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A study to determine the frequency of *van A* and *van B* genes in vancomycin-resistant *Enterococcus faecalis* strains obtained from clinical specimens in northwestern Iran

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Abstract

One of the significant global challenges today is the increasing prevalence of vancomycin-resistant enterococci (VRE). This study aims to understand the antibiotic resistance patterns, investigate the prevalence of VRE-causing genes in clinical samples, and evaluate the prevalence of enterococcal strains. For this study, 200 urine and blood samples were collected from patients visiting healthcare centers in Tabriz. Antibiotic susceptibility was determined using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, and the minimum inhibitory concentration (MIC) for vancomycin was assessed using the E-Test method. Additionally, polymerase chain reaction (PCR) techniques were employed for precise bacterial identification and to examine the presence of vancomycin resistance genes *van A* and *van B*. Molecular analysis revealed that 100 out of 200 samples (50%) belonged to *Enterococcus faecalis* (*E. faecalis*). The isolates exhibited the highest resistance to penicillin (83%), tetracycline (43%), and ciprofloxacin (41%), while they showed the greatest sensitivity to linezolid (87%), imipenem (85%), and teicoplanin (70%). A total of 31 samples were identified as resistant to vancomycin, with 18 strains (58.06%) containing the vanA genotype, 8 strains (25.81%) containing the vanB genotype, and 5 strains (16.13%) harboring both *van A* and *van B* genes. Given the high prevalence of VRE strains, it is essential to evaluate these organisms in all clinical samples.

Keywords: *Enterococcus faecalis*, vancomycin, blood, urine, infection, vancomycin resistance

1. Introduction

Enterococci are Gram-positive, catalase-negative bacteria that naturally inhabit the gastrointestinal tract but can also cause serious hospital-acquired infections, such as UTIs, surgical wound infections, bacteremia, endocarditis, and meningitis. They are highly resilient, tolerating bile salts, high salt concentrations, and extreme pH (9.6), with optimal growth at 10–45°C and survival at 60°C for 30 minutes. Recent overuse of antibiotics has led to rising global resistance, notably in vancomycin-resistant (VRE) and linezolid-resistant (LRE) strains (1-6). The first reported case of VRE in animals dates back to 1933, influenced by avoparcin. Later, in 1988, the first human case of VRE was reported in the United Kingdom (7). In the 1980s, immunological, biochemical, and genetic studies (including DNA similarity, rRNA analysis, and 16S rRNA sequencing) revealed distinct differences between Enterococcal and non-Enterococcal group D streptococci. These findings led to the reclassification of enterococcal strains into a separate genus, Enterococcus (8). The genus Enterococcus includes over 50 species, with *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) being the most significant members (9). The *E. faecalis* species is the dominant strain in this group, accounting for 85 to 90 percent of clinical isolates, while *E. faecium* contributes to about 5 to 15 percent of this statistic (10). Enterococci possess surface components such as polysaccharide capsules, pili, and adhesive substances that allow them to form biofilms and ultimately establish persistent infections. Other factors that exacerbate bacterial infection within the host include

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hemolysin/cytolysin, serine protease, bacteriocin, gelatinase, and toxic oxygen metabolites (TOM) produced by the bacteria that lead to cellular damage (7). Studies have clearly established that vancomycin resistance in enterococci is mediated by *van* genes. To date, several genotypes within the *van* group have been identified including *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*. The *vanA* and *vanB* genotypes have been predominant worldwide (2, 11). *E. faecalis* can transfer resistance genes via conjugative transposons, pheromone-responsive plasmids, or broad-host-range plasmids (12). Among the glycopeptide resistance genes in enterococci, the *vanA* gene is the most prevalent. This genotype confers high-level resistance to vancomycin and teicoplanin and is often located on plasmids transferred through transposon Tn1546. The *vanB* genotype exhibits similar functionality to *vanA*; however, their regulation differs as the *vanB* operon can be located on either chromosomes or plasmids (13-16). With reports of various Enterococcal strains emerging globally and the ongoing trend of acquiring resistance to multiple antibiotics, a significant reduction in therapeutic options for controlling these infections has occurred (17, 18). Therefore, the objective of this research is to evaluate the prevalence of Enterococcal strains, antibiotic resistance patterns, and identify the key genes responsible for vancomycin resistance (*vanA* and *vanB*) in clinical samples isolated from hospitalized patients in Tabriz city.

