The Antibiotic Susceptibility Pattern and Prevalence of vanA, vanB, and vanC Genes among Enterococcus faecalis Strains Isolated from Consumed Meat

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Abstract

Background: Enterococci play an important role in the spread of drug resistant genes and thus resistant strains. The dissemination of vancomycin-resistant Enterococci (VRE) strains is one of the crucial issues in hospitals worldwide, especially among those hospitalized patients. This study aimed to assess the antibiotic resistance pattern and the prevalence rates of vanA, vanB, and vanC genes among Enterococcus faecalis strains isolated from meat.

Materials and Methods: This cross-sectional study was performed on 181 isolates of E. faecalis isolated from consumed meat samples in Borujerd city. Antibiotic susceptibility testing was performed using the disk diffusion method according to CLSI criteria. The prevalence of vanA and vanB genes in vancomycin resistant E. faecalis strains was identified by PCR technique.

Results: Of 181 Enterococci isolates, 100 strains (55.25%) were E. faesitium, and 81 strains (44.75%) were E. faecalis. About 13 antibiotics were used in this study. The highest resistance was observed against erythromycin, linezolid, vancomycin, and penicillin antibiotics, and the lowest resistance was observed against meropenem; none of the isolates were resistant to nitrofurantoin and cefotaxime. A total of 68 isolates (83.95%) were resistant to vancomycin. Among the isolates, 38 isolates (46.9%) contained vanA gene, 21 isolates (25.9%) carried vanB gene, and 18 (22.2%) isolates contained vanA and vanB genes, but van C type was not detected in none of the isolates.

Conclusion: The presence of van gene in the majority of isolates is an indicator of resistant genes large reservoir in the strains rotation in the community. Furthermore, in order to limit the incidence of VRE, the use of antibiotics for human or animal should be taken with caution.

Key words: Enterococcus faecalis, Antibiotic resistance, Vancomycin, van A / B / C

1. Background

Enterococci are Gram-positive fermentative bacteria seen as a chain or single cocci (1). These bacteria have become appropriate strains for research regarding antibiotic-resistant genes transferred to other species, their permanent existence in the human and animal gut as normal flora, their clinical significance in causing infections, their ability to acquire multi-antibiotic resistance, their high capacity for horizontal genes transduction due to mutation, and receiving genetic components through plasmids or transposons transfer (2). These opportunistic pathogens are capable in causing various types of urogenital, endocarditis, meningitis, bloodstream infections, and infection in newborns (3). In addition to warm-blooded animals, Enterococci are present in water, soils, and vegetables (4). Studies have shown that 90 and 10% of the enterococcal infections in humans are caused by E. faesitium and E. faecium, respectively (5). These two Enterococci species are mostly found in foods such as cheese, fish, sausages, and cowpeas. Many antibiotics including beta-lactams, macrolides, aminoglycosides, and glycopeptides are used for the treatment of the enterococcal infections (6). The resistance of Enterococci to antimicrobial agents is intrinsically (low-level resistance to penicillins, cephalosporins, and aminoglycosides) or acquired (to glycopeptides and high concentrations of aminoglycosides) (7). Due to the spread of multidrug-resistant strains, vancomycin is nowadays as a selective drug for the eradication of drug-resistant enterococcal infections. This drug is one of the last line antibiotics consumed for the treatment of infections caused by Gram-positive multidrug resistant bacteria (8). According to the phenotype and genotype characteristics, there are eight types of vancomycin resistance-acquired genes, including vanA, vanB, vanC, vanE, vanD, vanG, vanM, vanL, and vanN (9).

