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PROOF RESEARCH PAPER

Occurrence of Vancomycine Resistance Genes in Enterococci Isolated from Cage Areas in Turkey

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Abstract

Antibiotic resistance and presence of the resistance genes were investigated in the Vancomycin Resistant Enterococci (VRE) isolated from sediment in cage areas. A total of 85 *Enterococcus faecium* strains were isolated from the sediment samples. The Minimum Inhibitory Concentration (MIC) tests were indicated that, 16.47% of the *E. faecium* strains resistant to vancomycin. Following the detection of antimicrobial susceptibilities of the isolates, occurrence of the vancomycin resistance genes (*vanA, vanB, vanC1*) were determined by PCR. At least one vancomycin resistance gene was found in 64.7% of the *E. faecium* strains. The *vanA, vanB* and *vanC1* genes were determined in 29.41%, 31.76% and 25.88% of the bacteria respectively. These results indicate fish cage farming can play an important role in the development of antibiotic resistance among VRE.

Keywords: Antimicrobial resistance, Enterococci, net cage, Turkey.

Introduction

In recent years, the aquaculture activities have developed significantly in Turkey, especially cage fish culture. Rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*), and sea bream (*Sparus aurata*) are the most intensively cultured fish among cultured species. Turkey produces approximately 200 000 tons of fish per year in net cages (TUIK, 2015).

Antibiotics and antibiotic resistant bacteria usually come into the aquatic environments from human and animal sources such as hospitals, fish and other animal farms (Baquero, Martinez, & Canton, 2008). In addition, aquaculture is polluted with some contaminants including land and water-derived bacteria, nutrients, heavy metals, feed debris and other chemicals (Sapkota *et al.* 2008). In aquaculture, during the presence of bacterial diseases, fish are usually treated with antibiotics. Antibiotics are rarely used in small amounts as a feed for growth promoters. However, overuse or misuse of antibiotics in the aquatic environmets is one of the major factors, result in occurrence of drug resistant bacteria and antibiotic resistance genes (ARGs). Consequently, the use of antibiotics sometimes fail in fish (Aoki & Takahashi,1997).

Enterococcus are known as fecal *streptococcus* and they are a part of human and animal interstinal flora. In recent years, various infections included septicemia, meningitis, endocarditis and genital tract infections were reported. Moreover, they have emerged as a causitive agents of nosocomial infection (Huycke, Sahm & Gilmore, 1998). The diseases caused by vancomycin resistant enterococci (VRE) have an increasing clinical significance especially in veterinary medicine (Seo *et al*. 2005; Bagcigil *et al*. 2015). There are different genes such as *vanA, vanB, vanC1,vanC2, vanD, vanE, vanG* encode vancomycin resistance among VRE. Two types of vancomycin resistance were reported. The first is intrinsic resistance which exhibits low level resistance. The second is acquired resistance, which is especially observed in *E. faecium* and *E. faecalis* strains. These strains can carry transferable *vanA* or *vanB* genes and demostrate high level resistance to vancomycin (Rice *et al*. 2003; Jung *et al*. 2007).

There are different studies on occurrence of VRE in various animal species especially companion animals (Bagcigil *et al*., 2015). According to our knowledge, this is the first study on ARGs in VRE isolated from sediment related to net cages in Turkey. In this study, we determined the presence of VRE and their resistance genes (*vanA, vanB* and *vanC1*) in net cage areas in Gumushane, Samsun, Trabzon and Ordu, Turkey.

Material and methods

Sediment Samples

The study areas were located in Gumushane, Samsun (fresh water) and Trabzon, Ordu (sea water) in Eastern Black Sea Region of Turkey. Four fish farms (two times from each station) were screened seasonally to examine *Enterococcus* spp. strains isolated from sediment samples between 2015 and 2016. For this purpose, sediment samples from each farm were aseptically collected below the open net cages with a grap (eckman grap, 15x15cm). The sample were collected from three cages in each station. Also, samples were collected from farther cages (1km distance) as a control. The samples were usually consisted of a kilogram sediment per farm. Five grams of sediment was diluted with 45 ml Laure Bertani Broth (Merck) and incubated for 18 h at 37˚C. A ml of it transferred into Bile Esculin Azide Agar (Merck). Further identification of presumptive *Enterococcus* spp. colonies were performed as described by Manero & Blanch (1999). Also, all strains of bacteria were identified by sequencing 16S rRNA genes as described below.