2. Materials and methods

2.1. Sample Collection and Identification:

In this study, 200 clinical samples (including blood and urine) were randomly collected from patients visiting hospitals and healthcare centers in Tabriz between March 2024 and August 2024. Patient demographic data, such as gender, age, and hospitalization status (hospitalized or outpatient), were also recorded. Initially, all samples were cultured on nutrient agar (Merck, Germany) and incubated at 37 °C for 24 hours. Colonies were identified at the genus level using Gram staining, catalase testing, disk resistance determination, optochin sensitivity, bile esculin growth, growth in 6.5% sodium chloride, and L-pyrrolidonyl-β-naphthylamide (PYR) hydrolysis tests. Additionally, the standard strain *E. faecalis* ATCC 19433 was used for quality control of the culture media and diagnostic tests (19). For final species determination, molecular identification was performed using polymerase chain reaction (PCR) techniques. In this study, bacterial DNA extraction was conducted using an extraction kit (Invitek Stratec Business) manufactured in Canada, and two pairs of primers were utilized for genus and species identification (Table 1).

The PCR reaction was performed in a final volume of 25 µl, which included 1 µl of each primer, 1 µl of dNTPs, 1 µl of template DNA, 1.7 µl of MgCl2, 2.5 µl of buffer, and 14.8 µl of distilled water, along with 1 unit of Taq DNA Polymerase (all consumables were provided by SinaGen, Iran). The thermal cycler program (Eppendorf serial number 46752 5332) consisted of 35 cycles with the following temperature conditions: an initial denaturation at 94 °C for 10 seconds, annealing at 64 °C for 15 seconds, elongation at 72 °C for 15 seconds, and a final elongation at 72 °C for 5 minutes. After completing the electrophoresis duration, the gel containing the PCR products was placed in a tank with ethidium bromide solution (produced by SinaGen) for 15 to 20 minutes. The presence of the desired bands was observed under ultraviolet (UV) light using a Gel Document device from ATP (serial number 001-020508), and photographs were taken and printed. Following the analysis of the obtained samples to confirm whether the cocci bacteria were indeed *E. faecalis*, two samples of the PCR products with four different readings were sent for sequencing to Macrogen in South Korea through Fazapajouh Tehran.

Table 1. Primers Used in This Study

Primer type	Product size (bp)	Sequence (5´-3´)	Reference
E. faecalis	941	ATCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	(20)

2.2. Antibiotic Sensitivity Testing:

To evaluate drug resistance, the standard Kirby-Bauer method was employed to assess the resistance of isolated strains against the following antibiotics: vancomycin (30 µg), ampicillin (10 µg), linezolid (30 µg), imipenem (10 µg), teicoplanin (30 µg), gentamicin (10 µg), penicillin (10 µg), ciprofloxacin (5 µg), tetracycline (30 µg), nitrofurantoin (300 µg), and dalfopristin (30 µg). These tests were conducted on Mueller-Hinton agar (Merck, Germany). The standard strain *E. faecalis* ATCC 19433 was used as a control for the disks. Additionally, the minimum inhibitory concentration (MIC) was determined using the E-Test method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines from 2018. The results were interpreted based on CLSI recommendations, where an MIC of vancomycin greater than 32 µg/µl was considered resistant, an MIC of approximately 6-12 µg/µl was categorized as intermediate, and an MIC of less than 4 µg/µl was deemed sensitive.

2.3. Identification of the *van* gene:

In this study, two pairs of primers were utilized (Table 2), which were employed together in a multiplex PCR reaction (21). The PCR reaction was conducted with a final volume of 25 µl, comprising 0.4 µl of each primer, 0.5 µl of dNTPs, 0.5 µl of template DNA, 1.5 µl of MgCl2, 2.5 µl of buffer, 18.7 µl of distilled water, and 0.5 units of Taq DNA Polymerase (SinaGen, Iran). The thermal cycler program included 35 cycles with the following temperature conditions: an initial denaturation at 94 °C for 1 minute, annealing at 57 °C for 1

minute, elongation at 72 °C for 1 minute, and a final elongation at 72 °C for 10 minutes. Subsequently, the PCR product was evaluated on a 1% agarose gel using electrophoresis. After completing the electrophoresis duration, the gel containing the PCR products was placed in a tank with ethidium bromide for 15 to 20 minutes and then observed under UV light to visualize the bands. *E. faecalis* ATCC 51299 with the *vanA* gene and *E. faecium* ATCC 51599 with the *vanB* gene were used as positive controls, while *E. faecalis* ATCC 29212, which is sensitive to vancomycin, served as a negative control. For statistical analysis of the data, version 20 of SPSS software and the chi-square test were employed, with a significance threshold set at $p < 0.05$.

