gene: *E. coli* (2), *Serratia liquefaciens, Citrobacter braakii* and *Enterobacter cloacae* (Figure 4). The *vanB* gene was not detected in any of the other isolates. In fact, the isolates with the *vanA* gene had a low MAR index.

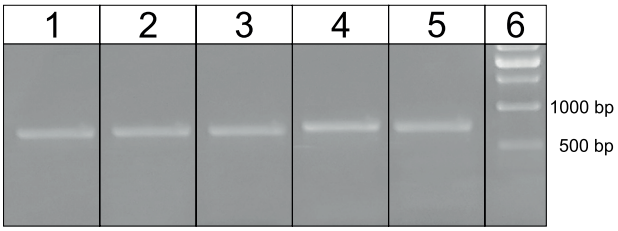

Figure 4

Detection of *Enterobacteriaceae vanA* gene coding for vancomycin resistance. Lane 1: *E. coli*, 2: *E. coli*, 3: *S. liquefaciens*, 4: *C. braakii*, 5: *E. cloacae*, 6: DNA size marker

Discussion

Coastal regions around the world are characterized by higher counts of indicator bacteria (Ramaiah et al. 2004; Kalkan & Altuğ 2015) and our findings are consistent with those of other scientists. In this study, the highest level of *Enterobacteriaceae* species was determined in the Sea of Marmara, as compared to the Istanbul and Canakkale Straits. This result is in accordance with the previous finding that characterizes the Sea of Marmara as having higher levels of bacterial loads compared to those of the Çanakkale Strait (Çardak et al. 2015). The Sea of Marmara is an intercontinental basin receiving discharges from the northeast, mainly through the Istanbul Strait and other river discharges which are under the influence of the most populated and developed socio-economic region in Turkey. Previous research also reported that most of the pathogenic species detected in the Sea of Marmara belonged to the *Enterobacteriaceae* family (Altuğ et al. 2012). In the present study, however, the levels of indicator bacteria were higher than that reported by Altuğ et al. (2012), indicating the increased deterioration of the marine environment in the region.

In this study, isolates showing resistance to the greatest number of antibiotics were identified from *E. coli* isolates. The level of antibiotic resistance found in the *E. coli* isolates was similar to that previously described in *E. coli* isolates from the Sea of Marmara, where 71.4% of the isolates showed resistance to one or more antibiotics. The level of *E. coli* β-lactam antibiotics resistance is in agreement with that described previously for GN bacteria isolated from beach sediment samples (Mudryk 2005). Significant levels of antibiotic resistance were also found in *Enterobacter*, *Salmonella* and *Enterococcus* isolates. Kimberly (2008) reported that in the U.S. state of Oklahoma, antibiotic resistance frequency of *Salmonella* isolates was higher than that of *E. coli*. This may be explained by the enhanced ability of *Salmonella* to survive in marine environments (Wait & Sobsey 2001). The higher resistance of *E. coli* isolates in the present study may be due to the continuous input of anthropological pollution into the Sea of Marmara. However, localities with lower levels of fecal indicators may also have a large number of resistant isolates (Altuğ & Balkıs 2009). This may be due to differential transport of nutrients by deep sea discharges that creates a nutrient-limited zone in surface waters where the growth of fecal indicators is limited but antibiotic-resistant isolates persist.

In this study, the highest frequency of bacterial resistance was to kanamycin, followed by vancomycin (glycopeptide) and ampicillin (β-lactam). The lowest frequency of resistance was to ceftazidime (20%) and gentamicin (25%). Similarly, β-lactam ampicillin- and penicillin-resistant bacterial isolates have been isolated from various marine environments (Hermansson et al. 1987; Mudryk 2005; Altuğ et al. 2010). Results on the frequencies of bacterial

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resistance to a given antibiotic differ considerably and frequency values of 29% for kanamycin (Algeria), 68% for vancomycin (Portugal) and 63% for ampicillin (China) have been reported (Alouache et al. 2012; Novais et al. 2005; Tao et al. 2010). Although such differences in the percentage of bacterial resistance to various antibiotics may reflect regional history of antibiotic use, the presence of bacterial resistance in the marine environment pose a threat to human health. A temporal change in bacterial resistance to different antibiotics has also been shown; for example, the previous study found the highest frequency of resistance to ampicillin in GN isolates from the Golden Horn Estuary (Istanbul Strait) and the Sea of Marmara (Altuğ et al. 2007). In addition, in the present study, the isolates that showed the highest resistance to kanamycin, vancomycin and ampicillin also displayed the lowest antibiotic resistance against gentamicin, ceftazidime and cefotaxime. The differences in the frequencies of resistance among various antimicrobial agents have been attributed to the presence of antibiotic-resistant plasmids in terrestrial bacteria entering into seawater (Vignesh et al. 2012).

The MAR index is commonly used to identify the level of bacterial resistance in a given population exposed to multiple sources of antimicrobial agents. The presence of multidrug-resistant isolates is alarming because infection with such isolates leads to a higher fatality rate than infection with antibiotic-sensitive isolates (Manjusha et al. 2005). The MAR index value >0.2 indicates the exposure to contamination sources having high risk levels of antibiotics, whereas values ≤0.2 indicate a low risk contamination (Pontes et al. 2009). In the present study, the MAR index of isolates from the Sea of Marmara (0.306-0.343) was higher compared to isolates obtained from the Canakkale Strait (0.29-0.316), reflecting higher contamination of antibiotic residues, possibly due to the discharges of wastewater into the Sea of Marmara. Multiple antibiotic resistance were also found in isolates of *Enterobacteriaceae* isolated from marine environments and ranged within 0.44-0.52 in Bangladesh, 0.19-0.45 in China and 0.4-0.8 in Egypt (Matyar 2012; Zahid et al. 2009; Tao et al. 2010).

In this study, resistance to vancomycin, the *vanA* gene has been detected in 5 different isolates. However, no resistance to the *vanB* gene was detected. It seems that bacteria have not been brought into contact with any *vanB* resistant isolates. This is in agreement with other reports showing that *vanA* resistance is more common in the environment than *vanB* resistance (Riberio et al. 2007). This is the first report confirming the presence of the *vanA* gene in different isolates from the aquatic environment in Turkey. In 2013, Turkey was the largest user of antibiotics in Europe, and vancomycin accounted for approximately 12% of all antibiotics used (TUIK, 2012).

In conclusion, our results indicated a temporal increase in the number of resistant isolates isolated from the seawater samples from the Sea of Marmara, the Istanbul Strait and the Çanakkale Strait. Bacterial pollution increases the levels of β-lactam antibiotic resistance among members of *Enterobacteriaceae* in the Sea of Marmara. We have found higher levels of kanamycin, vancomycin and amoxicillin/clavulanic acid resistance in isolates from the Sea of Marmara due to the extensive use of antibiotics for the treatment of bacterial infections. The increase in resistant isolates and coliform bacteria counts is associated with the deteriorating environmental conditions, possibly due to the increased rates of untreated wastewater discharges through rivers or surface runoff. Further research is required to determine potential steps aimed at reducing the input of antibiotic residues and to establish regulatory standards to control their dissemination into the marine environment.

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