



Study of *VanA*, *B*, *C*, *D*, *E* Genes in Vancomycin Resistant Enterococci Isolated from Retailed Dried Vegetables in Tehran, Iran

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Keywords: *Enterococcus* spp., Vancomycin, Van genes, Dried vegetables, Antibiotic resistance



Abstract

Background: *Enterococcus* spp. are resistant to many antimicrobials including vancomycin. They may be found in foods and water.

Objective: In the current study, *van* genes were investigated in vancomycin resistant enterococci (VRE) isolated from dried vegetables in Tehran, Iran.

Materials and Methods: In this study, 140 dried vegetable samples were collected from local retailers in Tehran, Iran, 2015. Bacteria were isolated using culture, biochemistry and molecular methods. Susceptibility of the enterococcal isolates was assessed to six antibiotics of ampicillin, chloramphenicol, erythromycin, gentamicin, tetracycline and vancomycin using Kirby-Bauer method. The prevalence of *vanA*, *B*, *C*, *D*, *E* genes was molecularly studied in VRE using polymerase chain reaction (PCR) and sequencing techniques.

Results: Of 140 dried vegetable samples, *Enterococcus* spp. strains were isolated from 84 samples (60%). Totally, 48% of the isolates were resistant to vancomycin. Of 41 vancomycin-resistant enterococcal isolates, *vanA* was found in 23 (56.1%), *vanB* in 8 (19.5%) and *vanC* in 2 (4.9%) isolates. No *vanD* or *vanE* was found in the isolates. Results have shown a high rate of contamination with *Enterococcus* spp., especially VRE, in dried vegetables in Tehran.

Conclusion: Therefore, further hygienic regulations such as personal training and food processing, transportation, storage and marketing must be routine in food industries and local retailers.

Keywords: *Enterococcus* spp., Vancomycin, Van genes, Dried vegetables, Antibiotic resistance

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Background

Enterococcus spp. are facultative anaerobic, Gram-positive, coccid form, non-motile (except *Enterococcus gallinarum* and *Enterococcus casseliflavus*) non-spore-forming bacteria belonging to Firmicutes division, Bacillus class, Lactobacillales order and Enterococcaceae family.^{1,2} The genus consists of more than 50 species now with *E. faecalis* and *E. faecium* as the most prevalent *Enterococcus* species.³ *Enterococcus* spp. belong to lactic acid bacteria (LAB) as some enterococcal species are currently used as probiotics in foods.^{4,5} Generally, *Enterococcus* spp. are not primary pathogens but they opportunistically cause various infections in humans and animals such as nosocomial and super infections.⁶ The bacteria most commonly infect biliary ducts, cardiovascular systems, gastrointestinal (GI) tracts, urogenital systems and burn wounds; however, they normally colonize GI tracts of humans and animals.⁷⁻⁹ *Enterococcus* spp. are inherently resistant to antibiotics such as low-level aminoglycoside, cephalosporins, clindamycin,

lincosamides, nalidixic acid and penicillins.^{10,11} Furthermore, they are resistant to antibiotics such as high-level aminoglycosides, chloramphenicol, clindamycin, erythromycin, fluoroquinolones, penicillins, tetracycline and vancomycin.¹² One of these antibiotics, vancomycin, is used as the first-line antibiotic in infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and is mediated by *van* genes. Vancomycin-resistant enterococci (VRE) can result in severe health problems in infected patients.^{13,14} Based on the highlighted bacterial risks for the public health, the aims of the current study included isolation of *Enterococcus* spp. from dried vegetables sold in Tehran, Iran, susceptibility assessment of the those isolates to six antibiotics of ampicillin, chloramphenicol, erythromycin, gentamicin, tetracycline and vancomycin and investigation of *vanA*, *B*, *C*, *D*, *E* genes usually responsible for the resistance to those antibiotics in VRE isolates.