In addition, an type of inherent resistance has been identified in van C gene (10) as an intrinsic property of motile Enterococci (casseliflavus, gallinarum, flavescens). Genes such as vanA, vanB, vanC, vanC2 / 3, vanG, vanG, and vanL are responsible for coding resistance to vancomycin, teicoplanin, and avoparcin. The most commonly found resistance is due to van A and van B genes which are carried on Tn1546 transposon and Tn5382 transposon, respectively (11). Antibiotics used in the livestock (such as avoparcin, bacitracin, spiramycin, tetracycline, and ampicillin) as a growth factor can act as mediators for the increase and spread of VRE in domestic animals; thus, vancomycin-resistant Enterococci (VRE) strains have the ability to be transferred to humans via infected food (12). During research in Europe and the United States, VRE strains were identified in pigs, breeding horses, cattle, sheep, chickens, and turkeys (13). Previous studies have shown that humans have a large amount of VRE in their stool and thus are considered as the carriers of which. But researchers were not able to find VRE in stool samples of those who had consumed plants for many years; therefore, the theory confirms that meat is infected with VRE source (14).

2. Objective

The aim of this study was to investigate the antibiotic resistance pattern and the prevalence rates of vanA, vanB, and vanC genes among Enterococcus faecalis strains isolated from meat.
### 3. Materials and methods

#### 3.1. Bacterial samples

This cross-sectional study was carried out on 181 Enterococcus isolates isolated from collected red meat of slaughterhouses in Boroujerd city during 2014-2015 years. All the samples were cultured in BHI broth (Merck) medium and incubated at 37°C for 24 h. After 24 hours, colonized bacteria were subjected to the following: staining with gram staining method, catalase test, bile esculin agar, PYR test, growth in BHI broth containing 6.5% sodium chloride, and growth at 45°C. Then, Enterococci isolates were identified by the acid production from mannitol, sorbitol, arabinose, and sucrose (1). Performing the related tests, 81 and 100 isolates of *E. faecalis* and *E. faecium* were identified, respectively.

#### 3.2. The antibiotic susceptibility testing

In antibiotic susceptibility testing, the following antibiotics prepared by ROSCO were used: vancomycin (30 μg/disc), ciprofloxacin (5 μg/disc), gentamicin (10 μg/disc), tetracycline (30 μg/disc), chloramphenicol (30 μg/disc), ampicillin (10 μg/disc), penicillin (10 μg/disc), erythromycin (15 μg/disc), lincomycin (30 μg/disc), nitrofurantoin (30 μg/disc), streptomycin (30 μg/disc), meropenem (10 μg/disc), and cefotaxime (30 μg/disc). The Kirby-Bauer disk diffusion method was performed in the Müller Hinton Agar medium (Merck). Phenotypic resistance was determined according to the CLSI 2015 guidelines. In this study, standard strains of *E. faecalis* ATCC 29212 were used as quality control.

#### 3.3. DNA isolation and PCR reaction

Following the determination of the vancomycin-resistant isolates, DNA extraction was performed using a DNA extraction kit (Sinagen). In order to detect *van A*, *van B*, and *van C* genes, the PCR reaction and their specific primers were used (Table 1).

#### 3.4. PCR amplification of *van genes*

PCR reaction was performed with a final volume of 25 μL, containing 12.5 μL of master mix (Sinagen), 7.5 μL of sterile distilled water, 1 μM of each primer, and 3 ng of template DNA. The PCR reaction was carried out in a thermocycler device (Bio Rad), the temperature and conditions are shown in Tables 2. PCR products were transferred to a 1.5% agarose gel containing ethidium bromide and electrophoresed with 0.5 X TBE buffer (Sina gene). To determine the size of PCR products, a 100-bp molecular marker (Merck) was used. *E. faecium* ATCC 51559 and *E. faecalis* ATCC 51299 were used as a positive control for *van genes* (prepared from the microbial bank of Pasteur Institute of Iran). The standard strain was purchased in the form of liofilization, and its passage was carried out in a nutrient broth medium and incubated at 37°C for 24 h.

#### 2.5. Data analysis

Data were analyzed using SPSS software version 21, where ANOVA tests were applied with 95% confidence interval and a significance level of p<0.05.

