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Frequency of Vancomycin and Aminoglycoside Resistance Genes in *Enterococcus* spp. Isolated from Chicken Raw Meat Samples

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ABSTRACT

Background: Enterococci are main normal microbial flora of both humans and animals and can survive in a diverse range of environments. These bacteria carry out aminoglycoside and vancomycin resistance genes and spread them in environment by many routs such as chicken meat products. The present study was aimed to determine the frequency of aminoglycoside and vancomycin resistant genes in *Enterococcus* species isolated from chicken meat specimens.

Methods: A total of 250 chicken raw meat specimens was prepared from slaughterhouses at Zanjan province, cultured at BHI broth and incubated at 37°C for 24h. The positive cultures were sub-cultured in blood agar plates and grown colonies identified using phenotypical and biochemical tests. Antibiotic susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) standards and PCR assays were performed to detect *vanA*, *vanB*, *aph* (2")1c, *aph* (2")1b, *aph* (2")1d, *ant*(3'), *aph*(3')IIIa, *ant*(4')1a, *ant*(6') and *aac*(6') genes.

Results: In total, 100 Enterococcus species isolated from 250 specimens and 35% of them belonged to E. faecalis and the others were E. faecium (65%). The prevalence of the *vanA*, *vanB*, *aph* (2")1c, *aph* (2")1b, *aph* (2")1d, *ant*(3'), *aph*(3')IIIa, *ant*(4')1a, *ant*(6') and *aac*(6') genes among the 100 *Enterococcus* species was 14%, 12%, 10%, 1%, 2%, 50%, 26%, 9%, 18% and 22%, respectively.

Conclusion: The current study revealed that the rate of antimicrobial resistance genes to aminoglycosides and vancomycin was worrying and health measurements in meat products industries must be performed to prevent spread of antimicrobial resistance elements among bacteria.

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Introduction

Enterococci are catalase negative and Gram-positive cocci which belong to *Enterococcaceae* family (1). Despite to existence up to sixty-one species, the most infections are associated with *E. faecalis* and *E. faecium* (2). Although these organisms have been usually regarded as low grade pathogens, however they are an important cause of nosocomial infections (3). Other infections caused by these species include endocarditis, bacteremia, intra-abdominal infections and urinary tract infections (4).

The clinical importance of enterococci comebacks to their ability to resist against antimicrobial agents. enterococci are always considered to be resistance against clindamycin, intrinsically. The expression of the penicillin-binding proteins with lower affinity to cephalosporins and the semi-synthetic penicillins leads to resistance to these antimicrobial agents and their usage in the clinical applications has been restricted (5).

Resistance to vancomycin among *Enterococcus* isolates is also a main concern in the public health and its rate is increasing annually (6, 7). The resistance to vancomycin is associated with several phenotypes including VanA, VanB, VanC, VanD, and VanE. VanA resistance phenotypes is associated with expression of some of transposon genes which produce an atypical peptidoglycan precursor ending in d-Ala–d-lactate instead of normal d-Ala–d-Ala unit (8). Enterococci also produce penicillin-binding proteins (PBPs), which result in a low-level intrinsic resistance to β-lactam agents because of low-affinity to these antibiotics (9).

Enterococci with high antimicrobial resistance phenotype have been distributing across the world. The existence of these bacteria in animals as normal flora and hence, contamination of their products such as meat, milk, can leads to human infections (10). Vancomycin-resistant enterococci (VRE) seem to be a main public health's concern because there are treated by routine therapeutic approaches unsuccessfully (11).

There limited data about prevalence and antimicrobial resistance trait of environmental and food related enterococci. Hence, the present study aimed to investigate the vancomycin and aminoglycoside resistance rate in *Enterococcus* species isolated from chicken raw meat specimen.

Materials and Methods

Preparation of Poultry meat and culture

In the present study, a total of 250 chicken meat specimens was collected from several slaughterhouses at Zanjan province. All of specimens were transferred to laboratory at 4°C without any deferment. The specimens were homogenized and cultured at Brain hart infusion broth(BHI) (Merck, Germany) and incubated at 37°C for 24h. The positive cultures were sub-cultured in blood agar plates (Merck, Germany) and following incubation for 24h, colonies identified using phenotypical and biochemical tests. The isolated strains were added into BHI broth with 20% glycerol and stored at -80°C.