Materials and Methods

Sampling

A total number of 140 dried vegetable samples, including 70 bulk and 70 packaged samples, were collected from the local retailers of Tehran, Iran, May–October 2015. The sample size was calculated based on the current prevalence of the bacteria in the region and included 10 bulk and 10 packaged samples of seven vegetables of coriander, dill, mint, parsley, tarragon, a mixture of chives, coriander and parsley and a mixture of chives, coriander, fenugreek and parsley.¹⁵ The samples were transferred to the Food Microbiology Laboratory of the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Bacterial Isolation

Bacterial isolation was carried out using regulations of Institute of Standards and Industrial Research of Iran (ISIRI) (Nos. 2198 and 5939) and a modified protocol originally described by Junco et al.^{16,17,18} Briefly, 5 g of each vegetable sample was added to 45 mL of peptone broth and mixed. After 10–20 minutes of incubation at room temperature, 1 mL of the mixture was added to 10 mL of bromocresol purple azide broth and incubated at 37°C for 24–48 hours to change the color of the media to yellow. Then, it was cultured on Kenner Fecal (KF) media containing 1% tetrazolium and incubated at 37°C for 24 to 48 hours. Suspected colonies (red or dark pink) were verified using biochemical tests, including catalase, oxidase, bile-esculin hydrolysis, growth in 6.5% NaCl, growth at 10 and 45°C, arginine dehydrogenase (ADH), pyrrolidonyl aminopeptidase (PYR), pigment formation, motility and arabinose, mannitol, raffinose, sorbitol, sorbose and sucrose carbohydrate fermentation tests.

Antibiotic Susceptibility Test

The antibiotic susceptibility scheme of the bacterial isolates was assessed using Kirby-Bauer (disk diffusion) method published by Arumugam et al.¹⁹ Briefly, bacterial suspensions of 0.5 McFarland Turbidity Standard were spread on Mueller-Hinton agar plates and then antibiotic disks (ampicillin, chloramphenicol, erythromycin, gentamicin, tetracycline and vancomycin) were placed carefully on the surface of the agar. Plates were incubated at 37°C for 24 hours and then diameters of growth inhibition zone were calculated and results were reported based on CLSI guidelines.²⁰

Polymerase Chain Reaction

The PCR of enterococcal isolates was carried out using specific primers and a modified protocol first described by Mazaheri Nezhad Fard et al (Table 1).²¹ One overnight-cultured colony of each isolate was suspended in 200 mL of sterile distilled water (DW) and heated at 95°C for 20 minutes. The mixture was centrifuged at 7500× g for 5 minutes and the supernatant was used as DNA template in PCR. To prepare a final volume of 20-mL PCR Master

Table 1. An Overview of the Genes and PCR Primers

Primer	Sequence (3' → 5')	bp*	Ref.
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCCGGCCGTTA	732	56
vanB	F: ATGGGAAGCCGATAGTC R: GATTTCGTTCCTCGACC	635	56
vanC	F: GGTATCAAGGAAACCTC R: CTTCCGCCATCATAGCT	822	56
vanD	F: CTCCTACGATTCTCTTG R: CGAGCAAGACCTTTAAG	439	56
vanE	F: ATCAAGTACAGTTAGTCT R: ACGATTCAAAGCTAACTG	941	56

*bp: base pair; ref.: reference.

Mix (Pishgam, Iran) for each sample, 1 µL of each primer in total concentration of 10 pmol was mixed with 12.5 µL of the Master Mix in a sterile microtube. Then, 3.5 µL of sterile DW was added to the mixture to make a total volume of 18 µL. Two µL of the extracted DNA was added to the mixture as a template and amplified using Peqlab Thermal Cycler (VWR, Germany). Conditions of cycles in the thermal cycler were as follows: Initial denaturation started at 94°C for 4 minutes followed by 30 cycles, each cycle included denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and elongation at 72°C for 3 minutes. Final elongation was carried out at 72°C for 4 minutes. PCR products were electrophoresed in 1% agarose gels and visualized under UV light. Molecularly identified isolates, harboring the highlighted *van* genes from previous studies by Tehran University of Medical Sciences, were used as positive controls. Positive DNA samples were rechecked by PCR.

Gene Sequencing

Amplified DNAs of the six enterococcal isolates, representing *vanA*, B, C, D, E genes, were sequenced using Sanger method and the results were compared with those from GenBank.