Table 2. Primers Used in This Study

Primer type	Sequence (5´-3´)	PCR product size (bp)	Reference
vanA	F: 5'AATACTGTTTGGGGGTTGCTC3'	734	(22)
	R: 5'CTTTTTCCGGCTCGACTTCCT3'		(22)
vanB	F: 5'GCGGGGAGGATGGTGCGA3'	420	(22)
	R: 5'GGAAGATACCGTGGCTCAAAC3'		(22)

3. Results

Out of the 200 collected samples, 169 samples (84.5%) were isolated from urine, while 31 samples (15.5%) were from blood. Among these, 120 samples (60%) were related to outpatient cases and 80 samples (40%) were from hospitalized patients. The average age of the patients was 37.4 ± 27 years, ranging from a minimum of 10 months to a maximum of 75 years. Of the obtained samples, 110 (55%) were from males and 90 (45%) were from females. After performing PCR, considering the expected size for genus and species, which is approximately 941 base pairs, the amplification resulting from the reaction with the specified primers confirmed a fragment of 941 base pairs. Out of the 200 Enterococcal samples tested, only 100 samples were identified as *E. faecalis* (Fig. 1). Among these, 62 samples were from males and 38 samples were from females, with 42 samples isolated from hospitalized patients and 58 samples from outpatients.

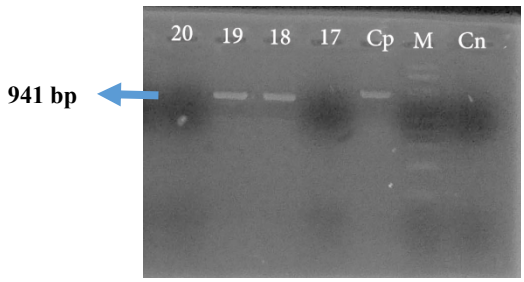

Fig. 1. Display of *E. faecalis* isolates (941 bp)
(M: 100 bp marker, Cn: negative control, Cp: positive control, 18 and 19: *E. faecalis* isolates)

Two samples, N70 and N32, were sent for sequencing to Macrogen, and both N70 and N32 were identified *as E. faecalis* using BLAST at the National Center for Biotechnology Information (NCBI). Based on the antibiotic sensitivity testing, *E. faecalis* exhibited the highest resistance to penicillin, tetracycline, and ciprofloxacin, while showing the greatest sensitivity to linezolid and imipenem (Table 3).

In this study, the determination of the MIC indicated that 31 samples were resistant to vancomycin. Among the resistant strains, 10 strains had an MIC of 48 µg/µl, 9 strains had an MIC of 64 µg/µl, and 12 strains had an MIC greater than 256 µg/µl (Figure 2). The vancomycin-resistant strains were isolated from urine samples with a frequency of 22 samples and from blood samples with a frequency of 9 samples. In this study, 18 vancomycin-resistant strains were isolated from outpatient cases and 13 strains were isolated from hospitalized patients.

Table 3. Percentage of Resistance and Sensitivity of Enterococcal Strains to Antibiotics (%)

Antibiotics	Sensitive	Intermediate	Resistant
Vancomycin	71	6	23
Ampicillin	65	3	32
Linezolid	87	4	9
Imipenem	85	1	14
Gentamicin	50	11	39
Teicoplanin	77	0	23
Penicillin	14	0	86
Ciprofloxacin	41	18	41
Tetracycline	54	3	43
Nitrofurantoin	70	18	12
Dalfopristin	59	11	31

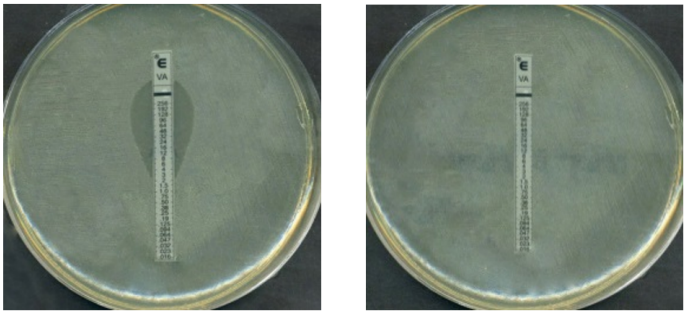

Fig. 2. Determination of Minimum Inhibitory Concentration (MIC) by E-Test

The results of the multiplex PCR indicated that 18 strains contained the *vanA* genotype, 8 strains contained the *vanB* genotype, and 5 strains contained both *vanA* and *vanB* genes (Figure 3). In this study, 88.89% of the *vanA* gene (16 strains) was observed in urine samples, while 11.2% (2 strains) was found in blood samples. The *vanB* gene was detected 100% in urine samples. Among the strains with the *vanA* gene, 61.12%

(11 strains) were from outpatient cases and 33.88% (7 strains) were from hospitalized patients. For the *vanB* gene, 75% (6 strains) were from outpatient cases and 25% (2 strains) were from hospitalized patients. Additionally, 40% (2 strains) of the strains carrying both *vanA* and *vanB* genes were isolated from outpatient cases, while 60% (3 strains) were isolated from hospitalized patients.