Antibiotic susceptibility testing

Antibiotic susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) standards using antibiotic discs purchased from MAST Company. The following antibiotics were selected for testing: gentamicin (GEN 120 µg), tobramycin (TOB 10 µg), ampicillin (AMP 10 µg), amikacin (AMK 30 µg), tetracycline (TET 30 µg), chloramphenicol (CLM 30 µg), neomycin (NEO, 100 µg) and vancomycin (VAN 30 µg). All isolates were cultured in Mueller-Hinton agar and incubated 37°C for 24h. Bacterial suspensions were provided from overnight cultures, adjusted to the 0.5 McFarland turbidity standard and then, the organisms were spread on the surface of a Muller Hinton agar

(Difco) plate using a cotton swab. Following 15 min, the antibiotic's disks were applied to the plates and incubated at 37°C for 18h. The diameter of the inhibition zone measured using a ruler.

DNA Extraction

All isolates were cultured on TSA broth (Merck, Germany) and incubated at 37°C for 24 h. The cultures were centrifuged at 4000 rpm for 5min, and genomics DNA extracted using Cinna Pure DNA extraction kit according manufacturer protocols (Tehran, Iran). The concentration of extracted DNA was calculated by an ND-1000 spectrophotometer (Nano Drop, Wilmington, DE, USA).

Polymerase chain reaction (PCR)

PCR was separately performed in optimized condition. Similar PCR reactions were applied to detect aph (3') IIIa, aac (6'), ant (6')Ia and ant (4')Ia genes. The assays carried out in a final 25ul reaction mixture containing 12.5 µL of 2× PCR Master mix, 10 pmol of both forward and reverse primers (Bioneer, Korea) (Table 1) and 50 ng DNA.

The PCRs were run at the following temperatures cycles: initial denaturation at 94°C, 5 min; 30 cycles of 94°C for 30s, [60°C for 45s (vanA and vanB), 54°C for 30s (aac(6'), aph(3')IIIa, aph(2')Ic, aph(2')Ib and aph(2')Id), 52.2°C for 60s (ant(3')III, ant(4')Ia and ant(6')Ia)], and 72°C for 1 min; and final extension at 72°C for 10 min using a thermocycler (Eppendorf Thermal cycler, Germany). The amplicons were electrophoresed using a 1% (W/V) agarose gel, stained with a DNA Safe Stain (CinnaGen, Iran) and finally visualized under a gel documentation system (Bio-Rad, Germany).

Result

In the present study, 100 *Enterococcus* spp. were isolated from 250 samples that belonged to *E. faecalis* (35) and *E. faecium* (65).

Antimicrobial susceptibility testing

All of *E. faecalis* and *E. faecium* isolates were evaluated for their antibiotic susceptibility profiles and the results shown in Figure 1. As show in Figure 1, high and low resistance rates were to tetracycline (70.3%) and neomycin (24%), respectively.

The frequency of vancomycin and aminoglycoside resistance genes

The prevalence of the vanA, vanB, aph (2'')1c, aph (2'')1b, aph (2'')1d, ant(3'), aph(3')IIIa, ant(4')1a, ant(6') and aac(6') genes among the 100 *Enterococcus* species was 14%, 12%, 10%, 1%, 2%, 50%, 26%, 9%, 18% and 22%, respectively. The electrophoresis Figures of vanA, vanB, aph(2'')-1d, aac(6'), ant(4')Ia and ant(6') amplicons are shown in figures 2-7.

Frequency of vanA gene among isolates

In the present study all isolates were screened for vanA gene and the positive amplicons observed in 18 isolates. The agarose gel electrophoresis for 6 positive isolates are shown on Figure 2.