Results

Out of 140 dried vegetable samples, *Enterococcus* spp. strains were isolated from 84 samples (60% totally), including 34 (48.6%) and 50 (71.4%) from bulk and packaged vegetable samples. Of these 84 contaminated samples, 15 samples (75%) belonged to parsley, 15 (75%) to coriander, 14 (70%) to tarragon, 12 (60%) to dill, 12 (60%) to mixed chives, coriander and parsley, 10 (50%) to mixed chives, coriander, fenugreek and parsley and 6 (30%) to mint. These included 61 *E. faecium* (72.6%), 10 *E. durans* (11.9%), 6 *E. gallinarum* (7.1%), 5 *E. faecalis* (6%), 1 *E. avium* (1.2%) and 1 *E. casseliflavus* (1.2%). In bulk samples, the most and the least prevalent samples belonged to *E. faecium* (70%) and *E. faecalis* (6%), respectively (Table 2). No *E. avium* and *E. casseliflavus* were isolated from the bulk samples. In packaged samples, the most prevalent species belonged to *E. faecium*

Table 2. Contamination of Retailed Dried Vegetable Samples with *Enterococcus* spp.

Bacteria	Bulk Sample (%)	Packaged Sample (%)
<i>E. faecium</i>	24 (70%)	37 (74%)
<i>E. faecalis</i>	2 (6%)	3 (6%)
<i>E. gallinarum</i>	3 (9%)	3 (6%)
<i>E. durans</i>	5 (15%)	5 (10%)
<i>E. avium</i>	0 (0%)	1 (2%)
<i>E. casseliflavus</i>	0 (0%)	1 (2%)
Total	34 (100%)	50 (100%)

Bulk, contamination of bulk samples; packaged, contamination of packaged samples.

(74%) and the least prevalent to *E. avium* (2%) and *E. casseliflavus* (2%) (Table 2). In bulk samples, the highest antibiotic resistance was seen to gentamicin (88%) and the least resistance to chloramphenicol (9%) and ampicillin (9%) (Table 3). In packaged samples, the highest and the lowest antibiotic resistance rates were seen to gentamicin (96%) and to chloramphenicol (0%) and ampicillin (0%), respectively (Table 3). Overall, 93% of the enterococcal isolates (n = 84) were resistant to gentamicin, 48% to vancomycin, 32% to erythromycin, 15% to tetracycline, 4% to chloramphenicol and 4% to ampicillin. Of 41 vancomycin-resistant isolates, 23 (56%) included *vanA*, 8 (20%) included *vanB* and 2 (5%) included *vanC* (Figures 1, 2 and 3). No *vanD* or *vanE* was found. The results of *van* gene sequencing showed a high rate of similarity to those from GenBank database (data not shown).

Discussion

Vegetables can be contaminated with bacteria such as *Enterococcus* spp. which can cause GI infections. Enterococci are mostly resistant to harsh environmental conditions such as heating and freezing.²² Furthermore, antibiotic resistance in *Enterococcus* spp. has been rapidly increased within the last few decades due to the extensive use of antibiotics.²³ This antibiotic resistance can be readily transferred to other bacteria within the intestinal microflora. Since no enterococci are allowed in food or water and because these bacteria are mostly found in intestines of humans and animals, food contamination with enterococci is a good sign of fecal contamination as well.²⁴ *Enterococcus* spp. are generally categorized as

Table 3. The Results of Bacterial Susceptibility Test

Antibiotic	R (%)	SR (%)	S (%)	Total (%)
Ampicillin	3 (4)	7 (8)	74 (88)	84 (100)
Chloramphenicol	3 (4)	9 (11)	71 (85)	84 (100)
Erythromycin	27 (32)	32 (38)	25 (30)	84 (100)
Gentamicin	78 (93)	3 (4)	3 (3)	84 (100)
Tetracycline	13 (15)	11 (13)	60 (72)	84 (100)
Vancomycin	41 (48)	20 (24)	23 (28)	84 (100)

R: No. of resistant isolates; SR: No. of semi-resistant isolates; S: No. of susceptible isolates.