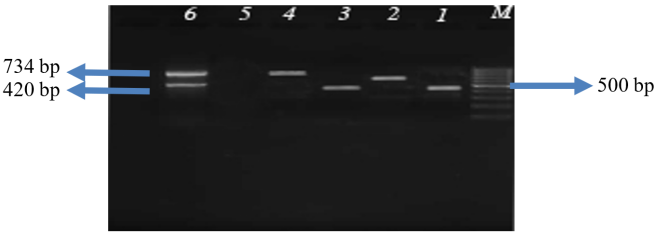

Fig. 3. Results of Amplification of Vancomycin-Resistant Enterococcus Genes by Multiplex PCR
M) indicates a 100 base pair marker, 1) *E. faecium* containing the *vanB* gene (positive control), 2) *E. faecalis* containing the *vanA* gene (positive control), 3) sample containing the *vanB* gene, 4) sample containing the *vanA* gene, 5) *E. faecalis* ATCC 29212, which is sensitive to vancomycin (negative control), 6) containing both *vanA* and *vanB* genes

4. Discussion

Enterococci are the second and third leading causes of urinary tract infections and bacteremia in humans (23). According to the present study, 50% of the 200 samples analyzed were identified as *E. faecalis*. The isolates exhibited the highest resistance to penicillin (83%), tetracycline (43%), and ciprofloxacin (41%), while they showed the greatest sensitivity to linezolid (87%), imipenem (85%), and teicoplanin (70%). Among the 31% of vancomycin-resistant samples, 58.06% contained the *vanA* genotype, 25.81% contained *vanB*, and 16.13% harbored both *vanA* and *vanB* genes. While our study identified vancomycin resistance in *E. faecalis* at significantly higher rates (23% by disk diffusion and 31% by Etest), Mohammadi et al. reported markedly lower resistance (9% by disk diffusion, with only 20% of these confirmed as resistant by Etest), highlighting both methodological differences in sensitivity and potential epidemiological variations in resistance prevalence across study populations and settings (24). Shahraki et al. reported an 18.6% vancomycin resistance rate using both Etest and antibiogram methods, which is lower than the findings of the current study (25). Our study's detection of significant VRE prevalence in outpatients (58.06%) contrasts with traditional hospital-associated epidemiology, suggesting potential community-acquired transmission through livestock exposure, asymptomatic colonization, or prior hospitalization. The outpatient VRE presence raises concerns about silent community spread and challenges empirical vancomycin use, necessitating enhanced outpatient surveillance, stricter antibiotic policies, and molecular typing to distinguish hospital versus community-associated clones for effective containment (26).

A study conducted by Sulaiman et al., found that 30% of all blood samples collected from children were *E. faecalis*, with 91.67% resistant to levofloxacin, 83.33% to amoxiclav, 66.67% to erythromycin, 58.33% to amikacin, 50% to ampicillin, 33.33% to cefotaxime and ceftriaxone, and finally, 25% resistant to vancomycin. Among the existing VRE samples, the *vanA* gene was identified in 89.88%, and the *vanB* gene in 77.78%, indicating that *vanA* is predominant, consistent with the findings of this study (27). According to research by Hosseini et al., which examined 48 enterococcal samples isolated from hospitalized and outpatient patients in southern Fars province, 27.09% of all samples belonged to *E. faecalis*. Overall, 43.75% of enterococcal isolates were resistant to vancomycin, with 40% carrying the *vanA* gene and 20% harboring the *vanB* gene (28). The statistical data from this research do not align with the results of the current study; however, similar to previous findings, the *vanA* gene is considered dominant. In a study by Malatrouni et al., conducted on 400 samples collected from patients in intensive care units (ICU) in Egypt, enterococcal isolates were identified in 12% of patients, with *E. faecalis* accounting for 66.7%. Among all enterococcal isolates, 41.7% were resistant to vancomycin, with the *vanA* gene observed in 85% of VRE samples, while the *vanB* gene was not found in any VRE isolates (29). Ultimately, the statistics and results obtained from this research exceed those of the current study and do not align; nevertheless, similar to findings from this study, the *vanA* gene remains predominant. According to data collected by Hammerum et al., between 2015 and 2022 in Denmark, among 4,862 clinical samples of VRE and Vancomycin-variable enterococci (VVE), approximately 60% were related to urine samples while the remainder were associated with other clinical specimens including blood and pus. About 2,504 samples (51%) contained *vanA E. faecium*, while 1,485 samples (30%) had *vanB E. faecium*; additionally, there were 62 cases (1.2%) with both *vanA* and *vanB* genes in *E. faecium*, 15 samples (0.3%) containing *vanA E. faecalis*, and 23 cases (0.4%) containing *vanB E. faecalis*. This comprehensive analysis highlights significant trends in antibiotic resistance among enterococci, particularly emphasizing the prevalence of *E. faecalis* as a major pathogen in urinary tract infections and its resistance patterns against various antibiotics across different studies and populations (30). According to a study conducted by Adimi et al., on 208 enterococcal isolates collected from clinical samples in three hospitals in Nigeria, 40.9% of the isolates were identified as VRE. In this study, *E. faecalis* constituted 28.2% of the VRE isolates. The *vanA* resistant phenotype was prevalent in 65.9% of the isolates (31). The high prevalence of the *vanA* gene in most studies may be attributed to the high transferability of transposons (28). According to a study by Dadashi et al., on human clinical samples worldwide, *E. faecalis* exhibited approximately 0.9% resistance to various antibiotics, with resistance to linezolid reported at 2.2%. The prevalence of linezolid-resistant *E. faecalis* was found to be 2.8% in Asia and 0.4% in Europe (32). In research conducted by Ghalavand et al., on 63 patients with catheter-associated urinary tract infections (CAUTI), 126 *E.*