Frequency of vanB gene among isolates

In the present study, the PCR reaction showed that only 12 isolates were positive for vanB gene. The agarose gel electrophoresis for 3 positive isolates are shown on Figure 3.

Frequency of aph(2")-1d gene among isolates

From 100 isolated strains, only 2 isolates were positive for aph(2")-1d gene (Figure 4).

Frequency of aac(6') gene among isolates

The molecular screening aac(6') gene among isolates showed that 22 isolates were positive for aac(6') gene with 368bp amplicon (Figure 5).

Frequency of ant(4')Ia gene among isolates

Out of 100 isolates, the intended amplicon (294bp) was shown in 9 strains that presented in Figure 6.

Frequency of ant (6') gene among isolates

Screening ant (6') gene among 100 isolates showed 18 positive isolates for the intended amplicon (688bp). The agarose gel electrophoresis for six positive isolates are shown on Figure 7.

Discussion

Although *Enterococcus* spp. are ubiquitous in nature and found in soil, waters, raw plant and animal products, however their importance in clinical cases comeback to their intrinsic resistance feature and abilities to resist against wide range of antimicrobial agents(16).

In the present study, out of 100 strains isolated from meat specimens, 35 isolates (35%) were positive for *E. faecalis* and 65 isolates belonged (65%) to *E. faecium*. Based on our data, the prevalence of *E. faecium* isolates was more than *E. faecalis* in the meat contamination.

Similar studies have been done worldwide. In the study by Kim et al. a total of 345 enterococci including 335 *E. faecalis* and 10 *E. faecium* obtained from meat products and the high-level ciprofloxacin-resistant (HLCR) seen in one *E. faecium* and 86 *E. faecium* isolates (17). The authors concluded that the food chain has a main role in enterococcal infections in humans.

In a study by Hayes et al. out of 1,357 enterococcal strains isolated from the retail meats, *E. faecium* (61%) and *E. faecalis* (29%) were the predominant species, respectively. The first species was more in ground turkey and *E. faecalis* was the predominant species recovered from pork chops. The antimicrobial testing results showed that all of the isolates were resistant to quinupristin-dalfopristin but the resistance to linezolid or vancomycin was not observed (18). In our study, a notable of resistance to vancomycin (25.9%) among enterococcal isolates was reached.

Antibiotic resistance is more common among Enterococcus species and our results also showed that the prevalence of the vanA, vanB, aph(2")1c, aph(2")1b, aph(2")1d, ant(3'), aph(3')IIIa, ant(4')1a, ant(6') and aac(6') genes in the isolated strains was 14%, 12%, 10%, 1%, 2%, 50%, 26%, 9%, 18% and 22%, respectively. In a study revealed that the frequency of vanA and vanB positive Enterococcus species, isolated from raw meat specimen, was 10.7% and 8.3% respectively. In addition, high rates of resistance were also observed for kanamycin, streptomycin and gentamycin (19).

In study by Pavia et al. resistance to vancomycin was observed in *E. faecium* (75%) and *E. faecalis* (40%) species, which have been isolated from various raw meat specimen (20). About of 65.4% isolates have been isolated from chicken meat and a high-rate resistance for streptomycin (88.9%) have also reported. Unlike the above-mentioned study, the isolates in our study have less resistance rate to vancomycin and streptomycin. The main reason to justify the high rate of the resistance against vancomycin comebacks to the fact that some producers applied antibiotics to livestock feed as feed additive (21). Although application of antibiotic in animal feed has some benefits but it also has several disadvantages consequences. The presence of plasmid related antimicrobial resistance genes among *Enterococcus* spp. led them act as reservoir of resistance determinants.

Table 1. Primers used in this study.