### 4. Results

#### 4.1. Bacterial isolates

A total of 181 isolates of Enterococci were isolated from red meat of slaughterhouses in Boroujerd city and identified with the conventional biochemical test and confirmed with 16sRNA genes evaluation by PCR method, of which 81 isolates (44.75%) were *E. faecalis*, and 100 isolates were *E. faecium*.

#### 4.2. The antibiotic susceptibility of isolates

All the 81 isolates of *E. faecalis* were enrolled in the antibiotic susceptibility testing with the disc diffusion method. The highest isolates resistance was observed against erythromycin (96.29%, n=78), linezolid (86.41%, n=70), vancomycin (83.95%, n=68), and penicillin (56.79%, n=46), whereas the lowest resistance was observed against meropenem (2.46%, n=2) and likewise, all were susceptible to nitrofurantoin and cefotaxime discs. The isolates antibiotic resistance profile is depicted in Table 3, and the resistance pattern of *E. faecalis* isolates from red meat is shown in Figure 1.

### Table 1. Primers used for the amplification of *van A*, *van B*, and *van C* genes in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon (bp)</th>
<th>Primer Sequence (5’ → 3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>van A</td>
<td>734</td>
<td>F: AATACTGTITGGGGGTGCT</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTITTTCGCCGTGCACTCCT</td>
<td></td>
</tr>
<tr>
<td>van B</td>
<td>420</td>
<td>F: CCGGGGGAGATTGTCG</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAAGATACCGTGCCCTCAA</td>
<td></td>
</tr>
<tr>
<td>Van C</td>
<td>485</td>
<td>F: ATGCCGTGATCCAAGGACTG</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGCAATGGTGGCTGG</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. PCR reaction thermal profile for the *van A*, *van B* and *van C*.

<table>
<thead>
<tr>
<th>Program</th>
<th>VanA</th>
<th>VanB</th>
<th>VanC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tempreture</td>
<td>Time</td>
<td>Cycles</td>
</tr>
<tr>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>58°C</td>
<td>1 min</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>
vancomycin-resistant Enterococcus faecalis from meat

Table 3. Antibiotic resistance pattern of E. faecalis isolates in this study.

<table>
<thead>
<tr>
<th>The Rate of Resistance, No (%)</th>
<th>Antibiotics Resistance Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 (28.17%)</td>
<td>Two antibiotics</td>
</tr>
<tr>
<td>44 (32.54%)</td>
<td>Three antibiotics</td>
</tr>
<tr>
<td>8 (87.9%)</td>
<td>Four antibiotics</td>
</tr>
<tr>
<td>7 (64.8%)</td>
<td>Five antibiotics</td>
</tr>
<tr>
<td>5 (17.6%)</td>
<td>Six antibiotics</td>
</tr>
<tr>
<td>4 (93.4%)</td>
<td>Seven antibiotics</td>
</tr>
<tr>
<td></td>
<td>All antibiotics</td>
</tr>
<tr>
<td></td>
<td>Susceptible to all antibiotics</td>
</tr>
</tbody>
</table>

Figure 1. The antibiotic resistance pattern of E. faecalis isolates from consumable meat.

4.3. PCR amplification of van A, van B, and van C genes

After examining the extracted genome of vancomycin-resistant E. faecalis (VRE), 68 strains (83.95%) were resistant to vancomycin, 38 (46.9%) amplified van A gene, 21 strains (25.9%) contained van B gene, and in 18 isolates (22.2%) both van A and B van genes were detected, but van C phenotype was not amplified in any of the strains by PCR (Figures 2 and 3).

Figure 2. Electrophoresis of the PCR product of van A gene, L: 100 bp ladder/marker, N: negative control E. faecalis ATCC51299, P: positive control E. faecium ATCC51559, and columns 1-4: van A gene.

Figure 3. Electrophoresis of PCR product of the van B gene, L: 100 bp marker, N: negative control E. faecium ATCC51559, P: positive control E. faecalis ATCC51299, and columns 1-4: the van B gene.