DNA preparation and sequencing of bacteria

Genomic DNA of all bacteria were extracted for the PCR assay (identification to bacteria and detection of ARGs) by QIAamp DNA mini kit (Qiagen), according to its instructions. RNA/DNA calculator (Qiaexpert) was used to measure optical density (average, A260/280 was 1.85 and DNA concentrations were adjust to 40 ng/µl). All bacteria were identified by DNA sequencing of their 16S rRNA genes. PCR was performed according to Marchesi *et al*. (1998) using primers of the highly conserved 5' and 3' regions of 16S rRNA gene and synthesised by Integrated DNA Technologies. PCR was performed with PCR master mix (Qiagen) in a thermal cycler (Applied Biosystems). PCR products were purified with a Nucleo Spin PCR purification kit (Invitrogen). The pure PCR product were sequenced according to the protocol of BigDye v3.1 (Applied Biosystems) and read on Applied 3500 genetic analyser (Applied Biosystems). The derived nucleotide sequences were analysed and aligned with the BioEdit Sequence Alignment Editor.

Antimicrobial Susceptibility Test

Following identification, *E. faecium* strains (n=85) were tested for their susceptibility to vancomycin. Minimum inhibitory concentration (MIC) levels for vacomycin were determined using Epsilometer procedure (E-test, Biotests). Commercial antibiotic test strep used in this study included vancomycin (256-0.016, µg/mL). Test was performed on Mueller Hinton agar (Oxoid). The breakpoints used for all strains were as defined by the CLSI 2014.

Detection of Vancomycin Resistance Genes

All isolates were tested induvidually for detection of genes encoding vancomycin resistance (*vanA, vanB, vanC1*) by PCR. The primers used in this study were shown in Table 1. Reaction mix and PCR assay was performed according to Capkin, Terzi & Altinok (2015). DNA amplification was performed in thermocycler (Applied Biosystems). Ten µl of PCR products were subjected to electrophoresis in 1.5% (w/v) agarose gel prepared with 1X TBE (Tris-Boric-Edta) buffer and run at 100 V for 1 h. Afterwards, stained PCR products (with ethidium bromide) viewed by UV transillumination. Sizes of the PCR products were determined by a 100 bp DNA ladder (Bio Basic).

Results

Isolated bacterial strains from sediment samples were identified at species level by 16S rRNA gene sequences. A total of 85 bacterial isolates were identified as *E. faecium* (Table 2). 16S rRNA gene sequences of *Enterecoccus faecium* strains were demonstrated ≥99% similarity with reference strains (Accession numbers: NC 017960.1) from Genbank.

Results of MIC testing indicated that, 25,92% (14/54) of the *E. faecium* isolated from bottom of net cages (*E. faecium*^{a}) were resistant to vancomycin (MIC≥256 µg/mL). After phenotypical detection of vancomycin resistance of *E. faecium* strains, ARGs of all bacteria were examined by individual PCR. The amplification of DNA fragments with various band intensities, and their sizes ranged between 536bp (*vanB*) and 1030bp (*vanA*) (Figure 1). Antibacterial resistance level of bacteria isolated from bottom of net cage (^{a}), were significantly higher than bacteria isolated from control points (^{b}). Presence of 3

Table 1. Primers used in the PCR reactions

Primer	Sequence (5′–3′)	Target gene	PCR product (bp)	References	Annealing temp. (Cº)
vanA-f	CATGAATAGAATAAAAGTTGCAATA	*vanA*	1030	Devriese *at al.*	51
vanA-r	CCCCTTTAACGCTAATACGATCAA			1996	
vanB-f	AAGCTATGCAAGAAGCCATG	*vanB*	536	Lopez *et al.* 2011	53
vanB-r	CCGACAATCAAATCATCCTC				
vanC1-f	GGTATCAAGGAAACCTC	*vanC*	822	Devriese *at al.*	50
vanC1-r	CTTCCGCCATCATAGCT			1996	

Table 2. Number of *E. faecium* strains at different sampling times and/or sampling points

Bacterial species	N	%	Sampling time		Sampling point			
			2015	2016	Gumushane	Samsun	Trabzon	Ordu
Enterecoccus faecium[a]	54	63.52	34	20	19	5	14	16
Enterecoccus faecium[b]	31	36.48	11	20	13	5	8	5
Total	85	100	45	40	32	10	22	21

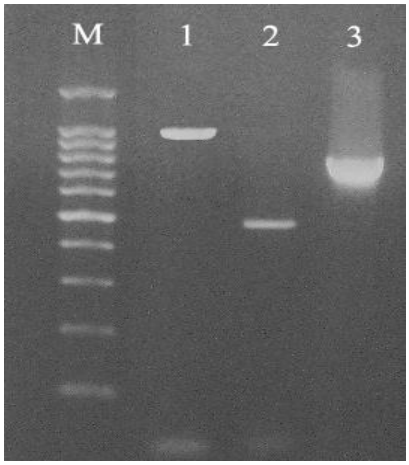

Figure 1. Gel electrophoresis image of different vancomycin resistance genes, M: 100-bp DNA Marker, 1-3*: VanA, VanB, VanC1*.

different ARGs were determined in 85 bacteria and the results of the PCR assays were summarized in Table 3. At least one vancomycin resistance gene was found in 64.7% of the *E. faecium* strains. The vancomycin resistance genes *vanA, vanB* and *vanC1* were determined in 29.41%, 31.76% and 25.88% of bacteria respectively.