Target gene	Primer sequence (5′→3′) (Forward and reverse primers)	Amplicon size (bp)	Reference
*van*A	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030	Clark et al.(12)
*van*B	GTG ACA AAC CGG AGG CGA GGA CCGCCATCCTCCTGCAAAAAA	433	Clark et al.(12)
aac(6')	AGGAATTTATCGAAAATGGTAGAAAAG CACAATCGACTAAAGAGTACCAATC	368	Vakulenko et al.(13)
aph(3')IIIa	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	Emaneini etal.(14)
aph(2')Ic	CCACAATGATAATGACTCAGTTCCC CCACAGCTTCCGATAGCAAGAG	444	Vakulenko et al.(13)
aph(2')Ib	CTTGGACGCTGAGATATATGAGCAC GTTTGTAGCAATTCAGAAACACCCTT	867	Vakulenko et al.(13)
aph(2')Id	GTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	642	Vakulenko et al.(13)
ant(3')III	CACGCTATTACGAACTATGA TAAGAAAGAACATCACCAACGA	526	Amini et al.(15)
ant(4')Ia	CAAACTGCTAAATCGGTAGAAGCC GGAAAGTTGACCAGACATTACGAACT	294	Vakulenko et al(13)
ant(6')Ia	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	Amini et al.(15)

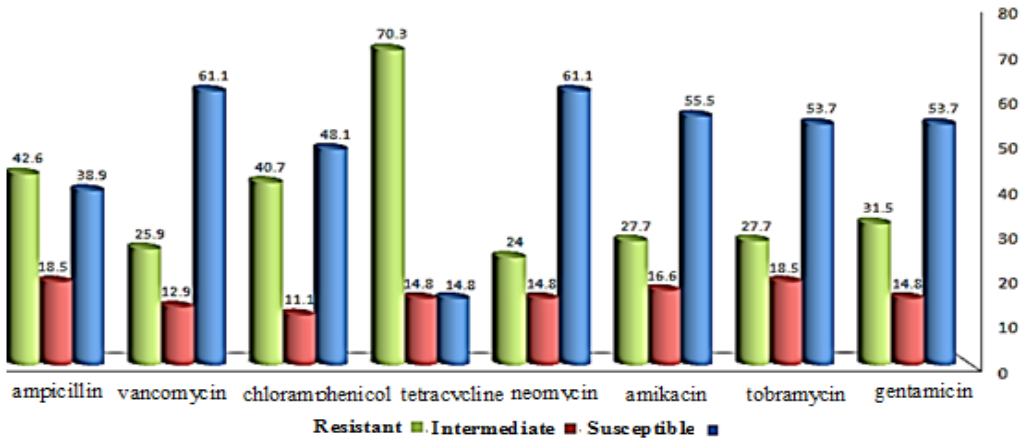

Figure 1. The frequency of antimicrobial resistance in *Enterococcus* isolates.

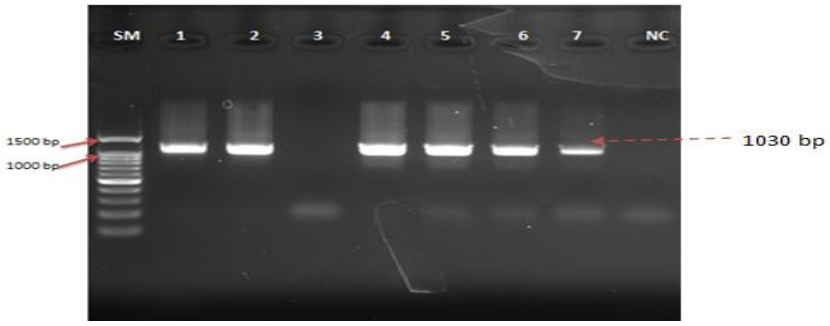

Figure 2. Electrophoresis of vanA amplicon on agarose gel 1%. NC; negative control and the lanes 1 to 2 and 4 to 7 are positive reactions (1030 bp in length).