5. Discussion

Food is one of the leading reservoirs for the transmission of antibiotic-resistant bacteria among human and animal populations (16). In this process, those antibiotic-resistance-encoding genes can be transmitted from microbial flora into
human pathogens (17). Enterococci spp are able to colonize in healthy animals, which is a concern due to the presence of strains carrying genes encoding antibiotic resistance and the possibility of spread of non-susceptible Enterococci species (18). Various surveys have demonstrated that primary vancomycin-resistant Staphylococcus aureus (VRSA) strains have probably received vancomycin-resistant gene (vanA) from Enterococcus strains which had contaminated food (19).

The consumption of antibiotics by humans, agricultural industries, and veterinary medicines used for livestock and poultry cultivated for human nutrition increase the resistance patterns and the emergence of multidrug resistant isolates and have made these strains as an epidemiological problem all over the world (20).

In this study, in addition to the prevalence rate of vanA gene, the resistance pattern and multiple resistance pattern of Enterococci spp isolates were investigated. Enterococcus isolates depicted the highest resistance to erythromycin 96.29% (78 samples) and the lowest resistance to meropenem (2.46%, n=2). This finding indicates the most commonly used antibiotics was intaken in one area, and that erythromycin has probably been used far more than other antibiotics in livestock breeding. Drug resistance pattern in various regions of Iran and the world is not the same due to such factors as genetic alterations in the isolates, difference in the amount of antibiotics used, and the difference in the antibiotics availability.

In this study, 82.9% of the isolates were MDR, and 6% of them demonstrated extensively drug resistant (XDR) phenotype. This phenotype is a potential crisis in the treatment of infections caused by these strains in humans.

Chingwaru and colleagues (2003) isolated E. faecalis strains from 46.1% of the meat samples, consistent with the frequency of this bacterium in the present study. In their study, although the prevalence of the residual pattern obtained by disc diffusion method showed 96% resistance to ampicillin, resistance to vancomycin was observed in 39.7% of the isolates (21), which was inconsistent with the present study results in which 83.95% resistance to vancomycin was an enhancing trend. In another study, the MIC method used for E. faecalis strains isolated from pork showed a higher concentration for the isolates than what is recommended for antibiotics like vancomycin (22).

Considering the similarity between the resistance pattern of the isolates such as Enterococi spp in hospital settings and environment, it is possible to reduce the risk of clinical resistance by controlling and preventing environmental contamination.

Rozanska et al. (2015) showed that 94 (119.68%) E. faecalis strains isolated from red meat were resistant to vancomycin, which is consistent with our results (23).

In a research conducted by Johnston (2004) in the United States on fresh meat products, 34% of the E. faecalis isolates were multidrug resistant (24) while in our research, the amount of multidrug resistant isolates were significantly higher (2.5 fold), highlighting the irrational use of antibiotics in the food industry.

6. Conclusion

The emergence of MDR enterococcal strains has led to a crisis in the medical community. Therefore, the following steps are recommended to be taken: the use of control methods for preventing the horizontal transmission of genes, the proper use of antibiotics, and taking into account the important role of livestock specimens as the reservoirs of resistance indices because these factors have a significant effect on decreasing trend of the resistance phenotype. Due to the transferability of vancomycin-resistant genes among animals, humans, and the environment, the contamination of consumable meat by VRE is a serious threat for the transfer of these resistant strains to humans. Resistance to glycopeptides, such as vancomycin and teicoplanin, limits the therapeutic selections, and there is another risk of resistance genes transfer to other bacteria such as Staphylococci strains. In order to limit the incidence of VRE, caution should be exercised in medicines consumption both by animal and human; also, precaution should be taken against the spread of Enterococci species by carefully monitoring the health and nutrition systems.

Conflict of interest
None to declare by the authors.

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Authors Contribution
MR.Mehraei designed the research, helped in data analysis and writing the paper; E.Madanipour performed experiments, wrote the paper, and analyzed the data; M.Mirzaei advised for PCR technique, extracted DNA.

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