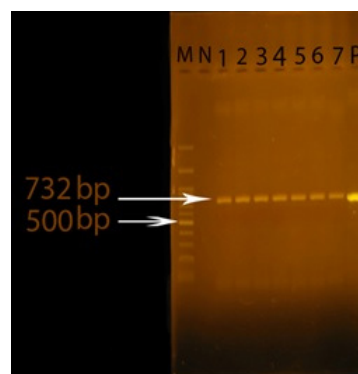


Figure 1. Gel Electrophoresis of *VanA* Gene. M: 100-bp ladder; N: Negative Control; Lanes 1, 2, 3, 4, 5, 6 and 7: 732-bp *VanA* Genes; P: Positive Control

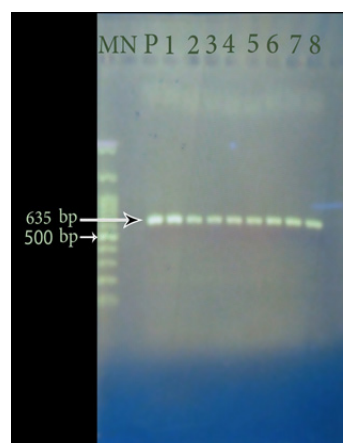


Figure 2. Gel Electrophoresis of *VanB* Gene. M: 100-bp ladder; N: Negative Control; P: Positive Control; Lanes 1, 2, 3, 4, 5, 6, 7 and 8: 635-bp *VanB* Genes.

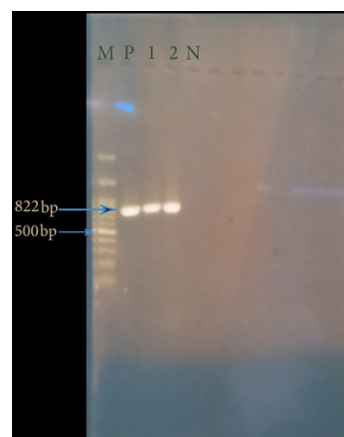


Figure 3. Gel Electrophoresis of *VanC* Gene. M: 100-bp ladder; P: Positive Control; Lanes 1 and 2: 822-bp *VanC* Genes; N: Negative Control.

fecal indicator bacteria (FIB). In the current study, of 140 dried vegetable samples, *Enterococcus* spp. were isolated from 84 samples, mostly *E. faecium* (72.6%). These results were similar to those reported by Johnston and Jaykus.²⁵ They isolated *Enterococcus* spp. from 300 leafy vegetables and reported that 52 and 21% of the isolates belonged

to *E. faecium* and *E. faecalis*, respectively. Torre et al (2010) showed that 105 (70%) out of 150 samples were contaminated with *Enterococcus* spp., mostly (62%) *E. faecium*.²⁶ In a study by Soltan Dallal et al on 100 various fresh vegetables, all were contaminated with enterococci.¹⁵ Similar to other studies, the higher resistance of *E. faecium* compared to *E. faecalis* in the present study can be explained by the natural extraordinary resistance of *E. faecium* strains to antimicrobials and ability to acquire resistance genes from mobile genetic elements (MGEs). In the present study, no *E. avium* and *E. casseliflavus* was isolated from the bulk samples. Since the prevalence of these species in packaged vegetables was low possibly due to the secondary contamination, lack of prevalence of the highlighted species is not unexpected. Furthermore, *E. avium* and *E. casseliflavus* are mostly isolated from birds.