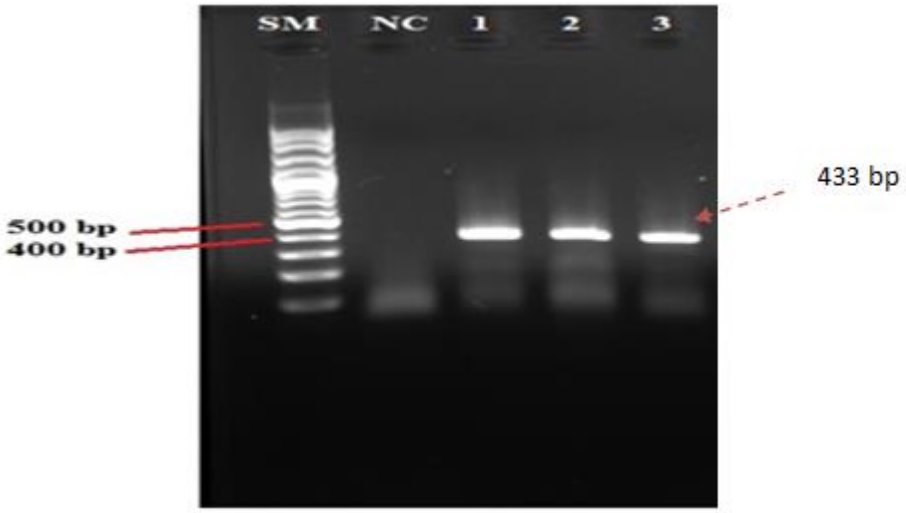

Figure 3. Electrophoresis of vanB amplicon on agarose gel 1%. NC; negative control and the lanes 1 to 3 are positive reactions with 433bp size.

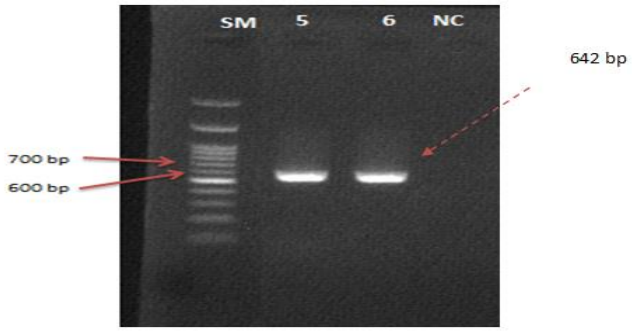

Figure 4. Electrophoresis of aph(2")-1d amplicon on agarose gel 1%. NC; negative control and the lanes 5 and 6 are positive reactions with 642bp size.

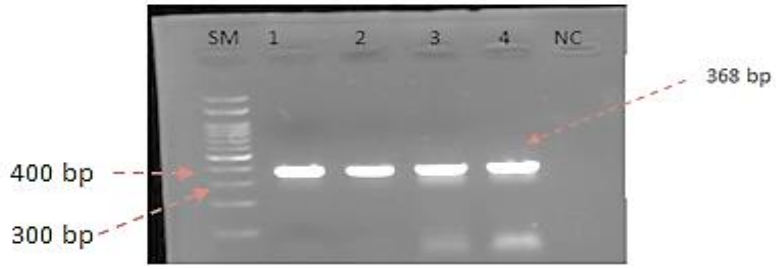

Figure 5. Electrophoresis of aac(6') amplicon on agarose gel 1%. NC; negative control and the lanes 1to 4 are positive reactions with 368bp size.

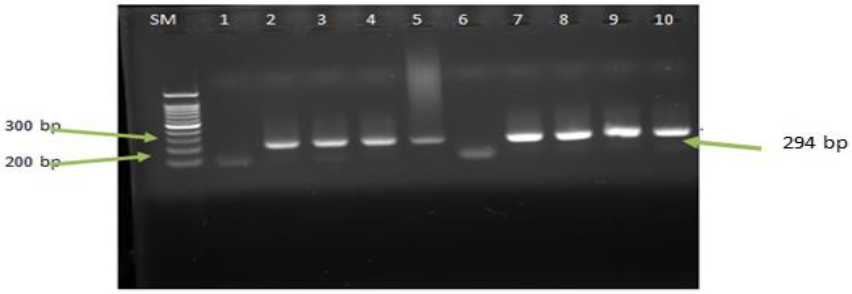

Figure 6. Electrophoresis of aph(2")-1d amplicon on agarose gel 1%. NC; negative control and the lanes 5 and 6 are positive reactions with 642bp size.

