Occurrence of Vancomycin 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 environments is one of the major factors, result in occurrence of drug resistant bacteria and antibiotic resistance genes (ARGs). Consequently, the use of antibiotics sometimes fails in fish (Aoki & Takahashi, 1997).

Enterococcus are known as fecal streptococcus and they are a part of human and animal intestinal flora. In recent years, various infections included septicemia, meningitis, endocarditis and genital tract infections were reported. Moreover, they have emerged as a causative 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; Bagecil 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 demonstrate 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 (Bagecil 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 grab (eckman grab, 15x15cm). The sample were collected from three cages in each station. Also, samples were collected from farther cages (1km distance) as control. The samples were usually consisted of 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 use 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 (1998) using primers of the highly conserved 5’ and 3’ regions of 16S rRNA gene and synthesised by Integrated DNA Technologies. PCR was performed according to Capki et al. (2011) 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).

Antimicrobial Susceptibility Test

Following identification, E. faecium strains (n=85) were tested for their susceptibility to vancomycin. Minimum inhibitory concentration (MIC) levels for vancomycin were determined using Epsilometer procedure (E-test, Biotests). Commercial antibiotic test strip 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 individually 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 Enterococcus 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 ) 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 (°), were significantly higher than bacteria isolated from control points (°°). Presence of 3

Table 1. Primers used in the PCR reactions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Target gene</th>
<th>PCR product (bp)</th>
<th>References</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA-f</td>
<td>CATGAATAGAATAAAAAGTGGCAATA</td>
<td>vanA</td>
<td>1030</td>
<td>Devriese et al. 1996</td>
<td>51</td>
</tr>
<tr>
<td>vanA-r</td>
<td>CCCCTT1TAACGCTATACATGCACAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vanB-f</td>
<td>AAGCTATGCAAGAAGCCATG</td>
<td>vanB</td>
<td>536</td>
<td>Lopez et al. 2011</td>
<td>53</td>
</tr>
<tr>
<td>vanB-r</td>
<td>CCGACAATCAATACATCCCTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vanC1-f</td>
<td>GGTATCAAGGAAACCTC</td>
<td>vanC</td>
<td>822</td>
<td>Devriese et al. 1996</td>
<td>50</td>
</tr>
<tr>
<td>vanC1-r</td>
<td>CTTCCGCCCATCAGCT</td>
<td></td>
<td></td>
<td></td>
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</table>
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. 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 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 *Staphylococcus*. 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>
<thead>
<tr>
<th>Bacterial species</th>
<th>N</th>
<th>%</th>
<th>Sampling time 2015</th>
<th>Sampling time 2016</th>
<th>Gumushane</th>
<th>Samsun</th>
<th>Trabzon</th>
<th>Ordu</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>54</td>
<td>63.52</td>
<td>34</td>
<td>20</td>
<td>19</td>
<td>5</td>
<td>14</td>
<td>16</td>
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<tr>
<td><em>Enterococcus faecium</em></td>
<td>31</td>
<td>36.48</td>
<td>11</td>
<td>20</td>
<td>13</td>
<td>5</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td>100</td>
<td>45</td>
<td>40</td>
<td>32</td>
<td>10</td>
<td>22</td>
<td>21</td>
</tr>
</tbody>
</table>

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