Discussion

It is known that, the use of antimicrobial drugs to treat bacterial infections in humans and various animals is the most appropriate method. However, the overuse of antimicrobial agents may lead to the acquirement of antibiotic resistant bacteria in the aquaculture environment (Cabello *et al*. 2013). The ARGs including *van* genes in aquaculture environments can be transferred horizontally among bacteria. Finally, they may transferred from human bacteria to fish pathogenic bacteria. Consequently, the presence of ARGs may restrict the efficiency of antibacterial treatment in aquatic environment (Schwartz *et al*. 2003; Muziasari *et al*. 2014). In this study, we detected ARGs in *E. faecium* isolated from sediment. *VanA, vanB* and *vanC1* genes which are responsible for resistance to vancomycin were investigated by PCR.

The presence of VRE especially in companion animals is becoming a high clinical concern due to transmission risk of VRE via close contact with farmers or their owners. In recent years, there were many studies on occurrence of VRE in different animal species and/or their products. The different isolation rates or diversity of *Enteroccoccus* species can be resulted from different breeding facilities and environmental factors (Seo *et al*., 2005; Cetinkaya *et al*., 2013; Bagcigil *et al*., 2015). Herrero *et al.* (2004) have investigated dogs for the presence of VRE. They have reported that *vanA* resistance gene was prevalent among the *E. faecium* isolates. Another study, Cetinkaya *et al*. (2013) investigate the prevalence of VRE in a animal-originated foods including milk, poultry and meat products. A small number of *Enterococcus* strains were isolated from these samples. All of strains had high levels of vancomycin resistance (MIC≥64 µg/mL). In this study, we identified 85 *E. faecium* strains. In *E. faecium* strains, among *van* genes, *vanB* was the most prevalant resistance gene (31.76%), followed by *vanA* (29.41%) and *vanC1* (25.88%).

In a previous study, the possible role of hospital and wastewater systems on occurrence of antibiotic resistant bacteria and resistance genes were investigated using indicator bacteria including *Enterococcus* and *Stapylococcus*. VRE and *vanA* resistance gene were detected by molecular methods. The *vanA* gene was commonly found in wastewater systems and drinking water biofilms. The results of this report proved possible gene transfer from wastewater system to the drinking water (Schwartz *et al*. 2003).

The role of the aquatic environment on occurrence of antibiotic resistant bacteria and their resistance genes, are not clear yet. Enterococci are naturally consisting bacteria from animal and human intestines. They are found in many aquatic

Table 3. Vancomycin resistance genes of *E. faecium* strains

Bacterial species	Sampling points	N	Antibiotic resistance genes		
			vanA	*vanB*	*VanC1*
E. faecium	A	54	16	21	14
E. faecium	B	31	9	6	8
Total		85	25	27	22

N: Number of bacteria
A: Bacteria which isolated from bottom of net cages
B: Bacteria which isolated from far away the cages as a control point

departments because of their tolerance to diffucult environmental conditions (Manero & Blanch, 1999; Schwartz *et al*. 2003). In this study, both phenotypic and molecular methods were used for detection of VRE and their resistance genes in sediment samples. According to our knowledge, this is the first work on ARGs in VRE isolated from sediment in Turkey. The detection of presence of *vanA*, *vanB* and *vanC1* genes reflecting the higher use of vancomycine in local environment including fish husbandry.

Many researchers have tried to explain the occurrence of antibiotic resistance in different environments including aquaculture systems. Schmidt *et al*. (2000) researched bacterial susceptibility for different antimicrobial agents around fish farms in Denmark. They determined the levels of antibiotic resistance among some of microorganisms isolated from sediment samples. They observed that, there was significant differences between influent and effluent water samples regarding the antibacterial resistance levels. In our work, antibacterial resistance level of bacteria isolated from bottom of net cage, were significantly higher than bacteria isolated from control points.

In conclusion, the results of this work revealed phenotypic and genotypic antibiotic resistance in *E. faecium* strains isolated from sediment. This results also provided insights to understanding resistance levels of VRE in aquatic environments. The frequency of resistance to vancomycin remained intermediate in *E. faecium* strains. Also, antibacterial resistance level of strains isolated from bottom of net cage, were significantly higher than strains isolated from far away cages.