faecalis isolates (63 urine samples and 63 fecal samples) were identified. Based on this study, *E. faecalis* isolated from urine and fecal samples showed resistance rates of 88.9% and 76.2%, respectively, against tetracycline, and 87.3% and 71.4%, respectively, against minocycline. According to the results obtained from this study, *E. faecium* showed no resistance to antibiotics such as linezolid, vancomycin, ampicillin, nitrofurantoin, and penicillin, which is contrary to the findings of the current study (33). Karna et al., reported a VRE prevalence of 25.3% among enterococcal samples in 2019, with the highest antimicrobial sensitivity recorded for linezolid (97.8%), teicoplanin (95.6%), and gentamicin (81.3%) (34), similar to the findings of this study where linezolid exhibited the highest sensitivity. In their study, Goudarzi et al. isolated 439 Enterococcus faecalis strains from 690 clinical samples, reporting 0% linezolid resistance and vanA/vanB gene prevalence rates of 72% and 22%, respectively. These findings are inconsistent with the results of our current study (35). The results from Moghimi Baghkhan et al. indicated that vancomycin resistance among enterococcal isolates in Iran was at 14% (36). The study reveals significant variations from reference data primarily due to geographic and population differences in patient demographics, sample sources, and regional antibiotic practices that shape resistance patterns, combined with methodological variations in study scale, techniques, and duration that influence data consistency, along with microbial genetic factors particularly the transposon driven *vanA* and *vanB* genotype showing location-dependent prevalence all of which collectively demonstrate antimicrobial resistance development through complex interactions between local microbiomes, clinical practices, and research methodologies, ultimately explaining apparent contradictions among similar studies.

The findings from this research and other studies indicate a high prevalence of VRE strains among hospitalized and outpatient patients. Variations in VRE prevalence across different studies may arise from factors such as sample size, duration of sample collection, age and gender of patients, as well as the diagnostic methods used for detecting VRE. Given that enterococci exhibit both intrinsic and acquired resistance to various antibiotics, conducting antibiotic susceptibility testing prior to drug administration is essential. Additionally, proper use of antimicrobial agents is recommended to prevent the emergence of more severe antibiotic resistance in bacteria. In summary, this research underscores the importance of continuous monitoring and identification of *E. faecalis* in clinical settings. Further investigations are necessary to clarify the underlying factors contributing to drug resistance and to develop more effective strategies for managing *E. faecalis* infections in clinical environments.

Ethical Statement

The study protocol was approved by the Clinical Research Ethics Committee of Islamic Azad University, Ahar Branch (Date: 2024, No: 22030507931003).

Conflict of interest

Authors declare that there is no conflict of interests.

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No to declare.

Authors' contributions

Concept: A.J.S., M.P., Design: A.J.S., Data collection or Processing: A.J.S., Analysis or Interpretation: S.V., M.F.S., G.K.I., F.G.I., K.S., K.H.K., Literature Search: S.V., M.F.S., G.K.I., F.G.I., K.S., K.H.K., Writing: S.V., M.F.S., G.K.I., F.G.I., K.S., K.H.K.

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