In this study, most isolates were resistant to the assessed antibiotics including 93% to gentamicin, 48% to vancomycin and 32% to erythromycin. In a study by Ben Said et al in Tunisia, 72.2% of the vegetable samples were contaminated with enterococci including 52.3% *E. faecium* and 6.15% *E. faecalis*, from which, 18% of the isolates were resistant to erythromycin, 15.4% to tetracycline, 7.7% to chloramphenicol and 6.15% to vancomycin.²⁷ Tyson et al in a study on antimicrobial resistance of enterococci isolated from meats in the United States, 2002–2014, reported a multiple drug resistance pattern of the isolates to antibiotics such as erythromycin, gentamicin and tetracycline.²⁸ Furlaneto-Maia et al reported a high rate of enterococcal resistance to erythromycin (86.7%), vancomycin (80.0%), tetracycline (43.35) and gentamicin (33.3%) in Brazil.²⁹ Bacterial antibiotic resistance has become an urgent health problem worldwide. Nowadays, the majority of bacteria are resistant to most routine antibiotics.^{30,31} Of these bacteria, *Enterococcus* spp., are repeatedly encountered as bacteria with intrinsic and acquired multiple resistance to most antibiotics, including vancomycin.³² Vancomycin is a glycopeptide antibiotic, active against most Gram-positive bacteria including enterococci.³³ The antibiotic mainly inhibits biosynthesis of the bacterial cell wall peptidoglycan, impairs RNA synthesis and alters the permeability of cytoplasmic membranes.³⁴ Resistance to glycopeptides was first described in enterococci, which is mediated by six *vanA–G* genes. Glycopeptide resistance is preferentially associated with *E. faecium* related *Enterococcus* spp.^{35,36} Enterococci can be transferred to humans throughout the food chain as well as oral-fecal routes.³⁷ These bacteria are able to transfer antibiotic resistance genes to other members of the microflora as well as bacterial pathogens via horizontal gene transfer.³⁸ Examples include *Streptococcus* spp., *Staphylococcus* spp. and *Listeria* spp. Recent studies have shown that human colonization with VRE occurs repeatedly in societies.³⁹ Moreover, high rates of VRE isolation have been reported from various foods such as vegetables, fruits, salads, meats and cheeses.^{40–43} Therefore,

these highlights address *Enterococcus* spp. as a medically important infectious agent in food, water and municipal sewage sources.

In the present study, *vanA*, *vanB* and *vanC* genes were found in VRE isolates, responsible for the bacterial resistance to vancomycin. No *vanD* or *vanE* was found. Six genes of *vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG* encode resistance to glycopeptides such as vancomycin.⁴⁴ These genes mediate the synthesis of abnormal precursors in bacterial peptidoglycan and hence reduce vancomycin affinity for binding to the peptidoglycan. The *vanA* and *vanB* genes are the most prevalent and predominant types of vancomycin resistance while *vanC*, *vanD* and *vanE* are usually less prevalent, as seen in the present study.^{45–47} Similarly, *vanA* and *vanB* were found in many studies by other researchers.^{48–50} In the current study, however, some VRE isolates (8 out of 41) did not carry *van* genes. Vancomycin resistance in these isolates might be associated with *vanG* (not investigated) or unknown genes rather than those described in the literature.⁵¹

Generally, microbial contamination rate of foods varies in different regions based on several parameters. They include the product and types of agriculture, geographical and environmental conditions, water source and local ecosystem. Furthermore, contamination of labors, devices, vehicles and packages are other parameters affecting the microbial contamination load in foods.⁵² However, one of the most important sources of bacterial contamination is water. This includes whether irrigation water or water used for washing agriculture products. Nowadays, urban wastewater is used to irrigate farms in many regions of the world, especially in undeveloped countries. To avoid contamination of foods and reduce its potential health hazards, improved regulations should be considered in different stages of food production, from farm to table. Moreover, the development of farming and food processing techniques can help.^{53–56}

Conclusion

In general, most vegetable samples collected in this study were contaminated with *Enterococcus* spp., including both bulk and packaged vegetables. This reveals a poor sanitation scheme during vegetable farming, production and processing. In conclusion, the bacterial antibiotic resistance has been increased significantly in recent decades due to inappropriate use of antibiotics; therefore, regular investigation of antibiotic resistance and the responsible genes in food bacteria is necessary. Furthermore, a better understanding of the mechanisms of resistance in foodborne antibiotic-resistant bacteria increases the ability of medical researchers to prevent consecutive hazards.

Authors' Contributions

RMNF: Study designing, advising, manuscript editing; MMSD: Study designing, supervising, manuscript editing; MA: Researching, carrying out experiments; ZR: Carrying out experiments.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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