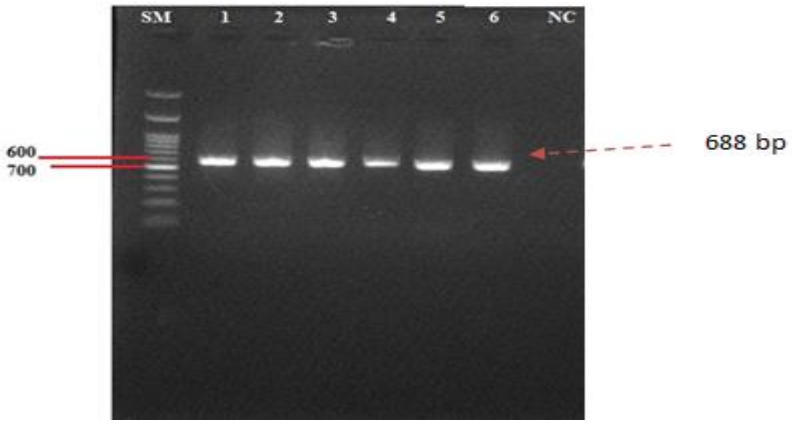

Figure 7. Electrophoresis of ant(6') amplicon on agarose gel 1%. Lane NC; negative control and the lanes 1 to 6 are positive reactions with 688bp size.

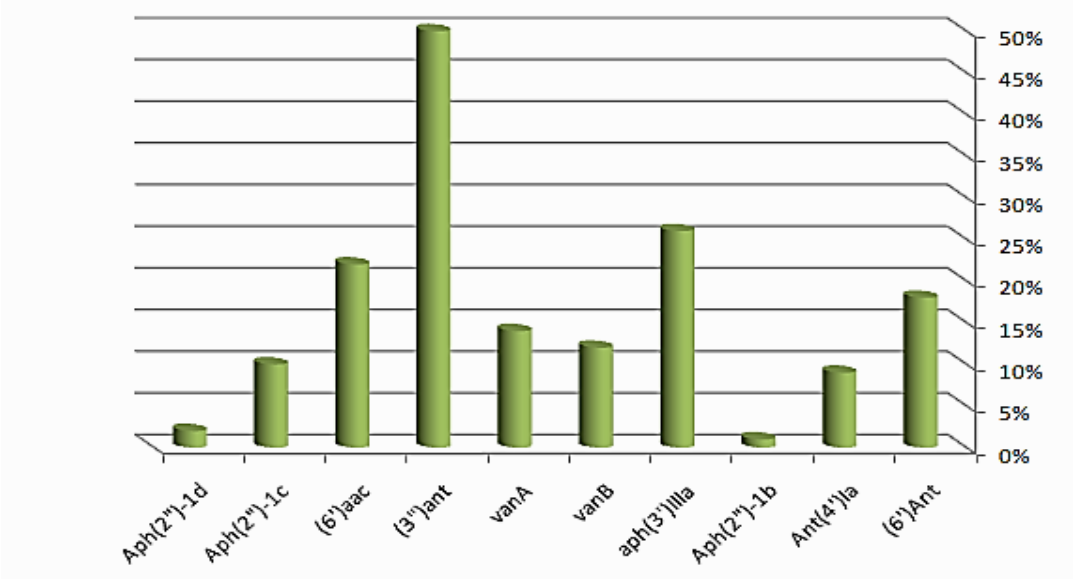

Figure 8. The frequency of antimicrobial resistanc genes among Enterococcus isolates. The highest frequency is related to ant(3') gene.

These determinants could spread easily by enterococcal-contaminated meat products and hence, it is important to evaluate epidemiological aspects and mobility of these determinants (22).

Conclusion

In conclusion, our data show that the rate of resistance to antimicrobial agents especially tetracycline is high and the appropriate health proceedings must be performed to prevent the extension of antibiotics-resistant bacteria in environment.

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The present project approved by research code: A-12-392-29 and ethical code IRZUMS.RE,1396.275.

Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors notified that there are no conflicts of interest.

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