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References

Aoki T & Takahashi A (1997). Class D tetracycline resistance determinants of R plasmids from the fish pathogens *Aeromonas hydrophila, Edwardsiella tarda,* and *Pasteurella piscicida*. Antimicrob Agents Ch 31: 1278–1280.

Bagcigil, AF., Ikiz, S. Ak, S. and Ozgur, NY (2015). Isolation of Vancomycin Resistant Enterococci from Animal Faeces, Detection of Antimicrobial Resistance Profiles and Vancomycin Resistant Genes. Kafkas Univ Vet Fak Derg, 21 (1): 87-94, 2015, DOI: 10.9775/kvfd.2014.11805.

Baquero F., Martinez JL. and Canton R (2008). Antibiotics and antibiotic resistance in water environments. Current Opinion in Biotechnology, 19: 260–265.

Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dolz H, *et al*. (2013). Antimicrobial use in aquaculture re-examined: Its relevance to antimicrobial resistance and to animal and human health. Environ Microbiol 15: 1917–1942.

Capkin E., Terzi E., Altinok I (2015). Occurrence of antibiotic resistance genes in culturable bacteria isolated from Turkish trout farms and their local aquatic environment. Dis Aquat Org, 114, 127–137.

Cetinkaya F, Elalmus T, Soyutemiz GE, Cıbık R (2013). Prevalence and antibiotic resistance of vancomycin-resistant enterococci in animal originated foods. *Turk J Vet Anim Sci*, 37, 588-593, 2013.

Clinical and Laboratory Standards Institute. 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement, M100-S24.

Devriese L, Pot B, Kersters K, Lauwers S, Haesebrouck F (1996). Acidification of methyl-α-D-glucopyranoside: A useful test to differentiate *Enterococcus casseliflavus* and *Enterococcus gallinarum* from *Enterococcus faecium* species group and from *Enterococcus faecalis*. *J Clin Microbiol*, 34, 2607-2608.

Herrero IA, Fernandez-Garayzabal JF, Moreno MA, Dominguez L (2004). Dogs should be included in surveillance programs for vancomycin - Resistant enterococci. *J Clin Microbiol*, 42, 1384-1385.

Huycke MM., Sahm DF. and Gilmore, MS. (1998). Multiple-Drug Resistant Enterococci: The Nature of the Problem and an Agenda for the Future, Synopses, 4, 2, 239-249.

Jung WK, Lim JY, Kwon NH, Kim JM, Hong SK, Koo HC, Kim SH, Park YH (2007). Vancomycin-resistant enterococci from animal sources in Korea. *Int J Food Microbiol*, 113, 102-117.

Lopez M, Tenorio C, Torres C (2011). Study of vancomycin resistance in faecal enterococci from healthy humans and dogs in Spain a decade after the avoparcin ban in Europe. *Zoonoses and Public Health*, 60, 160-167.

Manero A & Blanch AR (1999). Identification of *Enterococcus* spp. with a Biochemical Key, Applied and Environmental Microbiology, 65 (10), 4425–4430,

Marchesi J (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol, 64, 795–799.

Muziasari WI, Managaki S, Parnanen K, Karkman A, Lyra C, Tamminen M, Suziki S. and Virta M (2014) Sulphonamide and Trimethoprim Resistance Genes Persist in Sediments at Baltic Sea Aquaculture Farms but Are Not Detected in the Surrounding Environment. PLoS ONE 9(3): e92702. doi:10.1371/journal.pone.0092702

Rice EW, Boczek LA, Johnson CH, Messer JM. (2003). Detection of intrinsic vancomycn resistant enterococci in animal and human feces. *Diagn Microbiol Infec Dis*, 46, 155-158.

Sapkota A, Sapkota AR, Kucharski M, Burke J, McKenzie S, Walker P, Lawrence R (2008). Aquaculture practices and potential human health risks: current knowledge and future priorities. *Environ Int* 34: 1215−1226.

Schmidt, AS., Bruun, MS., Dalsgaard, I., Pedersen, K. and Larsen, JL (2000). Occurrence of Antimicrobial Resistance in Fish-Pathogenic and Environmental Bacteria Associated with Four Danish Rainbow Trout Farms. Applied And Environmental Microbiology, 66: 11, 4908–4915.

Schwartz T, Kohnen W, Jansen B, Obst U (2003). Detection of antibiotic–resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol Ecol, 43, 325–335.

Seo KS, Lim JY, Yoo HS, Bae WK, Park YH (2005). Comparison of vancomycin-resistant enterococci isolates from human, poultry and pigs in Korea. *Vet Microbiol*, 106, 225-233.

TUIK (2015). Turkish Statistical Institute, http://www.turkstat. gov.tr/Start.do, Access date: 04.02.2017.